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*"Life is like riding a bicycle.
In order to keep your balance,
you must keep moving."*

Albert Einstein.

ABBREVIATIONS

DHN	1,5-Dihydroxynaphthalene	dd	Double doublet
A	Acceptor	DODAB	Diocetadecyldimethylammonium bromide
Å	Angstrom	DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
a.u.	Arbitrary units	DPA	9,10 Diphenylanthracene
ABDA	9,10-anthracenediyl-bis(methylene)dimalonic acid	Dtxl	Docetaxel
AcOEt	Ethyl acetate	$\epsilon_A(\lambda)$	Molar absorptivity of the acceptor
AcOH	Acetic acid	EET	Excitation energy transfer
ACQ	Aggregation caused quenching	EPR	Enhanced permeability and retention effect
ADPA	Anthracene-9,10-dipropionic acid	ESA	Excited-state absorption
AFM	Atomic force microscopy	ESI	Electrospray ionisation
AIE, AIEE	Aggregation induced emission	ET	Energy transfer
AM1	Austin model 1	ETU	Energy transfer upconversion
AMBER	Assisted Model Building with Energy Refinement	ϕ , Q_D	Fluorescence quantum yield
ANI	4-amino-1,8-naphthalimide	FASOC	Folate-modified amphiphilic chitosan
AOT	Bis(2-ethylhexyl) sulfosuccinate	F_D	Fluorescence of the donor in acceptor absence
Boc	tert-Butyloxycarbonyl	$F_D(\lambda)$	Normalized donor fluorescence spectrum
BSA	Bovine serum albumin	F_{DA}	Fluorescence of the donor in acceptor presence
BuOH	Butanol	FDA	Food and drug administration
CBT	carbonyl-bridged triarylamine	ϕ_{ET}	Energy transfer quantum yield
CCD	Charged-coupled device	Fmoc	Fluorenylmethyloxycarbonyl
CD	Cyclodextrin, Circular Dicroism	FRET	Fluorescence resonance energy transfer
CHP	cholesterol-bearing pullulan	FTIR	Fourier transform infrared spectroscopy
CLSM	Confocal laser scanning microscopy	G''	Loss modulus
cmc	Critical micelle concentration	G'	Storage modulus
CPLs	Circular polarizing filters	Gly	Glicine
D	Donor	HEMA	Hydroxyethyl methacrylate
d	Doublet	HFIPA	1,1,1,3,3,3-Hexafluoro-2-propanol
DCC	dicyclohexylcarbodiimide	HLB	Hydrophilic lipophilic balance
DCC	N,N' -dicyclohexylcarbodiimide	HOMO	Highest occupied molecular orbital
DDOA	2,3-Didecyloxyanthracene	HP	Hematoporphyrin
DLS	Dynamic light scattering	HPS	1,1,2,3,4,5-Hexaphenylsilole
DMAP	Dimethylaminopyridine	HRMS	High resolution mass spectrometry
DME	Ethylene glycol dimethyl ether	HUVEC	Human Umbilical Vein Endothelial Cells
DMF	N,N-dimethylformamide	Hyp	Hypericin
DMSO	Dimethyl sulfoxide	IC_{50}	Half maximal inhibitory concentration
DNA	Desoxyribonucleic acid		

ICT	Pull-push charge transfer	OLEDs	Organic light emitting diodes
IR	Infrared	OPV	Oligo(p-phenylenevinyls)
IRQC	Infrared quantum counter	PA	Peptide amphiphile
ISC	Intersystem crossing	PA	Photon avalanche
$J(\lambda)$	Integral of normalized spectral overlap	PAAc	poly(acrylic acid)
κ^2	Transition dipole orientation	PAAm	polyacrylamide
K_{dexter}	Rate of energy transfer	PAN	polyacrylonitrile
K_s	Solubility product	PBO	poly(butylene oxide)
λ	Wavelength	PBS	Phosphate buffer saline
L	Sum of the van der Waals radii	PDS	pyridyl disulfide
λ_{ab}	Maximum absorption wavelength	PDT	Photodynamic therapy
LBG	N,N'-bis(octadecyl)-L-boc-glutamic diamide	PEG	Polyethyleneglycol
LED	Light emitting diode	PEI	polyethylenimine
λ_{em}	Maximum emission wavelength	PEMA	poly (ethyl methacrylate)
LMWGs	Low molecular weight gelators	PEO	poly(ethylene oxide)
LUMO	Lowest unoccupied molecular orbital	PF	propylene fumarate
m	Multiplet	PGEMA	poly(glucosylethyl methacrylate)
M540	Merocyanine 540	PHB	poly(hydroxy butyrate)
MB	Methylene Blue	PHPMA	poly(hydroxypropyl methacrylamide)
MCF-7/AZ	Human Caucasian breast adenocarcinoma	PI	Polydispersity index
mgc	Minimum gel concentration	PLA	Poly(lactic acid)
MR	Magnetic resonance	PLGA	poly(lactic-co-glycolic acid)
MRI	Magnetic resonance imaging	PM	Polymeric micelle
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	PMI	Perylene monoimide
MUC1	Mucin 1	PMMA	poly(methyl methacrylate)
N	Avogadro's number	PMT	Photomultiplier tube
Naf	Naphthalimide	PNIPAAm	poly(N-isopropyl acrylamide)
Ng's	Nanogels	PNP	Polymeric nanoparticle
NIBT	4-(5-hexyl-2,29-bithiophene)-naphthalimide	PNVP	poly (N-vinyl pyrrolidone)
NIR	Near infrared	PPO	poly(propyleneoxide)
nm	nanometres	PVA	poly(vinyl alcohol)
NMR	Nuclear magnetic resonance	PVA	poly(vinyl amine)
NONOate	1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium salt	PVAc	poly(vinyl acetate)
NPQ	Non-photochemical quenching	PVDF	polyvinylidene difluoride
NR	Nile red	q	Quadruplet
OA	Oleic acid	QDs	Quantum dots
OFETs	Organic field-effect transistors	r	distance between chromophores
		R_0	Förster distance
		RB	Rose bengal

r_{DA}	Donor-acceptor distance radii	w/w	Weight/weight
RE	Rare earth	WAXS	Wide angle X-ray scattering
RET	Resonance energy transfer	XRD	X-ray diffraction
Rhod123	Rhodamine 123	Z, Cbz	Benzyloxycarbonyl
RIM	Restriction of intramolecular motions	ZnPC	Zinc phthalocyanine
RIR	Restriction of intramolecular rotations	Φ_{Δ} , Φ_{102}	Singlet oxygen quantum yield
RIV	Restriction of intramolecular vibrations		
RNA	Ribonucleic acid		
ROS	Nuclear magnetic resonance		
rpm	Revolutions per minute		
s	Singlet		
SANS	Small-angle neutron scattering		
SAXS	Small angle X-ray scattering		
SDS	Sodium Dodecyl Sulfate		
SEM	Scanning electron microscopy		
SSET	Singlet-singlet energy transfer		
τ	Fluorescence lifetime		
t	Triplet		
τ_D	Lifetime of donor in absence of acceptor		
τ_{DA}	Lifetime of donor in presence of acceptor		
TEM	Transmission electron microscopy		
TFA	Trifluoroacetic acid		
THBA	Tetrahydro-5,5',-bidibenzo[a, d][7]annulenyldiene		
THF	Tetrahydrofuran		
TOF	Time of flight		
TPE	Tetraphenylethene		
Trp	Tryptophan		
TTA	Triplet-triplet annihilation		
TTET	Triplet-triplet energy transfer		
TTET	triplet-triplet energy transfer		
Tyr	Tyrosine		
UC	Upconversion		
UCL	Upconverted luminiscence		
UCMSNs	Upconverting mesoporus nanoparticles		
UCNPs	Upconverting nanoparticles		
UV	Ultraviolet		
V, Val	Valine		
Vis	Visible		

Compound Chart

4-amino-1,8-naphthalimide derivatives

1,8-naphthalimide derivatives

Acridine derivatives

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Chapter 1

General Introduction

Chapter 1: General introduction

1.1 Molecular self-assembly

How did we get here? How did matter managed to gain complexity to reach the levels of a thinking organism? How has this happened? What higher forms of complex matter are there to be evolved or created? Of course, we are not in a position to answer these fundamental philosophical questions because not enough data have been gathered to fill this critical knowledge gaps. However several authors agreed to point out a decisive event that may have led to this wonderfully organised biological world: Self-Assembly. Probably all came up by rudimentary self-assembled compartmentalization, i.e. by formation of a primitive membrane, vesicle or microsphere in a hydrophobic environment to achieve an outside and inside medium to physical restriction for subsequent cellular macromolecules.¹

Molecular self-assembly consists in the spontaneous and autonomous “bottom up” association of an ensemble of molecules without human intervention into supramolecular structures. Non-covalent interactions such as van der Waals, electrostatic, hydrophobic, coordination and hydrogen bonding are involved in the final assembly, which is stabilized by many, relatively weak non-covalent interactions distributed over the whole supramolecular assembly.² The self-assembly term has been bounded by Whitesides and Grzybowski to involve exclusively pre-existing components as distinct parts of a disordered structure requiring reversibility and controllable design of the components.³ A self-organization process requires the components to equilibrate between aggregated and non-aggregated states and to adjust their relative positions to form the supramolecular assemblies, additionally the characteristics of the components control how they interact with each other and thus the patterns and structures that emerge. Two main stages may be considered regarding the self-assembly and organization: (i) molecular recognition for the selective binding of the basic components; (ii) correct relative disposition and cooperative nucleation elongation growth through sequential and eventually hierarchical binding of multiple components (Figure 1).²

Figure 1. Different examples of self-assembly at molecular level. **a)** Lipid molecules self-assemble to form lamellar, tubular and vesicular structures, **b)** Proteins form lamellar, helical tubular and regular icosahedral structures. **c)** SDS@2β-CD assembles, in a protein-mimetic way, into lamellar, helical tubular and rhombic dodecahedral structures.⁴

1.1.1 Types of self-assembly

Two main types of self-assembly have been defined from a thermodynamic point of view: static and dynamic. Static self-assembly refers to the systems that are at equilibrium and do not dissipate energy. However static self-assembly may require activation energy to trigger the process, associated to operations such as stirring, sonication, heating-cooling etc. Most of the research in self-assembly has focused on the static type. Probably the most known self-assembly process is the 3D crystallization phenomenon. However several examples can be further depicted such as the formation of fibrillar assemblies of peptide amphiphiles in water (Figure 2), electrostatic self-assembly of polymeric microspheres on a charge-exchanging

substrate modified by wet stamping, electronic self-assembly of macroscopic 2D crystals whose formation is mediated by charges developed by contact electrification, capillary self-assembly of polymeric plates at an interface between two liquids or self-assembled polymeric microspheres of complex internal structures.^{5,6}

Figure 2. Example of static self-assembly of a peptide amphiphile (PA) adapted from reference.⁷ **A)** Chemical structure of PA highlighting the four structural domains (I–IV). **B)** Illustration of a self-assembled PA nanofiber, with red spheres representing water molecules. **C)** TEM image of PA nanofibers cast from aqueous solution. **D)** SEM image of a PA gel formed by mixing cell culture media with a PA solution.

Dynamic or dissipative self-assembly is observed in such systems whose interactions responsible for the formation of structures between components only occur if constant energy supply is provided. The energy is subsequently dissipated via entropy producing irreversible processes associated with the interactions of system's components. Examples of dynamic self-assembly include electrorheological fluids, magnetorheological fluids, magnetohydrodynamic self-assembly and more recently this dynamic behaviour has been implemented on supramolecular materials (Figure 3).^{8–10} These dynamic structures rely on a constant energy supply for survival and collapse when the flow of energy ceases.⁶

Figure 3. Transient anhydride formation by carbodiimide fuels. (a) Scheme of the chemical reaction network for the fuel driven formation of a transient anhydride bond. (b) Molecular structures of the dicarboxylates. (c) Molecular structures of the fuels.⁸

1.1.2 Biological inspiration

Mankind has been inspired by nature since the beginning of time. As usual, the most elegant, complex and adaptable examples of self-assembly are found in biological systems. Nature is plenty of ordered functional nanostructures formed by self-assembled biomolecular units. Beautiful examples can be extracted from the basic unit of life that can self-replicate: the cell (Figure 4). Almost all the machinery present inside the cell has been constructed or directed by self-assembly procedures. Examples include the ensemble of proteins and RNA in the formation of ribosome, the dynamic self-assembly of filaments of actin, DNA strands assembled into a double helix, microtubule filaments formed by short monomers of α and β -tubulin and the self-assembly of phospholipids, cholesterol, glycolipids, and protein building blocks to form the cellular membrane. Such non-covalent self assembly allows the cell to generate adaptable and reversible dynamic nanostructures.

Figure 4. Self-assembled cellular machinery.¹¹

1.1.3 Self-assembly and supramolecular chemistry

Self-assembly has shown to be a very good solution for designing and constructing structures larger than molecules, for example aggregates or several forms of organized matter cannot be synthesized bond by bond. The understanding of these processes were initiated by seminal works of Lehn, Cram and Pedersen, which supposed a ground-breaking event in the field of chemistry: the birth of supramolecular chemistry, also coined as “the chemistry beyond the molecules”. This work led them to be awarded later with the Nobel Prize in Chemistry in 1988.¹² Supramolecular chemistry aims to develop highly complex chemical systems from components interacting by noncovalent intermolecular forces. The development of supramolecular chemistry has supposed numerous developments in between the physics and biology giving place to a plethora of scientific, technologic findings and providing outstanding tools to unleash the creativity of scientists from all over the world.

Intrinsic advantages of supramolecular chemistry are based on the weak nature of the cohesive forces that binds the molecules together. For this reason, supramolecular materials can be highly modular, biodegradable, cytocompatible, dynamic and self-healing. Moreover, molecules can be chemically programmed to incorporate molecular components of suitable instructions for the generation of a well-defined supramolecular entity. Then, the information

coded in individual components can be translated at supramolecular level, arising new interesting features related with shape, surface properties, charge polarizability and magnetic dipole, among others.

A broad range of self-assembling systems have been explored so far, from the earliest simple synthetic examples of Pedersen and co-workers using cyclic polyethers for the recognition of metal cations¹³ to the complex liposomal systems designed by Torchillin.¹⁴ The increased knowledge of supramolecular interactions and structures enabled to develop a wide variety of applications related to the construction of chemical sensors, molecular cages, catalytic molecular gels, engineered nano-sized drug delivery systems and biomaterials for physical scaffolds or biological signalling (Figure 5).^{3,15-17}

Figure 5. Molecular units designed to form supramolecular architectures with mechanical (**A** and **B**), biological (**C** and **D**), and optical (**E** and **F**) or electronic functionalities (**G** and **H**).¹⁵

1.2 Molecular gels

The most comprehensive definition of gels was made in 1974 by Flory: “a substance is a gel if it has a continuous structure with macroscopic dimensions that is permanent on the time scale of an analytical experiment and is solid-like in its rheological behaviour”.^{18,19} In other words, gels are a colloidal state of matter in which a small amount of a solid-like microphase-separated component network (gelator) is able to immobilize the bulk flow of a larger amount of liquid-like phase. This microphase separation is induced by nucleation. As a result, the material is “solid-like” in its rheological behaviour: the storage (elastic) modulus (G') is larger than the loss (viscous) modulus (G'') over a shear frequency range within the linear viscoelastic region.

Although molecular gels have been described many years ago and a very wide range of materials have been recognised since 1860's to form molecular gels. The interest for this materials was eclipsed by its polymeric counterparts for many years, relegating these materials to a second plane until the interest for molecular gels experienced an explosion starting from 1990.²⁰ Polymeric gels are formed by networks of macromolecules. They have been found to present wide interesting properties such as swelling capabilities, physical integrity, biocompatibility and stimuli responsive behaviour finding very interesting applications (especially for hydrogels)²¹ which have shown to have great impact in industries such as biomedical and pharmaceutical, personal care, agriculture, construction and waste treatment.^{22–27}

The term (supra)molecular gels has been used to describe gels that consist of low molecular weight compounds (often referred to as low molecular weight gelators, LMWGs) which self-assemble in a given solvent to form a gel.²⁸ Organogels derived from LMWGs were first comprehensively reviewed by Terech and Weiss in 1997²⁹ while Hamilton and Estroff summarised the field of LMWG hydrogels in 2004.³⁰ A more recent review on molecular hydrogels as emerging biomaterials includes more than 600 molecules and 1000 references.³¹

The decisive event for molecular gel formation is the self-assembly of molecular building blocks, driven by complementary non-covalent interactions such as hydrogen bonding, π – π

interactions, metal-ligand coordination, van der Waals forces, hydrophobic effects, etc. In this way higher order structures are formed, most frequently in a quasi-one dimensional manner, yielding (nano)fibers that result in a sample spanning network. Nevertheless, other morphologies like (nano)ribbons, (nano)sheets or (nano)spheres also are described to create molecular gels. On the molecular level, the solvent is mobile within the gel-phase network. However, the flow of the bulk solvent is prevented as a consequence of capillary forces arising from solvent-gelator interactions. These gels are usually thermally reversible affording sol phases under the appropriate conditions. The sols consist of individual gelator molecules or aggregates which do not form a continuous network.³²

Molecular gelators discovery has often found to be a matter of serendipity. However the recent application of supramolecular principles to the design of new gelators has boosted the field. Diverse structural units have been reported to be successful gelating moieties: (i) Amide bonds, ureas and groups capable of undergo directional hydrogen bonding; (ii) aliphatic tails to engage in solvophobic interactions and van der Waals interactions and provide structural flexibility to pile up in a preferred direction; (iii) Aromatic moieties to undergo π - π stacking interactions.

Figure 6. Structure of reported low molecular weight gelators.

For gel formation, solvent is a key parameter. Moieties forming hydrogen bonding interactions are described to be the driving force for gelation in organic solvents. Hydrophobic and aromatic moieties are more important in the case of hydrogels because the hydrophobic effect is the dominating driving force (minimization of surfaces exposed to water, encompassing enthalpic interactions and entropic gain).³³ However, for the successful formation of a hydrogel, an accurate balance between hydrophobic (which drive the self-assembly) and hydrophilic (which ensure compatibility with water) structural motifs is required. Up to date, more than 2000 gelators with varied different structural motifs have been reported, including dendritic systems, nucleobases, metallogels, sugars, amides/peptides and ureas. Despite all this wide library of molecular gelators, nowadays it is still not possible to predict with certainty a priori if a given molecule is a molecular gelator. A proof of the complexity of the gelation process is the fact that very small changes in molecular structure of LMWGs lead the molecule to become a non-gelator. For example, changing one functional group to another will result in a change in the steric bulk of the molecule, the ability to pack, possibly the number of hydrogen bond donors or acceptors, the absolute solubility in a particular solvent, etc. Therefore, serendipity and library-based approaches are often used to discover new gelators,^{34,35} being the most successful approach for designing molecular gelators the chemical modification of known gelators to dial in specific properties to the gel system. Therefore, since a better understanding of the assembly processes and structures of known supramolecular gels is being developed, the design of new gelators is becoming more frequent.³⁶

Overall, the singular self-assembly organization and the nature of the cohesive forces by which the molecular gels are formed confers to these materials very interesting properties, in contrast to covalent organizations: dynamic behaviour, high degrees of structural order, self-healing properties and error correction, stimuli responsiveness to multiple external stimuli, easy synthesis of supramolecular materials, easy functionalization for introducing desired functionalities at molecular level to be translated (or even enhanced) to the gel.

1.2.1 Gelation process

Molecular gels are typically prepared by heating a (solid) gelator and a liquid component until a solution is formed and, subsequently, cooling the system below its sol-gel transition temperature. Other different gelation methods have been described so far such as pH shifting using acidic and basic gelators, sonication,³⁷ solvent polarity shift,³⁸ light irradiation,³⁹ salt^{40,41} and coordinating metals addition^{42,43} or enzymatic reactions among others.⁴⁴ The properties of the gels are demonstrated to be highly process dependent, which means that it is possible to access materials with very different properties from a single gelator.^{45,46}

The nano-scale network is formed by a combination of entanglement and bifurcation of the fiber bundles. This occurs via multiple hierarchical assemblies from molecule to network. First, as mentioned above, the molecular interactions must drive self-assembly, which controls the nanostructure. This nanostructure must favour formation of one-dimensional fibres over other possible structures. The self-assembly into such one-dimensional structures, as opposed to crystallisation, is driven by a balance of forces (Figure 7). Using Monte Carlo simulations, the stiffness of the 1-D aggregates formed upon gelator assembly and the strength of the intermolecular bonds between the resulting threads have been shown to be decisive for the formation of fibrillar networks. Should the threads be too stiff or their mutual attraction forces be too strong, disconnected bundles of threads would be formed instead of the connected network of bundles required for gel formation.⁴⁷ Additionally, it is important to note that a certain amount of the gelator is generally present in solution (sol fraction) that can be in dynamic exchange with the fibers.⁴⁸

Figure 7. Schematic cartoon showing the assembly of a LMWG into fibres, resulting in a network and gel formation. (a) LMWG dissolved in solution (b) self-assembly of LMWG starts to occur after application of a trigger (c) formation of the gel network by entanglement (d) a self-supporting bulk gel.⁴⁹

1.2.2 Properties and characterization

Different techniques have been used to characterize molecular gels (Figure 8).^{50,51} The nature of molecular interactions can be unravelled by different techniques such as NMR spectroscopy, IR spectroscopy, WAXS (wide angle X-ray scattering), UV-Vis absorption and fluorescence. These techniques permit to determine properties such as solubility, packing modes, composition, thermodynamic parameters and shed light on conformational dispositions as well as giving clues of molecular environment. Furthermore, these techniques allow carrying on time-dependent experiments to investigate the kinetics of the gel formation/disruption. For investigating higher structural levels circular dichroism (CD) and X-ray based techniques such as SAXS (small angle X-ray scattering), and SANS (small-angle neutron scattering) are useful and offer interesting descriptions about the self assembly process, supramolecular organization and supramolecular chirality. Valuable information about fibrillar network morphologies are provided by different microscopic techniques such as SEM (scanning electron microscopy) and TEM (Transmission electron microscopy). Despite the fact that these two techniques present the highest resolution up to sub-nanometer scale, they require the drying of the material introducing possible artefacts in the observation. The preferred microscopic techniques are those in which the observation can be carried out with the minimum sample perturbation, for example cryo-TEM, wet AFM (atomic force microscopy), and confocal laser scanning microscopy, which allows to perform three-dimensional imaging. For bulk macroscopic material characterization the rheology is the most used technique and provides useful information about the structure of the assemblies like cross-linking density, their dynamics and even about the self-assembly mechanisms.^{51,52}

Figure 8. Low-molecular-weight gels form as a result of assembly across a range of length scales; different techniques are appropriate for analyzing the structures formed at each length scale.⁵³

1.2.2.1 Stimuli responsiveness

Probably one of the differentiating features of molecular gels over its polymeric counterparts is their intrinsic stimuli-responsiveness. This important feature permits to expand the applications in a very creative way by introducing sensitive groups to many different stimuli. The obvious and most studied stimuli are temperature and concentration. However, other triggers that require chemical planning and design have been nicely described, for example pH shifts are used to trigger the aggregation of gelators containing acidic or basic moieties which are soluble when presenting neat charges and aggregate when neutral.^{54,55} Other stimuli are the changes in ionic strength by addition of salts or metal ions or the formation/breakage of covalent bonds. Examples include enzymatic phosphorylation/dephosphorylation to expose or protect sensitive groups that induce self-assembly,⁴⁴ redox reactions (cysteine residues in peptide gelators)⁵⁶ and the use of photolabile gelators.^{39,57}

1.2.3 Applications

Classically, gels have been used in numerous industrial applications; they are important in areas such as cosmetics, food processing, lubrication and hydrometallurgy. Gels also have been used with environmental purposes, for example in the control and recovery of spilled or cooking oils. Additionally, they have been used in military context as aviation fuels and explosive technologies and are extensively used among other applications, for macromolecular separations due to steric and hydrodynamic interactions between permeating macromolecules and the disordered fibrous media.²⁹

A plethora of interesting applications has raised fruit of the extensive studies on molecular gels, revealing them as very interesting materials (Figure 9). For example, molecular gels have been studied in wide variety of fields including templates for hard materials,⁵⁸ photonics, magnetic (molecular-based ferromagnetism) and electronic (electronic conduction or semiconduction of donor-acceptor conjugated systems) materials,⁵⁹ catalysis,⁶⁰ sensors (temperature, humidity and (bio)chemical agents),⁶¹ molecular and chiral recognition,^{62,63} controlled loading and release⁶⁴ and tissue engineering.⁶⁵

Figure 9. Overview of applications for molecular gels.⁵³

1.3 References

- (1) Trevors, J. T.; Psenner, R. *FEMS Microbiol. Rev.* **2001**, 25 (5), 573.
- (2) Lehn, J.-M. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, 99 (8), 4763.
- (3) Whitesides, G. M. *Science (80-.)*. **2002**, 295 (5564), 2418.
- (4) Felip-León, C.; Segarra-Maset, M. D.; Escuder, B.; Miravet, J. F. *Tetrahedron Lett.* **2015**, 56 (9), 1132.
- (5) Palmer, L. C.; Stupp, S. I. *Acc. Chem. Res.* **2008**, 41 (12), 1674.
- (6) Fialkowski, M.; Bishop, K. J. M.; Klajn, R.; Smoukov, S. K.; Campbell, C. J.; Grzybowski, B. A. *J. Phys. Chem. B* **2006**, 110 (6), 2482.
- (7) Matson, J. B.; Zha, R. H.; Stupp, S. I. *Curr. Opin. Solid State Mater. Sci.* **2011**, 15 (6), 225.
- (8) Tena-Solsona, M.; Rieß, B.; Grötsch, R. K.; Löhrer, F. C.; Wanzke, C.; Käsdorf, B.; Bausch, A. R.; Müller-Buschbaum, P.; Lieleg, O.; Boekhoven, J. *Nat. Commun.* **2017**, 8 (May), 15895.
- (9) Klingenberg, D. J. *AIChE J.* **2001**, 47 (2), 246.
- (10) Whitesides, G. M.; Grzybowski, B. A.; Stone, H. A. *Nature* **2000**, 405 (6790), 1033.
- (11) Petkau-Milroy, K.; Brunsveld, L. *Org. Biomol. Chem.* **2013**, 11 (2), 219.
- (12) Lehn, J. M. *Acc. Chem. Res.* **1978**, 11 (2), 49.
- (13) Pedersen, C. J. *J. Am. Chem. Soc.* **1967**, 89 (26), 7017.
- (14) Torchilin, V. P. *Nat. Rev. Drug Discov.* **2005**, 4 (2), 145.
- (15) Aida, T.; Meijer, E. W.; Stupp, S. I. *Science (80-.)*. **2012**, 335 (6070), 813.
- (16) Dong, R.; Zhou, Y.; Huang, X.; Zhu, X.; Lu, Y.; Shen, J. *Adv. Mater.* **2015**, 27 (3), 498.
- (17) Stephanopoulos, N.; Ortony, J. H.; Stupp, S. I. *Acta Mater.* **2013**, 61 (3), 912.
- (18) Almdal, K.; Dyre, J.; Hvidt, S.; Kramer, O. *Polym. Gels Networks* **1993**, 1 (1), 5.
- (19) Flory, P. J. *Discuss Faraday* **1974**, 57 (7), 7.
- (20) Weiss, R. G. *J. Am. Chem. Soc.* **2014**, 136 (21), 7519.
- (21) Peppas, N. A. *Curr. Opin. Colloid Interface Sci.* **1997**, 2, 531.
- (22) Ahn, S.; Kasi, R. M.; Kim, S.-C.; Sharma, N.; Zhou, Y. *Soft Matter* **2008**, 4 (6), 1151.
- (23) Jagur-Grodzinski, J. *Polym. Adv. Technol.* **2009**, 21 (1), 27.
- (24) Vermonden, T.; Censi, R.; Hennink, W. E. *Chem. Rev.* **2012**, 112 (5), 2853.
- (25) Li, J.; Mooney, D. J. *Nat. Rev. Mater.* **2016**, 1 (12), 16071.

- (26) Rogovina, L. Z.; Vasil'ev, V. G.; Braudo, E. E. *Polym. Sci. Ser. C* **2008**, 50 (1), 85.
- (27) Thakur, V. K.; Thakur, M. K.; Voicu, S. I. *Polymer Gels Perspectives and Applications*; Springer Verlag, 2018.
- (28) Buerkle, L. E.; Rowan, S. J. *Chem. Soc. Rev.* **2012**, 41 (18), 6089.
- (29) Terech, P.; Weiss, R. G. *Chem. Rev.* **1997**, 97 (8), 3133.
- (30) Estroff, L. A.; Hamilton*, A. D. *Chem. Rev.* **2004**, 104 (3), 1201.
- (31) Du, X.; Zhou, J.; Shi, J.; Xu, B. *Chem. Rev.* **2015**, 115 (24), 13165.
- (32) Hardy, J. G.; Hirst, A. R.; Smith, D. K. *Soft Matter* **2012**, 8 (12), 3399.
- (33) Chandler, D. *Nature* **2005**, 437 (7059), 640.
- (34) Raeburn, J.; Zamith Cardoso, A.; Adams, D. J. *Chem. Soc. Rev.* **2013**, 42 (12), 5143.
- (35) Van Esch, J. H. *Langmuir* **2009**, 25 (15), 8392.
- (36) Hirst, A. R.; Escuder, B.; Miravet, J. F.; Smith, D. K. *Angew. Chemie - Int. Ed.* **2008**, 47 (42), 8002.
- (37) Cravotto, G.; Cintas, P. *Chem. Soc. Rev.* **2009**, 38 (9), 2684.
- (38) Raeburn, J.; Mendoza-Cuenca, C.; Cattoz, B. N.; Little, M. A.; Terry, A. E.; Zamith Cardoso, A.; Griffiths, P. C.; Adams, D. J. *Soft Matter* **2015**, 11 (5), 927.
- (39) Draper, E. R.; Adams, D. J. *Chem. Commun.* **2016**, 52 (52), 8196.
- (40) Nebot, V. J.; Ojeda-Flores, J. J.; Smets, J.; Fernández-Prieto, S.; Escuder, B.; Miravet, J. F. *Chem. - A Eur. J.* **2014**, 20 (44), 14465.
- (41) Segarra-Maset, M. D.; Nebot, V. J.; Miravet, J. F.; Escuder, B. *Chem. Soc. Rev.* **2013**, 42 (17), 7086.
- (42) Piepenbrock, M.-O. M.; Lloyd, G. O.; Clarke, N.; Steed, J. W. *Chem. Rev.* **2010**, 110 (4), 1960.
- (43) Zou, R.; Wang, Q.; Wu, J.; Wu, J.; Schmuck, C.; Tian, H. *Chem. Soc. Rev.* **2015**, 44 (15), 5200.
- (44) Ulijn, R. V. J. *Mater. Chem.* **2006**, 16 (23), 2217.
- (45) Díaz-Oltra, S.; Berdugo, C.; Miravet, J. F.; Escuder, B. *New J. Chem.* **2015**, 39 (5), 3785.
- (46) Colquhoun, C.; Draper, E. R.; Schweins, R.; Marcello, M.; Vadukul, D.; Serpell, L. C.; Adams, D. J. *Soft Matter* **2017**, 13 (9), 1914.
- (47) Sayar, M.; Stupp, S. I. *Phys. Rev. E* **2005**, 72 (1), 11803.
- (48) Escuder, B.; Llusar, M.; Miravet, J. F. *J. Org. Chem* **2006**, 71 (20), 7747.

- (49) Raeburn, J.; Adams, D. J. *Chem. Commun.* **2015**, 51 (25), 5170.
- (50) Nebot, V. J.; Smith, D. K. In *Functional molecular gels*; Escuder, B., Miravet, J. F., Eds.; 2013; pp 30–66.
- (51) Yu, G.; Yan, X.; Han, C.; Huang, F. *Chem. Soc. Rev.* **2013**, 42 (16), 6697.
- (52) Piepenbrock, M.-O. M.; Lloyd, G. O.; Clarke, N.; Steed, J. W. *Chem. Rev.* **2010**, 110 (4), 1960.
- (53) Draper, E. R.; Adams, D. J. *Chem* **2017**, 3 (3), 390.
- (54) Morris, K. L.; Chen, L.; Raeburn, J.; Sellick, O. R.; Cotanda, P.; Paul, A.; Griffiths, P. C.; King, S. M.; O'Reilly, R. K.; Serpell, L. C.; Adams, D. J. *Nat. Commun.* **2013**, 4:1480.
- (55) Lin, B. F.; Megley, K. A.; Viswanathan, N.; Krogstad, D. V.; Drews, L. B.; Kade, M. J.; Qian, Y.; Tirrell, M. V. *J. Mater. Chem.* **2012**, 22 (37), 19447.
- (56) Ren, C.; Song, Z.; Zheng, W.; Chen, X.; Wang, L.; Kong, D.; Yang, Z. *Chem. Commun.* **2011**, 47 (5), 1619.
- (57) Yang, R.; Peng, S.; Hughes, T. C. *Soft Matter* **2014**, 10 (13), 2188.
- (58) Roy, G.; Miravet, J. F.; Escuder, B.; Sanchez, C.; Llusar, M.; Zhao, Y.; Wang, Z.; Kim, F.; Yan, H. *J. Mater. Chem.* **2006**, 16 (19), 1817.
- (59) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. *Chem. Rev.* **2014**, 114 (4), 1973.
- (60) Escuder, B.; Rodríguez-Llansola, F.; Miravet, J. F. *New J. Chem.* **2010**, 34 (6), 1044.
- (61) Wang, S.; Xue, P.; Wang, P.; Yao, B. *New J. Chem.* **2015**, 39 (9), 6874.
- (62) Dickert, F. L.; Haunschild, A. *Adv. Mater.* **1993**, 5 (12), 887.
- (63) James, T. D.; Kawabata, H.; Ludwig, R.; Murata, K.; Shinkai, S. *Tetrahedron* **1995**, 51 (2), 555.
- (64) Vintiloiu, A.; Leroux, J.-C. *J. Control. Release* **2008**, 125 (3), 179.
- (65) Hirst, A. R.; Escuder, B.; Miravet, J. F.; Smith, D. K. *Angew. Chemie - Int. Ed.* **2008**, 47 (42), 8002.

Chapter 2

Objectives of the thesis

Chapter 2: Objectives of the thesis

The general objective of the thesis is the study and development of self-assembled low molecular weight molecules for photonic and biomedical applications.

Specific objectives are tackled in each chapter as follows:

1. Synthesis and characterization of new amino acid-derived low molecular weight families of gelators bearing fluorescent moieties (mainly 4-amino-1,8-naphthalimide, 1,8-naphthalimide and acridine) will be carried out.
2. Evaluation of the energy transfer process that takes place on reversible self-assembled molecular gel as possible mimicker of natural systems. Design of orthogonal stimuli-responsive self-assembled networks of donor-acceptor gelators. These self-assembled networks are pretended to unify in a simple way, and as a proof of concept, the function of the protein scaffolds (photoprotection, orientation and approximation) and the pigments (light absorption and energy transfer) present in photosynthetic systems in one smart material. The system to be studied differs from previously reported related cases in the fact that fibrillization of both donor and acceptor is a requisite for the light manipulation. This evidence together with the reversible nature of molecular gels could afford tunable photonic soft materials. (Chapter 3).
3. Development of a novel, versatile, stimuli-responsive gelator undergoing aggregation induced emission phenomena upon fibrillization will be studied. Formation of self-assembled gels of a 1,8-naphthalimide gelator in aqueous media is unprecedented. The fluorescence emission enhancement, ability to form films and strong gels, thixotropic behaviour and self-healing features will be investigated. Additionally, photophysical study of different compounds bearing this 1,8-naphthalimide moiety which self-assemble in different morphologies will be explored in order to point out the importance of the spatial arrangements of the 1,8-naphthalimide units in the

aggregates to explain the aggregation induced emission behaviour. Finally, the aggregation-triggered emission for potential sensing of solvent polarity and temperature will be studied (Chapter 4).

4. Miniaturization of macroscopic molecular gels into molecular nano- and microgels will be studied. Two different strategies will be explored to synthesize nanogels of 5 different compounds of different molecular nature: two derived from 4-amino-1,8-naphthalimides, two derived from 1,8-naphthalimide, and one from acridine. The stimuli-responsiveness of the molecular nano(micro) gels and its ability to load and release actives as well as act as intracellular carriers will be studied as proof of concept. Molecular nanogels may represent an interesting alternative to polymeric analogues in nanomedicine taking into account their intrinsic reversibility and stimuli-responsive nature (Chapter 5).
5. In the sixth chapter fundamental study of a new hybrid material based on the structural and energetic relationships between two areas of interest: Fluorescent molecular gels and upconverting nanoparticles (UCNPs) will be evaluated. This new system is aimed to manipulate the light reversibly as a result of an optical communication between UCNPs and the photoactive gel network via efficient excitation energy transfer. This energy transfer is expected to afford a transformation of the light emitted from the nanoparticles. Hybrid UCNPs-gel systems are envisaged to be used in applications related to IR-based sensing or light manipulation in general. Further studies will be carried out regarding the miniaturization of these hybrid materials. Tricomponent nanomaterials will be synthesized composed by molecular nanogel + photosensitizer + upconverting nanoparticles. This new nanomaterials are expected to be studied and assayed to act potentially as a photodynamic therapy agents in deep tissues, since nanogel acts as a “molecular glue” to approximate the upconverting nanoparticles and the photosensitizers. This approximation will facilitate the energy transfer between them for efficient production of singlet oxygen upon IR irradiation (Chapter 6).

6. The study of 4-amino-1,8-naphthalimide moiety in self-assembled systems as nitric oxide (NO) sensor is studied. Structure-activity relationship regarding the supramolecular organization will be studied (Chapter 7).

Figure 1. Overview of the chapters and its topics tackled on the present thesis.

Chapter 3

Light harvesting smart molecular gels derived of 1,8-naphthalimide and acridine

Chapter 3: Light harvesting smart molecular gels derived from 1,8-naphthalimide and acridine

3.1 Motivation

Since many years ago, curious people have been surprised by the Nature as a magnificent problem-solver. Millions of years of evolution changed the world and the world changed the nature by the survival of the fittest.¹ Primary organisms developed solutions to transform CO₂ into glucose using sunlight and a reducing agent or source of electrons, first by using reducing agents as H₂ or H₂S (abundant in the ancient atmosphere), and then, approximately 2400 million years ago, by using H₂O. This fascinating compendium of processes is what we call photosynthesis. Derived from these complex chemical reactions, O₂ was created as reaction subproduct and these phenomena changed the world drastically, forcing the whole Earth to evolve to support a highly oxidizing atmosphere.²

In the photosynthetic process,³ the affordance of chemical energy from sunlight takes place on thylakoid membranes inside the chloroplasts (Figure 1) where the photosystems I and II perform the leading role. These photosystems are large **self-assembled and auto-organized** protein flocks responsible for carefully orchestrate the delicate photophysical processes and chemical reactions that take place on the photosynthetic process.^{4,5}

Figure 1. Left: TEM image of chloroplast from *Nicotiana tabacum* (thylakoid membrane highlighted).⁶ Right: Spatial distribution of the complexes inside the membrane.⁷

The photosystems can be divided into three key players: Protein backbone assemblies, light harvesting pigments and reaction centres.

The first step on the photosynthetic process is the absorption of light that is able to energize photosynthetic pigments and promote electrons to higher energy levels. It is important to say that only the electronic promotions that take place on the reaction centre are efficient to produce chemical bonds from light energy. Of course, Nature does not rely only on the probability of a photon to reach the tiny central chlorophyll pigment on the reaction centre, it has developed an antenna network and an electron transfer chain that provides **energy funnelling** towards the reaction centre via **excitation energy transfer**⁸ (EET), where is captured to create activated electrons and initiate the chain reaction. To maximize this absorption-transfer process, Nature introduced via self-assembly on the surface of the plant leaves highly absorbing-molecules (pigments) on key positions surrounding the reaction centre. These pigments are classified by chemical nature on three big groups (Figure 2).⁹

Chlorophylls: Composed of a magnesium cation on the center of a chlorin ring (large heterocyclic aromatic ring composed by 3 pyrroles and 1 pyrroline) and different side chains. The absorption of this kind of molecules is on the blue and red regions of the visible spectrum.

Phycobilins: Composed by opened tetrapyrrole chain usually bonded to water-soluble proteins (phycobiliproteins). They are efficient absorbers on the red, yellow and green portion of visible spectra.

Carotenoids: They are mainly tetraterpenoids (40 carbon atoms) formed by a polyene hydrocarbon chain terminated by rings that may contain oxygen (called xanthophylls) or not (called carotenes). They have efficient absorption on wavelengths ranging from violet to green light.

Once the molecular excitation has taken place, it is mandatory to be transferred to the reaction centres via excitation energy transfer (EET). At this stage is where the protein assemblies come to play, they are in charge of closely approximate the pigments (donor-acceptor molecules) and dispose them with high density distribution and the adequate orientation in order to maximize EET and minimize self-quenching.^{7,10} Besides, the protein

assemblies play a crucial role on the protection of the whole system from the photodamage produced by reactive oxygen species (ROS) when high light conditions are present. The photosynthetic organisms have developed several mechanisms to do so and incorporated stress sensors in the smart matrix, which induce self-quenching. In most cases the stress sensors work because they undergo protonation of ionisable moieties. The pH in the lumen decreases in the presence of ROS, the matrix stress sensors protonate and conformational changes are induced to produce pigment-pigment self-quenching (non-photochemical quenching (NPQ) mechanisms). Other protective mechanism consists on photoisomerization of sensible bonds and its corresponding conformational change, that again produces quenching on the pigment energy transfer efficiency.^{11,12}

Figure 2. Photosynthetic pigments: Chlorophylls, bacteriochlorophylls phycobilins and carotenoids. **a)** Substituted tetrapyrroles, general structure of Chlorophylls. **b)** Representative structure of phycobilins and carotenoids. **c)** Normalized absorption spectra of representative photosynthetic pigments. **d)** Normalized absorption spectra of representative phycobilins and carotenoids.⁹

The next step happens on the reaction centre (Figure 3) where a chlorophyll molecule receive this energy and one of its electrons is promoted to a higher energy level. This energized electron jumps through several other pigmented molecules, until reaches the plastoquinone B which is released from the photosystem and it delivers its electrons to the next link in the electron-transfer chain. Finally, the reaction centre has to replace the promoted electron and it takes it from a water molecule, after this, the reaction centre is ready to absorb another photon and start the process again.

Figure 3. **Left** and **right** figure: Photosystem II and Photosystem I, reaction centres indicated by arrows, other pigments as carotenoids and chlorophylls are represented on orange and green respectively, triangular assemblies correspond to light harvesting proteins. Pictures adapted from PDB source.¹³ **Central** image: High-resolution AFM topographs of the supramolecular assembly of the photosynthetic complexes in *Rsp. Photometricum*.¹⁴ Example of energy funnelling through the central chlorophyll is depicted.

Several approaches have been made so far with notable success for mimicking the natural light-harvesting mechanism^{7,15,16} and to convert sunlight into electric energy.^{17,18} Nevertheless the economic factor and the versatility of photosynthetic organisms to self-heal and reproduce themselves is still beating the artificial devices.

The first chapter of the present thesis gets inspired by the photosynthetic process and takes a little insight on the energy transfer processes that occurs on reversible self-assembled molecular gels as possible mimickers of natural systems. The idea is to build orthogonal stimuli-responsive self-assembled networks from donor-acceptor gelators. This approach unifies in a simple way, and as a proof of concept, the function of the protein scaffolds (photoprotection, orientation and approximation) and the pigments (light absorption and energy transfer) in one smart material.

3.2 Introduction

As we mentioned above, EET plays a crucial role in photosynthesis. In photosynthetic centres, the proper spatial disposition of donor and acceptor units provokes energy funnelling toward the reaction centre. Inspired by the natural systems, there is a growing interest in the development of materials for the capture and storage of solar energy. Such materials are constituted by the appropriate arrangement of energy donors and acceptors.^{19–21} For this

purpose, supramolecular assemblies have been studied extensively.^{22,23} Photoinduced energy transfer processes are at the front-line of research, provided their potential applications in diverse cutting-edge fields ranging from optoelectronics to tailored nanomedicine.^{24–29}

3.2.1 Energy transfer mechanisms

The energy transfer has been extensively studied since late 1940s.³⁰ Several types of spin-allowed excitation energy-transfer processes are recognized. i) Singlet-singlet energy transfer (SSET), an electronically excited singlet donor produces an electronically excited singlet acceptor. ii) Triplet-triplet energy transfer (TTET), a triplet excited donor produces triplet species. iii) Triplet-triplet annihilation (TTA), two triplet excited donors are converted into two singlets. In any of these processes the donor and acceptor can exchange energy by fluorescence (or Förster) resonance energy transfer (FRET), by electron exchange (Dexter mechanism) or both.

Figure 4. Different excitation energy transfer processes

3.2.1.1 Förster resonance energy transfer

FRET is an electrostatically mediated energy transfer, whereby long-distance dipole-dipole interactions between the fluorescent donor and the fluorescent or nonfluorescent acceptor decrease the emission intensity of the former by transferring the energy to the latter.^{31–33}

According to the Förster theory, the energy transfer efficiency (Φ_{ET}) is determined by the following expression:

$$\Phi_{ET} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

where R_0 is the Förster distance (where the transfer efficiency is 50%) and r is the distance between chromophores. R_0 is calculated as:

$$R_0^6 = \frac{9000(\ln 10)\kappa^2 Q_D}{128\pi^5 N n^4} \int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda$$

Where n is the refraction index of the medium, N is the Avogadro's number, κ^2 (describes the transition dipole orientation and equals 2/3 for a random distribution of interacting dipoles), Q_D is the quantum yield of the donor and $\int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda$ is the overlapping integral that can be calculated from the emission and absorption spectra of donor and acceptor respectively. The term λ corresponds to the wavelength of light (nm), $\varepsilon_A(\lambda)$ is the molar absorptivity of the acceptor at that wavelength ($M^{-1}\cdot cm^{-1}$) and $F_D(\lambda)$ is the normalized donor fluorescence spectrum. Practically, the Φ_{ET} can be calculated as a ratio of the fluorescence intensities or of the fluorescence lifetimes of the donor in the absence and presence of the acceptor (F_D and F_{DA} or τ_D and τ_{DA} , respectively):

$$\Phi_{ET} = 1 - \frac{F_{DA}}{F_D} = 1 - \frac{\tau_{DA}}{\tau_D}$$

This strong distance dependence makes this energy transfer process very useful to measure short distances between chromophores (10–100 Å). Moreover, it has been demonstrated as a very powerful tool as “molecular ruler” in immunoassay kits in the biochemical and biomedical field.^{34–36}

3.2.1.2 Electron exchange (dexter interaction)

Dexter interaction, is short-range mechanism occurring at very low distances ($<10 \text{ \AA}$).³⁷ This energy transfer involves the movement and exchange of an electron from the LUMO of electronically excited donor (D^*) to the LUMO of acceptor (A) simultaneously with the movement of an electron between the HOMOs of A to D^* . The rate of energy transfer, k_{dexter} , is determined by the following equation:

$$k_{\text{dexter}} = \frac{2\pi}{\hbar} KJ(\lambda) \exp\left(\frac{-2r_{\text{DA}}}{L}\right)$$

Where K is an empirical factor related to specific orbital interactions, $J(\lambda)$ is the integral of normalized spectral overlap of the donor emission and acceptor absorption, r_{DA} is donor/acceptor separation, and L is the sum of the van der Waals radii of the donor and acceptor. In conclusion, Dexter energy transfer rate depends on spectral overlap, but it decays with donor–acceptor separation exponentially. As a result, Dexter interactions are limited to short distances ($<10 \text{ \AA}$).

3.2.2 Self-sorted systems vs co-aggregated systems

The preparation of multicomponent molecular gels expands tremendously their tunable properties.^{38–40} Specially interesting is the case of gels composed of orthogonal fibrillary networks as a result of a self-sorting process (Figure 5).

Figure 5. Cartoon showing (a) self-sorting, (b) social co-assembly and (c) random co-assembly of two different gelators. The primary fibres formed in (a) can then further assemble in many ways. Here, we show two hypothetical ways in which self-sorted fibres (red and blue) could interact. (d) Intimate wrapping; (e) interactions only occur at a small number of specific points.⁴¹

For example, Smith et al. have shown self-sorting of molecular gelators in several examples.^{42–}

⁴⁴ Noticeably, self-sorting was reported by Adams et al. in aqueous environment by means of a controlled pH change of the system.^{39,45} Related to this, self-sorting between molecular gelators (1,3,5-cyclohexyltricarboxamide derived gelators) and surfactants (DDOA and DOPC, DODAB) was analyzed by Brizard and coworkers demonstrating orthogonal self-assembly of hydro-gelators and surfactants in order to produce a new sort of nanostructures (self-assembling interpenetrating networks and “gellosomes”) which are unattainable by the self-assembly of a single component. This new much more complex architectures show new features which arise from the interactions between self-assembled objects rather than specific intermolecular interactions, giving the first step for (in a very preliminary way) bio-mimicking the complexity and functionality of natural systems (Figure 6).^{46–48}

Figure 6. Left: Schematic representation of hydrogelators based on 1,3,5-cyclohexyltricarboxamide, which self-assemble. Representation of the orthogonal self-assembly strategy using hydrogelators and surfactants to create more complex and compartmentalized structures are also shown. Right: Cryo-TEM images of unilamellar **a)** DOPC vesicles coexisting with a network of well-defined fibers of molecule 1; **b)** DODAB vesicles with thicker fibers of 3; **c)** and **d)** DOPC vesicles deformed by the growth of fibers of 1 directly contained in their aqueous compartment.⁴⁸

One step forward has been described recently in separate reports from Smith et al. and Adams et al., in which they describe orthogonal gels which can assemble/disassemble selectively by the application of different external stimuli. The presence of photoswitchable groups in the

low molecular weight gelator structure allows to achieve photopatterned gels from orthogonal two-component aqueous systems (Figure 7).^{49,50}

Figure 7. Schematic of assembly process. Spatially resolved multicomponent gels are formed by a three-stage process. A slow pH decrease leads to a sequential assembly and a self-sorted gel. One of the fibrous network is then removed by irradiation with light.⁴⁹

The use of two component fibrillar networks has also applications in organocatalysis; recently Singh and co-workers described a two component self-sorted fibrillar network containing mutually incompatible proline and carboxylic catalytic groups. The formation of this two separate networks allowed to spatially separate these groups acting as an enzyme-like microreactor, allowing one-pot tandem reactions catalyzed by basic and acidic moieties for deacetalization and subsequent aldol reaction.⁵¹ It can be noted that the control over the orthogonal assembly of multi-component gelators expands enormously their applications and gives improved versatility to an already very versatile system by itself.

3.2.3 Energy transfer on synthetic self-assembled systems.

A number of reports have described the incorporation of photonic functionalities to molecular gels.⁵² Several examples of gelators with chromophore units have been used as photocatalysts by Stupp et al. for hydrogen production.^{53,54} In those systems the gelator matrix is composed by self-assembled perylene monoimide ribbons acting as a light harvesting system that

incorporates in it a nickel organocatalyst and ascorbic acid (acting as proton source and sacrificial electron donor) in a close spatial disposition to achieve efficient hydrogen production (Figure 8).

Figure 8. Left: H₂ evolution experiments. Right: Scheme of gel formed by perylene monoimide (PMI) chromophore amphiphile (inset) showing nanoribbons (in red) that trap the catalyst and ascorbic acid for photoinduced hydrogen production (Image adapted from ref. 53).

In other example, energy transfer has been reported in single supramolecular nanofibers presenting large coherent long-range transport making this systems promising candidates for efficient solar energy transport and conversion (Figure 9).⁵⁵

Figure 9. Left: Schematic long-range energy transport along single supramolecular nanofibres. **a)** Widefield photoluminescence image. **b)** Photoluminescence image of the nanofibre from the boxed region upon confocal excitation of its bottom left end (filled green circle), demonstrating highly efficient energy transport over 4 μm. Right: Chemical structure of fibres, carbonyl-bridged triarylamine in green (CBT) core; amide moieties in blue; 4-(5-hexyl-2,29-bithiophene)-naphthalimide (NIBT) periphery in yellow. (image adapted from ref. 37)

Energy transfer has also been studied in molecular gels formed by photoactive fibres which contained entrapped dyes. In particular, the emission of white light using a cascade of energy transfer processes in molecular gels has received special attention.⁵⁶ For this purpose

Desvergne and Del Guerso nicely described self-assembled [n]-acene fibers doped with tetracene derivatives (Figure 10).^{57–59}

Figure 10. Fluorescence confocal microscopy image of a WL-emitting gel (8+9+10).⁵⁹

Oligo(p-phenylenevinylenes) and related compounds have been also used by Ajayaghosh et al. for EET modulation and tuneable fluorescence emission (Figure 11).^{60–63}

Figure 11. (a) Molecular structure of energy transfer donor (OPV6) and acceptor (OPV7), (b) emission spectra ($\lambda_{\text{ex}} = 380$ nm) and corresponding fluorescence microscopy images of OPV6 in the presence of 0, 2, and 20 mol% OPV7, and (c) schematic representation of the FRET process within the coassemblies of OPV6 (yellow) and OPV7 (red) under different donor–acceptor compositions. (From ref. 42).

The adsorption of dyes in molecular gel fibres has been also exploited to prepare photon upconversion matrices.⁶⁴ In a different approach, photoactive components are mixed in the gel fibers, (non-orthogonal fibrillization) producing efficient EET.^{65–68} In a representative work, Huang et al. designed and synthesized three gelators with different emissions (red, green and blue) that presented efficient energy transfer when gelation upon self-assembly occurs. They also demonstrated that the ET effect played a crucial role in providing the tuneable colours in mixed gel states (Figure 12).

Figure 12. Normalized fluorescent spectra and colours of gels in n-octanol under excitation with 365 nm light.⁶⁵

Only a few reports have addressed the preparation of self-sorted photoactive fibres, and all of them are aimed to develop photoinduced electron transfer. For this purpose, electron deficient (n-type) and electron rich (p-type) units are present in the gelators (Figure 13).^{69–75}

Figure 13. Possible interactions between p-type donor and n-type acceptor leading to different possible hierarchical structures.⁷¹

3.3 Light harvesting two component orthogonal gels

Distinctly from the precedents cited above, here, a new photoactive multicomponent material comprised by two separate self-assembled fibers showing efficient and stimuli responsive EET is presented. Tuneability is linked to the selective disassembly of one of the orthogonal fibrillar networks. Moreover, the system exhibits a notable Stokes-Shift of *ca.* 200 nm, taking into account the excitation and emission wavelength maxima of the two component material: 350 and 540 nm, respectively.

Figure 14. Left: Graphical summary of system under study. Right: Molecular structure of designed gelators derived from 4-amino-1,8-naphthalimide and acridine.

3.3.1 System design

Two different gelators were designed taking into account their capability to self-sort into orthogonal fibrillar networks because of their distinct chemical nature. One of them (**3.1** or **1**) contains an aromatic-peptide architecture. A priori, this architecture is known to be a good candidate for hydrogelator synthesis considering literature precedents. Simple aminoacid or dipeptide derivatives containing a hydrophobic aromatic unit such as, for example, Fmoc or naphthyl are described to be good supramolecular hydrogelators.⁷⁶ The other compound (**3.2** or **2**) is a bolaamphiphilic molecule derived from valine, related to gelators widely studied in our group.^{77–82} Both compounds were also designed for containing complementary

chromophores (1,8-naphthalimide and acridine) which met the required spectral overlap for the occurrence of Förster's Resonance Energy Transfer (FRET).

The complex system under study is composed by two new photoactive gelators. They are selected because they represent a suitable donor-acceptor pair for energy transfer processes and orthogonal fibrillization is presumed due structural difference between them. The introduction of these two chromophores, 1,8-naphthalimide and acridine, was synthetically simple via commercial products (Figure 15).

Figure 15. Commercial compounds for introducing the complementary fluorophores. 4-nitro-1,8-naphthalic anhydride (**left**) and acridine-9-carboxylic acid (**right**).

3.3.2 Gelator synthesis

An extensive library of compounds was synthesized in order to select the gelator that suited our requirements. Synthesized compounds can be organized in three different groups derived from 1,8-naphthalimide moiety: i) Glycine or non amino acid derivatives; ii) valine derivatives; iii) glycine-valine derivatives and one derived from acridine. The preparation of 4-amino-1,8-naphthalimide derivatives included conventional peptide synthesis, using sequentially amino acid activation with N-hydroxysuccinimide and DCC in THF, activation of amino acid and amine coupling in THF, hydrogenolysis with Pd/C catalyst in CH₃OH, introduction of 4-nitro-1,8-naphthalic anhydride in the N-terminal group and final reduction step of the nitro- moiety to amino- group again by hydrogenolysis with Pd/C catalyst in CH₃OH. For acridine derivatives, introduction of acridine was achieved starting by acridine-9-carboxylic acid activation by oxalyl chloride and DMF (cat.) to obtain acridine-9-carbonyl chloride. Finally the addition of the corresponding diamine over the activated acid chloride in THF and Et₃N yield the desired compounds. Further synthetic information can be found in synthesis section 3.5.1.

3.3.2.1 Glycine or non amino acid compounds

Figure 16. General structure of 4-amino-1,8-naphthalimide glycine or non amino acid derivatives.

Different 4-amino-1,8-naphthalimide derivatives were synthesized containing only one glycine amino acid residue (Figure 16) with a carboxylic acid ending (compound **3.13**) or a methoxyethyl ester (compound **3.11**). Other compounds were easily synthesized bearing an aliphatic chain (compound **3.12** and **3.14**). All of these compounds showed poor ability to gelate in organic solvents or water. For example, compound **3.14** tends to form gels in cyclohexane, hexane or toluene with m.g.c of 17, 34 and 79 mM, respectively, and nanoparticles in aqueous solvent containing 10% DMSO at 10 μ M (see chapter 7). Compound **3.13** showed good solubility in basic water but non-directional aggregation in acidic water forming amorphous aggregates (Figure 17).

Figure 17. Glycine and no amino acid compounds bearing 4-amino-1,8-naphthalimide moiety.

3.3.2.2 Valine derivatives

Figure 18. General structure of 4-amino-1,8-naphthalimide-valine derivatives.

The valine moiety is known to be a good gelator amino acid because it provides good hydrophobic/hydrophilic balance and aggregation packing (Figure 18).⁸³ For this reason, the gelation ability of 4-amino-1,8-naphthalimide-valine derivatives has been investigated in several compounds. Compounds containing different tails such as aliphatic or cyclic chains (compounds **3.19**, **3.20** and **3.21**), methoxyethyl ester or methoxyethyl amide (compounds **3.15**, **3.18**), ionisable moieties such as carboxylic acid or alcohol groups (compounds **3.17** and **3.16**) have been synthesized (Figure 19). Unfortunately these molecules showed very poor solubility and null ability to form fibrillary aggregates. Consequently none of the compounds tested formed gels at concentrations below 30 mM (determined by the tube inversion method). The presence of valine amino acid is thought to confer too much rigidity, preventing the molecule to adopt the correct disposition for 1D growth in fibrillar structures and, consequently, precipitating in amorphous aggregates upon cooling down to room temperature.

Figure 19. Valine compounds bearing 4-amino-1,8-naphthalimide moiety.

3.3.2.3 Glycine-valine derivatives

Figure 20. General structure of 4-amino-1,8-naphthalimide-valine-glycine derivatives

The inability of the non-aminoacidic and valine derivatives to form gels in several solvents lead us to expand the peptide chain by adding a glycine derivative to confer structural flexibility and improve the possible packing. Moreover it also adds extra hydrogen bonding donor/acceptor moieties (Figure 20). Again, different tails were added to the fluorophore-Gly-Val core by amide or ester bond link (Figure 21). Finally, these compounds showed good gelating behaviour, both in organic and aqueous solutions ($\text{H}_2\text{O}/\text{DMSO}$), by heating-cooling method. However, only compound **3.26** formed a pure hydrogel by pH shift method at 30 mM. The best gelating performance in all tested solvents was achieved by compound **3.1**, reaching the “supergelator” status with a mcg of 1.4 mM in CH_3CN .

Figure 21. Glycine-valine derivatives bearing 4-amino-1,8-naphthalimide moiety.

3.3.2.4 Acridine derivatives

Bolaamphiphilic acridine derivatives (Figure 22) were synthesized in the laboratory by Dr. Santiago Díaz-Oltra, showing good gelating abilities in CH₃CN. For example, compound **3.2** presented a mgc of 2.9 mM in CH₃CN which was the selected solvent for further studies.

Figure 22. Bolaamphiphilic valine compounds bearing bis-acridine moieties.

3.3.3 Characterization

The aggregation process of the selected compounds was investigated by several techniques, firstly individually and then when forming part of a complex system.

3.3.3.1 4-amino-1,8-Naphthalimide derivative (3.1)

- *Minimum gelator concentration*

Compound **1** was selected as a component of the smart light harvesting material because of its ability to form stable optically transparent/translucent gels in several solvents at very low concentration (compound **1** is a supragelator (<1% w/w) in CH₃CN; table 1).

Table 1: Minimum gelator concentration (mgc) values for **1** in different solvents calculated by the tube inversion method

Solvent	mgc / mM	Aspect
CH ₃ OH	15	Translucent
Toluene	24	Transparent
CH ₃ CN	1.4	Translucent
H ₂ O/DMSO (6:1)	3.6	Opaque

- Absorption and emission spectroscopy*

Besides, the 4-amino-1,8-naphthalimide moiety present in **1** is widely used in photonic systems because its stability and high fluorescence quantum yield. Absorption and fluorescence spectra are shown in figure 23. It must be noted that the long wavelength emission of 4-amino-1,8-naphthalimide chromophore is due to an internal pull-push charge transfer process (ICT) which would be favored under solvating conditions.⁸⁴

Figure 23. UV-Vis and emission spectra of diluted samples of compound **1** in CH₃CN (λ_{ex} = 360 nm).

UV-vis technique has demonstrated to be a very powerful tool to observe and follow aggregation processes. The energy levels of the chromophores are strongly modified by the environment that surrounds them. Solvent changes, the presence of interacting molecules or aggregation phenomena have an observable effect on the UV-Vis spectra by shifting or displacing the absorption maximum peaks. For example, aggregation of compound **1** can be followed since a notable peak shift ($\Delta\lambda_{\text{abs}} = 17 \text{ nm}$) and a shoulder appearance at $\lambda = 475 \text{ nm}$ is observed upon increasing concentration from its soluble state at 0.02 mg/mL ($\lambda_{\text{abs}} = 419 \text{ nm}$) to its aggregated state ($\lambda_{\text{abs}} = 436 \text{ nm}$) in CH_3CN (Figure 24).

Figure 24. UV-Vis spectra of compound **1** at different concentrations at 25°C . Measured in 1 mm pathlength cuvette in CH_3CN ; $[\mathbf{1}] = 0.02, 0.1, 0.4$ and 0.6 mg/mL .

This bathochromic shift of absorption maximum could be ascribable to J-aggregates. The formation of J-aggregates implies bathochromic shifts and special “head to tail” one dimensional organization. For this kind of aggregates three head to tail spatial dispositions could occur: i) ladder-type ii) staircase-type and iii) brickwork type.^{85,86} In this case, no further information about the particular organisation of the aggregates of **1** can be investigated since the aggregates showed poor crystallinity.

Further insight on the aggregation behaviour can be investigated considering that molecular gels are stimuli-responsive. Aggregation/disaggregation process can be controlled with temperature decrease/increase (Figure 25). For example, when temperature is lowered, peak shift from 424 nm to 436 nm is observed, as well as hypochromism, which is also detected at 259 nm . This hypochromic behaviour is similar to the aggregation process experienced by the DNA biomacromolecule.⁸⁷

Figure 25. UV-Vis spectra of compound **1** (0.6 mg/ml) at different temperatures. Measured in 1 mm path length quartz cuvette in CH_3CN .

The aggregation process also can be followed by fluorescence spectroscopy. In this case, the fluorescence of the chromophore is quenched by aggregation. This phenomenon is known as aggregation caused quenching (ACQ),⁸⁸ in which the fluorescence is quenched due to the increased efficiency of non-radiative relaxation pathways (further discussion on this topic will be carried out in chapter 4). As can be seen on Figure 26 and Figure 27, the fluorescence of **1** in DMSO, where the molecule is in solution, shows $\lambda_{\text{em max}} = 532 \text{ nm}$. As H_2O is added to the system, aggregation takes place and the fluorescence is quenched significantly, while $\lambda_{\text{em max}}$ shifts to 539.5 nm. Similar effects have been described in the literature. For instance, Zhong et al. report a derivative of 4-amino-1,8-naphthalimide which displays very low emissive properties when encapsulated in the hydrophobic cavity of a cyclodextrin. However, the emission of such a compound is boosted upon decomplexation and exposure to the solvating polar environment.⁸⁹

Figure 26. Fluorescence spectra of compound **1** (0.17 mg/ml) at different water content in DMSO. Measured in 1 cm path length cuvette at 25 °C.

Figure 27. Normalized fluorescence emission spectra of **1** (0.17 mg/ml) in DMSO/H₂O mixtures at 25 °C, λ_{ex} = 420 nm λ_{em} = 536 nm for **1**. Measured in suprasil quartz triangular cuvette. 85 μ L of stock solution in DMSO is diluted with the corresponding amount of DMSO and then, with water. Final V = 500 μ L

An analogue behavior is observed when temperature is increased from 25 to 70 °C, producing the progressive disassembly of the fibrillar network (see Figure 28). It has to be noted that the disassembly of the gel formed by **1** does not result in a shift of the maximum emission wavelength upon going from 25 to 75 °C. This effect is explained taking into account that the emission of the gel at 25 °C predominantly comes from the small fraction of free, non-aggregated molecules that coexist with the gel fibers, as a result of the above-mentioned aggregation induced emission quenching experienced by **1**. This phenomenon is counterintuitive since typically fluorescence undergoes quenching when temperature increases because an increase of collisional and rotational relaxation pathways.⁹⁰ However, the ACQ upon aggregation overcomes this effect, and molecules released from the fibres are much more emissive than in the self-assembled state.

Figure 28. Fluorescence spectra of compound **1** (0.17 mg/ml) at different temperatures in CH₃CN. Measured in 1cm path length cuvette.

- *Computational aggregation models*

Tentative models for the self-assembly of the molecular gelator **1** are proposed in Figure 29. Based on our experience and the literature on related molecules,^{78,91,92} hydrogen bonding and π - π interactions are proposed to be the driving force for 1D sorting and fibre formation.

Figure 29. Left: Possible hydrogen bonding scaffold for aggregation of **1**. Right: Energy minimized molecular mechanics models (MACROMODEL, AMBER force field) of stacks of compounds **1**. Dotted lines indicate close contacts due to hydrogen bonding or aromatic π - π stacking.

- *Electron microscopy*

Morphology of the fibrillar network of **1** has been investigated by TEM (Figure 30) and SEM (Figure 31) revealing nanofibrils of *ca.* 10 ± 2 nm width that come together to form larger fibrillar bundles. Several twisted chiral fibres (left handed) also have been detected, indicating the transference of the molecular quirkality of **1** to the supramolecular level.

Figure 30. Transmission electron microscopy of compound **1** xerogels in CH₃CN.

Figure 31. Scanning electron microscopy of compound **1** xerogels in CH₃CN.

- *Confocal microscopy*

As we mentioned above, fluorescence is a powerful tool to describe this kind of materials, for this reason confocal laser scanning microscopy (CLSM) is an ideal technique because it combines fluorescence with microscopy; however it has some optical resolution limitations

(diffraction resolution 200-250 nm).⁹³ CLSM images of gels of **1** (2.44 mM) in CH₃CN are presented in Figure 32. Several fibre bundles can be observed (white arrows) in the periphery of the bulk gel material in the yellow light. Differently from the EM techniques, the confocal fluorescence microscopy does not require any drying or manipulation of the samples: the gels are placed on a glass slide and observed directly without distorting the original macroscopic architecture. The gel structure can be clearly distinguished from the solvent wells and background because of the very poor solubility of compound **1** in CH₃CN (see NMR solubility calculation below). Microspectroscopy can also be performed in order to see the fluorescence spectra of the area of interest presenting $\lambda_{em\ max} \approx 550\ nm$ (Figure 33).

Figure 32. Confocal laser images of compound **1** gels in CH₃CN.

Figure 33. Confocal laser emission microspectroscopy of compound **1** gels in CH_3CN .

- Solubility and thermodynamic parameters (NMR)*

NMR experiments were carried out in order to assess the thermodynamic values of gel formation of **1** in CH_3CN and its solubility at different temperatures. NMR experiments consist on introducing an internal standard with known concentration in a concentric NMR tube and performing several NMR spectra at different temperatures (Figure 34 left). By this procedure it is possible to calculate gelator concentration and its solubility constants (K_s) (Figure 34 right).⁹⁴

Figure 34. Left: NMR spectrum of **1** (1.7 mM) in CD_3CN at different temperatures. Right: K_s evolution with the temperature calculated from NMR spectroscopy.

The following solubility equilibrium is considered, being K_s the solubility constant.

$$K_s = [\mathbf{1}]$$

Using the linear form of Van't Hoff equation (eq. 1) and considering that the solubilisation enthalpy is constant on the working temperature range, the thermodynamic values of the gel-sol transition can be calculated by plotting $\ln K_s$ vs $1/T$. ΔH and ΔS can be determined from linear slope and intercept respectively.

$$\ln K_s = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \text{ (eq. 1)}$$

Table 2: Thermodynamic values of solubilisation process of **1** in CH_3CN

Compound	Solubility 30°C / mM	ΔH / $\text{kJ}\cdot\text{mol}^{-1}$	ΔS / $\text{J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$
1	0.13	17.3 ± 0.2	40.0 ± 0.7

Obviously, the solubilisation process is an endothermic process and has a positive enthalpy variation, which indicates that energy has to be provided to the system to solubilise it. The entropy change is also positive which means that the solubilised molecules present more molecular disorder (presence of more degrees of freedom associated to molecular motions and rotations) that when forming self-assembled fibres.

3.3.3.2 Acridine derivative (2)

- *Minimum gel concentration, UV-vis and emission spectroscopy*

The acridine unit introduced in compound **2** is a fluorophore with elevated emission quantum yields used for a variety of applications.⁹⁵ It is a good candidate as energy donor because it has absorption band at 360 nm and emits in the 400-500 nm region (Figure 35), where the acceptor **1** absorbs, and also forms translucent gels in CH_3CN with a mgc of 2.9 mM.

Figure 35. Absorption and emission spectra of **2** in CH_3CN ($\lambda_{\text{ex}} = 360 \text{ nm}$).

- pH-responsive behaviour

Moreover, the acridine derivative has a pK_a of *ca.* 3.5 in aqueous media and can be easily protonated by addition of a weak acid (i.e. CH_3COOH) in CH_3CN . This feature confers reversible stimuli-responsiveness because when it is on the protonated state, the gel disassembles and changes its colour from white to yellow, which can be followed by the increase in absorption band from 400 to 475 nm (Figure 36a). Fluorescence emission was also notably modified upon acridine protonation since big bathochromic shift on emission peak was noticed from 420 (blue emission) to 490 nm (green emission) (Figure 36b).

Figure 36a. UV-Vis spectra of a gel of **2** (1 mg/ml, 1.46 mM) in CH_3CN upon addition of increasing amounts of glacial acetic acid. $\Delta\text{Vol} = 10 \mu\text{l}$; $[\text{AcOH}] = 0.6$ to 3.9 M .

Figure 36b. Normalized fluorescence spectra of a gel of **2** (2 mg/ml, 2.92 mM) before and after addition of 50 μ L of glacial acetic acid 17 M (λ_{exc} = 360 nm). Inset: Pictures under UV light (λ_{exc} = 365 nm) before (gel) and after (solution) H^+ addition.

- *Computational aggregation models*

Tentative models for the self-assembly of the molecular gelator **2** are proposed in Figure 37. Based on our experience and the literature describing related molecules, hydrogen bonding and π - π stacking interactions are proposed, as in the case of **1**, to be the driving force of 1D sorting and fibre formation.

Figure 37. **Right:** Possible hydrogen bonding scaffold for aggregation of **1**. **Left:** Energy minimized molecular mechanics models (MACROMODEL, AMBER force field) of stacks of compounds **2**. Dotted lines indicate close contacts due to hydrogen bonding or aromatic stacking.

- *Electron microscopy*

Morphology of the fibrillar network of **2** was investigated by transmission electron microscopy and scanning electron microscopy (Figure 38 and 39) and revealed that is similar to **1** but presenting wider fibres that **1**, of *ca.* 35 ± 13 nm.

Figure 38. Transmission electron microscopy of xerogels of **2** from CH₃CN.

Figure 39. Scanning electron microscopy of xerogels of **2** from CH₃CN.

- *Confocal laser microspectroscopy*

Fibrillar networks of **2** have also been observed by CLSM, revealing blue-emitting fibre boundles. Microspectroscopy showed that $\lambda_{em\ max}$ of the acridine gel is at 455 nm (Figure 40).

Figure 40. Confocal laser microspectroscopy of compound **2** gels in CH₃CN.

- *Solubility and thermodynamic parameters (NMR)*

Solubility of acridine derivative **2** was also determined in CH₃CN by the procedure described above for compound **1** (section 3.3.3.1). Based on the adquisition of ¹H NMR spectra at different temperatures, determination of the solubility constant (K_s) is performed.

Analogously, from the corresponding Van't Hoff plot (eq. 1) the determination of thermodynamic parameters ΔH and ΔS were carried out for the gel-sol transition (Figure 41).

Figure 41. Left: NMR spectrum of **2** (2.27 mM) in CH_3CN at different temperatures. Right: K_s evolution with the temperature calculated from NMR spectroscopy

Regarding the thermodynamic parameters, it has to be highlighted that while ΔH values are similar to those for the solubilization process of **1**, the solubility of **2** is 5 times higher than in the case of **1** at 30 °C. As we will see later, because of this behaviour it is possible to disrupt selectively the network of **2** by temperature increase without disturbing appreciably the fibrillar network of **1**.

Table 3: Thermodynamic values of solubilisation process of **2** in CH_3CN

Compound	Solubility 30°C / mM	ΔH / $\text{kJ}\cdot\text{mol}^{-1}$	ΔS / $\text{J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$
2	0.38	25.8 ± 0.3	29.8 ± 0.7

3.3.3.3 Orthogonal self-sorted bicomponent system formed by **1** and **2**

A complex system was prepared by mixing donor (**2**) and acceptor (**1**) gelators (Figure 42). This mixture was designed taking into account their capability to self-sort into orthogonal fibrillar networks and the required spectral overlap for the occurrence of Förster's Resonance Energy Transfer (FRET).^{32,96,97}

Figure 42. Structure of the studied compounds and overlap of their absorption and emission spectra in CH_3CN . Inset: picture of a molecular gel of compound **1** under UV light. ($\lambda_{\text{exc}} = 360 \text{ nm}$ for **1** and **2**)

The spectral overlap integral is calculated⁹⁸ to be $J(\lambda) = 1.55 \times 10^{-14} \text{ M}^{-1} \cdot \text{cm}^{-1} \cdot \text{nm}^4$, which predicts a Förster's distance $R_0 = 21 \text{ Å}$ for a Coulombic mechanism of energy transfer.

In order to determine the energy transfer efficiency of the studied system, theoretical parameters were calculated. The Förster distance R_0 is defined as the distance where the transfer efficiency is 50%. The energy transfer efficiency between 2 chromophores is calculated by means of next equation:

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \quad (\text{eq. 2})$$

E = FRET efficiency

R_0 = Förster distance

r = Distance between chromophores

For the Förster distance calculation (eq. 4) is necessary to know the value of integral overlap between donor emission spectrum and acceptor absorption spectrum $J(\lambda)$. $J(\lambda)$ is calculated using equation 3 and acceptor and donor absorption and emission spectra. Donor emission spectrum has to be integrated prior to area calculation (A). After that the original spectra has to be corrected by a constant factor $(A)^{-1}$ to obtain $F_D(\lambda)$. Moreover the molar extinction coefficient of the acceptor for the λ relevant range extracted from the absorption acceptor spectrum. Finally, according to equation 4, knowing $J(\lambda)$ donor fluorescence quantum yield, using physical constants (n = refraction index, N = Avogadro's number) and taking $\kappa^2 = 2/3$, we are able to calculate R_0 .

$$J(\lambda) = \int_0^{\infty} F_d(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \quad (\text{eq. 3})$$

$$R_0^6 = \frac{9000(\ln 10) \kappa^2 Q_D}{128 \pi^5 N n^4} \int_0^{\infty} F_d(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \quad (\text{eq. 4})$$

The calculation of the parameters were made by using a python script (http://www.pymolwiki.org/index.php/Forster_distance_calculator). As input we introduced the emission and excitation spectrum of donor and acceptor chromophores (in .txt extension), the molar extinction coefficient of the acceptor in $\lambda_{\max}$ ($M^{-1}cm^{-1}$), $\lambda_{\max}$ where the acceptor molar extinction coefficient is maximum, the donor quantum yield, the refraction index and the κ^2 . The program gives as output the overlap integral and the Förster distance (R_0) of our system:

$$R_0 = 20.9 \text{ \AA}$$

$$J(\lambda) = 1.54877 \cdot 10^{14}$$

Hybrid gel formation with mixtures of **1** and **2** could be efficiently carried out yielding translucent gels in CH_3CN by cooling down hot solutions containing both components. Several evidences point out the expected orthogonal self-assembly which is predicted from the structural differences of the compounds:

- a) Gelation capability is not altered in the mixtures. Taking into account that mgc values of **1** and **2** are respectively 1.4 and 2.9 mM, gels were formed for mixtures of both gelators containing half of their mgc values (0.7 and 1.45 mM), pointing out the existence of separated fibrillar networks.
- b) Raman spectra of the hybrid gel correspond to the sum of the isolated fibrillary networks with no new signals assignable to a new type of fibrillary assemblies (Figure 43).

Figure 43. Raman spectra of xerogels from single component gels and mixed equimolecular gel of **1 + 2**.

- c) XRD of the two component xerogel gel can be correlated to a sum of the separated xerogels, although in all cases the samples presented broad diffraction patterns, indicating poor crystallinity (Figure 44).

Figure 44. Powder WAXRD. Comparison of mixed xerogel (**1 + 2**) and single component xerogels (**1** and **2**).

- d) Significant differences could be observed when comparing electron microscopy images of single- and two-component xerogels, which in all cases showed fibrillary structures. However, it is possible to differentiate enriched domains of **1** and enriched domains of **2** (yellow circles and blue circles respectively in Figure 45) and mixed domains, as is commonly observed in many cases for molecular gelators.

Figure 45. Electron microscopy images showing differentiated and mixed domains indicating orthogonal assembly (Yellow (**1**) Blue (**2**) yellow+blue (**1+2**)) **Top row:** TEM images of mixed xerogels. **Bottom row:** SEM images of mixed xerogels.

- e) Rheological measurements revealed that mixed gels have similar viscoelastic properties to the single component gel of **2**, and improved storage and loss module in comparison to the gel of **1**, however these results cannot be unambiguously ascribed to the mentioned orthogonality (Figure 46).

Figure 46. Comparative of stress sweep (1 Hz) between gels in CH_3CN of compounds **1** and **2** and their two-component gel at 25 °C. Concentration of the gelators was 3 mM. The storage modulus (G'), loss modulus (G'') and oscillatory stress are in logarithmic scale.

- f) Selective disassembly of the fibrillary network formed by **2** from a two-component gel. This process was monitored by NMR and occurs following a temperature increase or acidification of the medium. As seen in Figure 47, the two-component gel releases selectively compound **2** upon heating from 30 to 70 °C, affording, according to NMR data, full solubilization of the network formed by **2** and only partial solubilization of that formed by **1**. Indeed, in the experiment represented in Figure 40 at 70 °C, a self-supported gel formed exclusively by **1** was obtained.

Figure 47. Variation of the solubility with the temperature, as determined by ^1H NMR, of a two-component gel formed by an equimolar mixture of **1** and **2** (3 mM each) in CD_3CN .

- g) Addition of acetic acid to a two-component gel also disassembles preferentially those aggregates formed by compound **2**, which is transformed in its soluble, easily detected by NMR, acridinium derivative (it should be noted that the pK_a values of acridine derivatives⁹⁹ and carboxylic acids¹⁰⁰ in CH₃CN do not predict full proton transfer, therefore, rather than protonation of acridine it is more accurate to use the term acridine–acetic acid complex).¹⁰¹ As seen in Figure 48, the NMR spectrum of a gel formed by **1** + **2** shows only a minimal amount of soluble, nonfibrillized, compound **2**. Addition of acetic acid produced full solubilization of **2**, as calculated using an internal standard for integration, but only partial solubilization (most likely due to polarity increase) of compound **1**. Again, full removal of compound **2** from the two-component gel produced in this case a self-sustained molecular gel formed exclusively by compound **1**.

Figure 48. Partial ¹H NMR spectra of a two-component gel formed by **1** + **2** (3 mM each) in CD₃CN, before and after addition of acetic acid (final concentration 1.8 M). The signals for compounds **1** and **2** correspond, respectively, to the protons attached to the chiral center of the glycine and valine amino acid moieties.

3.3.4 Energy transfer in self-assembled orthogonal fibrillar networks

Once the orthogonality of mixed fibrillary networks was demonstrated, the hybrid gels were studied as light harvesting systems by measuring the energy transfer from the acridine units to the naphthalimide ones. For this purpose, the emission spectra of samples containing only **2** and a mixture of **1** and **2** were compared using a spectrofluorometer with a front-face measurement setup and confocal laser scanning microscopy (CLSM). Energy transfer between the two chromophores was calculated by the following equation:

$$E_t = 1 - \left(\frac{F_{DA}}{F_D} \right) \quad (\text{eq. 5})$$

E_t = FRET efficiency

F_{DA} = Fluorescence of the donor in acceptor presence

F_D = Fluorescence of the donor in acceptor absence

As shown in Figure 49, a significant energy transfer process is detected for two-component samples. Notably, the graph in Figure 49 reveals that when a gel of acridine derivative **2** is mixed with increasing amounts of **1** a critical point is reached where energy transfer takes place. The critical point is coincident with the aggregation onset of **1**, revealing that only fibrillary aggregates of **1** can act efficiently as acceptors. For diluted concentrations of donor **1** no energy transfer at all is observed. This fact should be ascribed to the lack of interaction of free molecules of **1** with the fibers formed by **2**. However, fibrillization of **1** must provoke intimate fibrillary entanglement with fibers of **2**, affording a quite efficient energy transfer process.

Highly remarkable is that this process does not take place at all for systems with both components in solution (Figure 49, bottom left). Similarly, quite poor or null energy transfer is measured in the presence of one of the fibrillary networks and the complementary fluorophore in solution.

Figure 49. Top: graph representing the FRET efficiency of systems formed by a gel of donor **2** (2.5 mM) and variable amounts of acceptor **1** in CH₃CN. Bottom left: Overlap of emission spectra of diluted, nonaggregated samples of donor **2** and equimolar mixture of **1** and **2** ($c = 0.2$ mM). Bottom right: Overlap of emission spectra of gel samples of donor **2** and equimolar mixture of **1** and **2** ($c = 3$ mM; $\lambda_{\text{ex}} = 360$ nm).

Most likely solvophobic interactions together with π -stacking between electron-rich naphthalimide and electron-poor acridine units could be responsible of intimate contact between fibers formed by **1** and **2**. A simplified energy minimized semiempirical (AM1) model of such stacking is shown at Figure 50 supporting the feasibility of such structural arrangement.

Figure 50. Energy minimized semiempirical (AM1) model of a possible stacking of compounds **1** and **2**. Left: Space-filling, right: sticks. Dotted lines indicate hydrogen bonding.

Additional experiments with confocal microscopy allowed visualization of the energy transfer described above. As shown in Figure 51, the mixed gel shows a much brighter emission of yellowish light when compared with a single component of **1** under the same conditions. Although the evidence shown above points to an orthogonal fibrillary assembly, one might wonder if the observed excitation energy transfer could result from the incorporation of some units of **1** in the fibers of **2**. Regarding this aspect, in some areas of the confocal images corresponding to the interface of gel and solution, isolated domains of compound **2** could be observed (Figure 51, bottom right), which present no excitation transfer affording the original emission of **2**.

Figure 51. **Top:** Emission spectra obtained by confocal microscopy for gels formed by **1**, **2** and their mixture in CH₃CN, $\lambda_{\text{ex}} = 405$ nm. **Bottom:** Pictures of the gels of **1** (left) and **1 + 2** (right) obtained by confocal microscopy; $\lambda_{\text{ex}} = 405$ nm, $\lambda_{\text{obs}} = 550$ nm.

This observation discards that the light harvesting process arises from fibers of **2** doped with compound **1**. The light harvesting phenomena is quantified in the emission spectrum recorded with the confocal microscope upon irradiation at 405 nm. As shown in the spectra at Figure 51, the emission of acridine units at 450 nm in the mixed gel is fully suppressed and that due to naphthalimide moieties at 550 nm is boosted in agreement with the results obtained by the in-cuvette experiments described above.

3.3.5 Energy transfer on nanostructured two-component orthogonal xerogels from 1 and 2

The EET process was also preserved and nicely visualized in the absence of solvent when hybrid xerogels were studied. As seen in Figure 52, a 96% energy transfer from the acridine to the naphthalimide moiety occurs for a molar ratio up to 2:1 acridine:naphthalimide. Remarkably in this case, the EET process also produces a significant increase in the emission of naphthalimide units from **1**. Quantitative measurements of the energy transfer processes indicate that for an excitation at 360 nm, the hybrid xerogels present an emission intensity which is *ca.* 3 times that of the single component xerogel formed by acceptor **1**.

Figure 52. Fluorescence emission of xerogels of **1**, **2** and **1 + 2** mixtures in CH_3CN . Excitation: 360 nm. **A)** Mixed xerogel at a 2:1 acridine / naphthalimide ratio. **B)** Mixed xerogel at a 5:1 acridine/naphthalimide ratio. **C)** Overlap of normalized emission spectra of xerogel and ground solid samples from equimolar mixtures of **1** and **2** ($\lambda_{ex} = 360$ nm). **D)** Energy transfer efficiency on xerogels when increasing acridine proportion measured by decreasing of peak located 420 nm ($\lambda_{ex} = 360$ nm). **E)** Fluorescence emission intensity of xerogels when increasing acridine proportion, measured by the increase of peak located 530 nm.

This effect can be observed with the naked eye: the hybrid xerogel has a bright yellowish colour under UV light, whereas mechanically grounded mixture of both solids (no fibrillization process) shows a completely different appearance with a bluish emission (Figure 53).

Figure 53. Comparison of pictures of solids and microstructured samples by self-assembly. Under visible (left) and UV light (right 365 nm).

3.3.6 Stimuli-responsiveness of the two component materials formed by 1 and 2

Furthermore, an interesting fact is that the basic nature of compound **2** enables the disruption of the energy transfer by protonation of the acridine units. This affords soluble acridinium chromophores which lack the spectral overlap and the fibrillar-close disposition required for the excitation energy transfer to take place efficiently (Figure 54).

Figure 54. Scheme of pH-responsive energy transfer. When **2** is protonated, the emission of donor is shifted to longer wavelengths, interrupting the energy transfer by breaking the necessary spectral overlap and the fibre intimate contact.

Quenching of the energy transfer and leaching kinetics of protonated **2** are observed by means of fluorescence spectroscopy (Figure 55). When monitoring the fluorescence intensity variation at 533nm (emission of **1**) and at 490 nm (emission of acridinium cation $\mathbf{2-H^+}$) it can be observed how upon addition of CH_3COOH to the gel in CH_3CN , the emission at 533 nm is vanished as soon as the emission at 490 nm raises, corresponding to the energy transfer quenching and release of solubilized acridinium cation species.

Figure 55. Variation of fluorescence intensity upon addition of CH_3COOH in **1** + **2** mixed gel in CH_3CN . $\lambda_{\text{ex}} = 365 \text{ nm}$.

Additionally, confocal microscopy nicely visualizes the emission tuning achieved in this way. When a mixed gel was acidified with aqueous HCl, the emission of acceptor **1** dropped dramatically as a result of acridine chromophore donor protonation. The emission is only restored partially after a subsequent basification due to the leaching of some acridinium derivatives out of the gel network (Figure 56 right). As a result of the acidification, the gel bulk shows emission of **1** around 560 nm and the solution presents emission at 500 nm because of the presence of acridinium units $\mathbf{2-H^+}$ dissolved (Figure 56 right).

Notably, acid-base tuning of the emission is more efficient in the absence of solvent, namely in xerogels. As revealed in Figure 57, upon exposition of the mixed xerogel to acetic acid vapors emission of compound **1** is blocked to a large extent. Then, addition of NH_3 fumes deprotonate the acridinium derivative restoring the efficient energy transfer process. Presumably, in this case the lack of solvent prevents migration of the acridinium species and allows for a much more efficient recovery of the energy transfer. Also, rather than the acid-promoted fibrillary disassembly observed in gels, here most likely fibers of compound **2** are preserved after

becoming protonated or complexed with acetic. As observed in the pictures of Figure 50, after restoring the energy transfer process by addition of ammonia, the material distribution on the slide is essentially the same, refusing the possibility of fiber disassembly and reassembly. Therefore, these systems can be considered as stimuli responsive, namely, smart, soft photonic materials.

Figure 56. Left: Variation of fluorescence emission spectra of mixed xerogels of **1 + 2** in CH_3CN obtained by confocal microscopy upon acid/base addition ($\lambda_{\text{ex}} = 405 \text{ nm}$). Centre: Picture showing the image obtained by confocal microscopy of the acidified gel; circles indicate the areas where the emission spectra were collected ($\lambda_{\text{obs}} = 550 \text{ nm}$). Right: microspectroscopy recorded in both areas of interest (solution and gel) marked as circles in the central picture.

Figure 57. Top: Pictures of a xerogel formed by an equimolar mixture of **1** and **2** upon treatment with acetic acid and ammonia vapors ($\lambda_{\text{ex}} = 360 \text{ nm}$). Bottom: Fluorescence emission spectra ($\lambda_{\text{ex}} 360 \text{ nm}$) upon acidification/basification steps.

3.4 Conclusions

In summary, orthogonal fibrillization resulting in gel formation of photoactive compounds is reported. A very efficient EET between complementary fluorophores, acridine and 4-amino-1,8-naphthalimide, has been shown. The system described here differs from previously reported related cases in that fibrillization of both donor and acceptor is a requisite for the light manipulation. This fact together with the reversible nature of molecular gels affords tunable photonic soft materials. As schematized in Figure 58, the two-component gels when the system is irradiated at 360 nm, acridine moieties emission at 420 nm is removed and, as a result of excitation energy transfer, the emission of naphthalimide acceptor units at 550 nm is boosted compared to that obtained irradiating at 420 nm (big and small sun cartoons in Figure 58). Acidification provokes the formation of acridinium species, resulting in a dramatic reduction of naphthalimide emission and appearance of an emission at 490 nm from acridinium moieties. EET is observed both for wet gels and xerogels but the tuning is fully reversible only in the case of xerogels, which recover their initial emission properties after basification. Light harvesting phenomena is nicely visualized even with the naked eye for xerogels, which show a yellowish emission under a lamp irradiating at 365 nm. Overall, the efficient EET process among reversibly self-assembled fluorophores could open new paths for the development of smart systems with a wide variety of envisaged applications. It must be also noted that this kind of materials could be of practical interest for the development of white light emitting devices,⁵⁶ taking into account that the emission over a wide range of wavelengths (400–650 nm) could be tuned by adjusting the proportion of donor and acceptor concentrations.

Figure 58. Schematic representation of the tunable emission properties of the studied systems.

3.5 Experimental section

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3.5.1 Synthetic routes

3.5.1.1 Glycine-Valine amide derivatives bearing ANI moiety

Scheme 1. Synthetic route for the synthesis of glycine-valine derivatives bearing 4-amino-1,8-naphthalimide moiety. *i)* NHS, DCC, THF, 0 °C, 4 h. *iii_a)* THF, 4 h, 25 °C. *iii_b)* Et₃N, C₃H₅ClO₂, THF, 12h. *iv)* H₂, Pd/C, MeOH, 25 °C, 4 h. *v)* 4-nitro-1,8-naphthalic anhydride, CH₃OH, 75 °C, 8 h. *vi)* H₂, Pd/C, MeOH, 12 h.

3.5.1.2 Glycine-Valine esters and acid derivatives bearing ANI moiety

Scheme 2. Synthetic route for the synthesis of glycine-valine esters and acids bearing 4-amino-1,8-naphthalimide moiety. *ii*) DMAP, THF, reflux 60 °C, 12 h. *iii_b*) Et₃N, C₃H₅ClO₂, THF, 12h. *iv*) H₂, Pd/C, MeOH, 25 °C, 4 h. *v*) 4-nitro-1,8-naphthalic anhydride, CH₃OH, 75 °C, 8 h. *vi*) H₂, Pd/C, MeOH, 12 h. *vii*) LiOH, H₂O/THF 25 °C, 8 h.

3.5.1.3 Valine derivatives bearing ANI moiety

Scheme 3. Synthetic route for the synthesis of valine derivatives bearing 4-amino-1,8-naphthalimide moiety. *v*) 4-nitro-1,8-naphthalic anhydride, CH₃OH, 75 °C, 8 h. *vi*) H₂, Pd/C, MeOH, 12 h.

3.5.1.4 Valine and glycine esters and acid derivatives bearing ANI moiety

Scheme 4. Synthetic route for the synthesis of valine or glycine derivatives bearing 4-amino-1,8-naphthalimide moiety.

ii) DMAP, THF, reflux 60 °C, 12 h. **iv)** H_2 , Pd/C, MeOH, 25 °C, 4 h. **v)** 4-nitro-1,8-naphthalic anhydride, CH_3OH , 75 °C, 8 h.

vi) H_2 , Pd/C, MeOH, 12 h. **vii)** LiOH, $\text{H}_2\text{O}/\text{THF}$ 25 °C, 8 h.

3.5.1.5 Non amino acid ANI derivatives

Scheme 5. Synthetic route for the synthesis of non amino acid derivatives bearing 4-amino-1,8-naphthalimide moiety.

v) 4-nitro-1,8-naphthalic anhydride, CH_3OH , 75 °C, 8 h. **vi)** H_2 , Pd/C, MeOH, 12 h.

3.5.1.6 Acridine derivatives

Scheme 6. Synthetic route for the synthesis of valine derivatives bearing acridine moiety. *iii_a)* THF, 4 h, 25 °C. *iv)* H_2 , Pd/C, MeOH, 25 °C, 4 h. *viii)* CH_2Cl_2 , 0 °C, Oxalyl chloride, DMF (cat.), 18h, then, THF, 16 h.

Synthetic conditions and details are depicted in next section.

3.5.2 General synthetic procedures

3.5.2.1 General procedure for amino acid activation with NHS (i)

A solution of Carbobenzyloxy-L-amino acid (60 mmol) and Nhydroxysuccinimide (NHS) (60 mmol, 1.0 eq.) in dry THF (200 mL) was added dropwise under N_2 at 0 °C to a solution of N,N'-dicyclohexylcarbodiimide (60.6 mmol, 1.01 eq.) in dry THF (150 mL). The mixture was further stirred for 4 h at 0 °C. After this time, the mixture was filtered under vacuum, and the filtrate was removed under reduced pressure. Finally the crude residue was purified by crystallization in isopropanol to yield the activated ester.

3.5.2.2 General procedure for esterification between amino-acid and alcohol (ii)

A solution DMAP (17 mmol, 1.03 eq) in THF is added with an ice bath to a solution of the corresponding alcohol in THF (17 mmol, 1.03 eq) and stir until the addition is complete. After 5 minutes a solution of Z-activated amino acid (16.5 mmol) is added dropwise under stirring and the system heated to reflux at 60 °C for 12 hours. The crude is then vacuum dried and oily mixture is obtained. The product is dissolved with CH_2Cl_2 and washed several times with HCl 0.1

M and then with an aqueous solution of saturated NH_4Cl . The organic layer is dried with anhydrous MgSO_4 and dried in the rotary evaporator.

3.5.2.3 General procedure for coupling amino-acid and amine (iii)

a) In a representative example, 3.4 mmol activated ester was dissolved in 50 mL of THF. Then a solution of the correspondent amine in THF, 3.4 mmol was added at once at 25 °C under vigorous stirring. After 4 h the solvent was evaporated. To isolate the product, the solid residue was washed thrice with aqueous KOH 0.1 M, then with distilled water and then dried overnight at 60 °C.

b) A solution of Et_3N (2.30 mL, 16.3 mmol, 1 eq) in THF (40 mL) was added dropwise to a solution of commercially available carbobenzyloxy-L-amino acid (4.125 g, 16.22 mmol) in THF (60 mL) under N_2 flow on an ice bath (0 °C). To this mixture, an ice bath cooled solution of ethyl chloroformate (1.70 mL, 17.7 mmol, 1.1 eq) in THF (50 mL) was added dropwise. The reaction was left at 0 °C for 30 minutes. Finally, a solution of the free-amine derivatives (19.8 mmol, 1.2 eq.) in THF (50 mL) was added dropwise for 15 minutes at 0 °C. The reaction was left for 12 hours at room temperature. After this time, the solvent was removed under reduced pressure and the residue was poured onto HCl 0.1 M. The mixture was sonicated during 5 minutes. It was filtered off under vacuum, and the residue was washed with water KOH 0.1 M and H_2O until pH = 7. The residue was dried under reduced pressure at 50 °C overnight.

3.5.2.4 General procedure for deprotection of carbobenzyloxy group (iv)

Palladium catalyst (10% w/w) was suspended in CH_3OH (250 mL) and stirred under H_2 at room temperature for 10 min. Subsequently, a solution of carbobenzyloxy amino compound in CH_3OH (150 mL) was added via syringe, followed by stirring under H_2 at room temperature for 2–4 h. The reaction mixture was then filtered through HPLC filters (nylon, 0.46 μm), and the solvent was removed under reduced pressure to yield the respective amine.

3.5.2.5 General procedure for preparing naphthalimide derivatives (v)

4-Nitro-1,8-Naphthalic anhydride or 1,8-Naphthalic anhydride (498.2 mg, 1.94 mmol, 1 eq.) was suspended in a closed opaque vial with CH₃OH (15 mL) and vigorous stirring at room temperature. A solution of free-amino derivative (1.95 mmol) in CH₃OH (15 mL) was added dropwise. The vial was closed and the reaction mixture was heated to 75 °C for 8 hours. Dissolution of the suspension was observed as the temperature increased and appearance of voluminous solid was observed. After this time, the reaction mixture was left at room temperature and the brown solid was filtered through sintered funnel and washed with hot CH₃OH several times until a light brown solid was obtained.

3.5.2.6 General procedure for reduction of nitro moieties (vi)

Palladium catalyst (10% w/w) was suspended in CH₃OH (50 mL) and stirred under H₂ at room temperature for 10 min. Subsequently, a suspension of 4-nitro-1,8-naphthalimide derivatives (1.17 mmol) in CH₃OH (50 mL) was added via syringe, followed by stirring under H₂ at room temperature for 12 h. Solution changed the color almost immediately from brown suspension to green/yellow suspension. The palladium catalyst was removed by filtering with celite. To recover the entire product, washes with DMSO were performed. The CH₃OH was evaporated and the DMSO solution was lyophilized to obtain pure product.

3.5.2.7 General procedure for base hydrolysis (vii)

A solution suspension of naphthalimide derived ester (1.33 mmol) in THF (60 mL) at r.t. was mixed with a solution of LiOH (50 mg, 2.1 mmol, 1.6 eq) in water (20 mL) and stirred at room temperature for several hours until obtain a transparent solution. The reaction was followed by TLC (hexane/ethyl acetate 1:1). Then the reaction mixture was evaporated *in vacuo* to remove the THF and the crude product was washed with diethyl ether. The aqueous phase was then acidified with HCl 1 M and voluminous solid precipitated. The solid was then filtered off and washed with saturated NH₄Cl_(aq) and dried under vacuum at 60 °C.

3.5.2.8 General procedure for acridine coupling (viii)

To a suspension of acridine-9-carboxylic acid (529 mg, 2.3 mmol) in anhydrous dichloromethane (6 mL) at 0 °C and under N₂ atmosphere, a solution of oxalyl chloride (0.61 mL, 4.6 mmol) in anhydrous dichloromethane (3 mL) was added dropwise and 1-2 drops of dimethylformamide was added as catalyst. The reaction was stirred for 18 hours at room temperature. This resulted in complete dissolution of the starting material to give a strongly coloured yellow solution. Then, the reaction mixture was evaporated *in vacuo* to give crude acridine-9-carbonyl chloride as a yellow-green solid. This solid was suspended in anhydrous tetrahydrofuran (35 mL) under N₂ atmosphere and cool down to 0 °C. A solution of diamine derivatives (1 mmol) and triethylamine (2.8 mL, 20 mmol) in anhydrous THF (40 mL) was added dropping to the former suspension. The reaction was stirred for 1h at 0°C and for 16 h more at room temperature to give a strongly coloured red suspension. Then, the reaction mixture was evaporated *in vacuo*. The resulting solid was sonicated with aqueous NaOH 1 M (20 mL) and filtered off *in vacuo*, this washing was repeated once again. Then the solid was washed first with water until neutral pH and finally with cold ether. The solid obtained was dried at 60 °C *in vacuo* to yield pure product.

3.5.3 Synthesized compounds

Compound 3.3

NMR spectra were consistent with those described in the literature⁷⁸

¹H NMR (300 MHz, CDCl₃) δ (ppm): 1.02 (d, 6H, J = 7.4 Hz), 1.06 (d, 6H, J = 7.7 Hz), 2.31 (m, 1H), 2.77 (s, 4H), 4.66 (dd, 1H, J = 4.7 Hz), 5.13 (s, 2H), 5.43 (d, 1H, J = 9.5 Hz), 7.28-7.41 (m, 5H). ¹³C NMR (126 MHz, CDCl₃) δ (ppm): 17.2, 18.7, 25.5, 31.5, 57.4, 67.2, 128.0, 128.1, 128.3, 135.8, 155.6, 167.5, 168.6.

Compound 3.4

NMR spectra were consistent with those described in the literature.¹⁰²

¹H NMR (300 MHz, *d*₆-DMSO) δ (ppm): 7.86 (t, *J* = 4.9 Hz, 1H), 7.46 – 7.24 (m, 5H), 7.17 (d, *J* = 8.9 Hz, 1H), 5.03 (s, 2H), 3.78 (t, *J* = 7.9 Hz, 1H), 3.18 – 2.87 (m, 2H), 2.04 – 1.79 (m, 1H), 1.51 – 1.20 (m, 2H), 0.96 – 0.62 (m, 9H). ¹³C NMR (75 MHz, CDCl₃) δ (ppm): 11.2, 17.9, 19.2, 22.7, 30.1, 41.2, 60.7, 67.0, 128.0, 128.2, 128.5, 136.3, 156.4, 171.1. ESI-MS *m/z* = 293.4 (M + H⁺).

Compound 3.5

NMR spectra were consistent with those described in the literature.¹⁰²

¹H NMR (500 MHz, CDCl₃) δ (ppm): 0.77 (d, 3H, *J*=6.5 Hz), 0.87 (t, 3H, *J*=8.5 Hz), 0.92 (d, 3H, *J*=7.5 Hz), 1.34 (br s, 2H), 1.47 (m, 2H), 2.22 (m, 1H), 3.16 (m, 3H), 7.28 (br s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 11.4, 16.1, 19.7, 23.0, 30.9, 40.7, 60.3, 174.3

Compound 3.6

¹H NMR (300 MHz, CDCl₃) δ (ppm): 7.93 (t, *J* = 4.9 Hz, 1H), 7.68 (d, *J* = 8.8 Hz, 1H), 7.42 (t, *J* = 5.7 Hz, 1H), 7.38 – 7.24 (m, 5H), 5.01 (s, 2H), 4.09 (t, *J* = 7.8 Hz, 1H), 3.66 (d, *J* = 6.0 Hz, 2H), 3.13 – 2.86 (m, 2H), 2.00 – 1.81 (m, 1H), 1.38 (sext, *J* = 6.5 Hz, 2H), 0.91 – 0.72 (m, 9H). ¹³C

NMR (75 MHz, d_6 -DMSO) δ (ppm): 171.1, 169.4, 156.9, 137.5, 128.8, 128.2, 128.1, 65.9, 58.1, 46.0, 43.9, 36.8, 31.1, 29.5, 19.6, 18.5.

Compound 3.7

^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.72 (d, $J = 7.4$ Hz, 1H), 6.10 (t, $J = 5.4$ Hz, 1H), 4.14 (t, $J = 8.5$ Hz, 1H), 3.38 (s, 2H), 3.33 – 3.09 (m, 2H), 2.24 – 2.08 (m, 1H), 1.60 – 1.45 (sext, $J = 7.2$ Hz, 2H), 1.01 – 0.84 (m, 9H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 172.8, 171.0, 57.5, 44.8, 40.66, 31.5, 22.7, 19.6, 18.5, 11.8. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{10}\text{H}_{22}\text{N}_3\text{O}_2^+$ 216.1712; found 216.1710 $[\text{M}+\text{H}]^+$ ($\Delta=1.2$ ppm).

Compound 3.8

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.89 (d, $J = 8.7$ Hz, 1H), 8.71 (d, $J = 7.3$ Hz, 1H), 8.67 (d, $J = 8.1$ Hz, 1H), 8.43 (d, $J = 8.0$ Hz, 1H), 8.02 (dd, $J = 8.7, 7.3$ Hz, 1H), 6.72 (d, $J = 8.4$ Hz, 1H), 6.33 (t, $J = 5.9$ Hz, 1H), 4.88 (AB system, $J = 15.7$ Hz, 2H), 4.04 (t, $J = 8.3$ Hz, 1H), 3.34 – 3.06 (m, 2H), 2.07 (m, 1H), 1.54 (sext, $J = 7.3$ Hz, 2H), 0.99 (d, $J = 7.1$ Hz, 3H), 0.96 (d, $J = 7.1$ Hz, 3H), 0.91 (t, $J = 7.3$ Hz, 3H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 171.7, 168.5, 164.1, 163.1, 150.1, 133.2, 130.6, 130.4, 130.1, 129.2, 125.8, 123.9, 123.7, 121.8, 60.0, 42.8, 41.7, 30.8, 22.0, 18.5, 17.7, 10.7. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{22}\text{H}_{24}\text{N}_4\text{O}_6\text{Na}^+$ 463.1594; found 463.1587 $[\text{M}+\text{Na}]^+$ ($\Delta=1.5$ ppm).

Compound 3.1

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.62 (d, J = 8.4 Hz, 1H), 8.40 (d, J = 7.3 Hz, 1H), 8.18 (dd, J = 8.5, 7.0 Hz, 2H), 7.91 (t, J = 5.4 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.43 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.71 (s, 2H), 4.13 (t, J = 7.9 Hz, 1H), 3.19 – 2.90 (m, 2H), 1.96 (m, 1H), 1.42 (sext, J = 7.3 Hz, 2H), 0.99 – 0.75 (m, 9H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 170.6, 167.0, 163.7, 162.7, 152.8, 134.0, 131.0, 129.8, 129.4, 123.9, 121.7, 119.4, 108.2, 107.4, 57.9, 41.9, 40.3, 30.7, 22.2, 19.2, 18.2, 11.4. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{22}\text{H}_{26}\text{N}_4\text{O}_4\text{Na}^+$ 433.1852; found 433.1859 $[\text{M}+\text{Na}]^+$ (Δ =1.6ppm).

Compound 3.11

^1H NMR (500 MHz, CD_3OD) δ (ppm): 8.03 – 7.98 (m, 2H), 7.76 (d, J = 8.4 Hz, 1H), 7.14 (d, J = 7.2 Hz, 1H), 6.38 (d, J = 8.4 Hz, 1H), 3.87 – 3.83 (m, 2H), 3.19 – 3.14 (m, 2H), 2.89 (s, 3H), 2.87 – 2.84 (m, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ (ppm): 168.9, 164.5, 163.7, 153.4, 134.3, 131.3, 130.2, 129.1, 123.8, 121.5, 119.7, 108.3, 107.6, 69.9, 64.1, 57.7, 40.7. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_5\text{Na}^+$ 351.0958; found 351.0960 $[\text{M}+\text{Na}]^+$ (Δ = 0.9 ppm).

Compound 3.12

^1H NMR (500 MHz, d_6 -DMSO presence of CH_3OH) δ (ppm): 8.60 (d, $J = 8.3$ Hz, 1H), 8.42 (d, $J = 7.2$ Hz, 1H), 8.19 (d, $J = 8.4$ Hz, 1H), 7.65 (t, $J = 7.3$ Hz, 1H), 7.40 (s, 2H), 6.84 (d, $J = 8.4$ Hz, 1H), 3.97 (t, $J = 7.3$ Hz, 2H), 1.68 – 1.55 (m, 2H), 0.90 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 163.8, 162.9, 133.9, 129.7, 129.2, 123.9, 121.8, 119.4, 108.2, 107.6, 40.7, 21.0, 11.4. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{15}\text{H}_{15}\text{N}_2\text{O}_2 + 255.2839$; found 255.1133 $[\text{M}+\text{H}]^+$ ($\Delta = 0.4$ ppm).

Compound 3.13

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.67 (dd, $J = 8.4, 1.0$ Hz, 1H), 8.44 (dd, $J = 7.3, 1.0$ Hz, 1H), 8.20 (d, $J = 8.4$ Hz, 1H), 7.68 (dd, $J = 8.3, 7.4$ Hz, 1H), 7.55 (s, 2H), 6.87 (d, $J = 8.4$ Hz, 1H), 4.67 (s, 2H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 170.2, 164.0, 163.0, 160.0, 153.6, 134.7, 131.8, 130.2, 124.5, 121.9, 119.9, 108.8, 107.4, 41.4. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{14}\text{H}_{10}\text{N}_2\text{O}_4\text{Na} + 293.0538$; found 293.0536 $[\text{M}+\text{Na}]^+$ ($\Delta = 0.7$ ppm).

Compound 3.14

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.59 (d, $J = 8.2$ Hz, 1H), 8.40 (d, $J = 7.1$ Hz, 1H), 8.17 (d, $J = 8.3$ Hz, 1H), 7.62 (t, $J = 7.7$ Hz, 1H), 7.41 (s, 2H), 6.83 (d, $J = 8.3$ Hz, 1H), 3.97 (t, $J = 6.9$ Hz, 2H), 1.57 (s, 2H), 1.21 (d, $J = 38.2$ Hz, 19H), 0.81 (s, 3H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 164.2, 163.4, 153.1, 134.3, 131.4, 130.1, 129.7, 124.4, 122.3, 119.8, 108.6, 108.1, 31.8, 29.5, 29.5, 29.4, 29.4, 29.2, 29.2, 28.1, 27.0, 22.5, 14.4. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{24}\text{H}_{32}\text{N}_2\text{O}_2\text{Na} + 403.2361$; found 403.2364 $[\text{M}+\text{H}]^+$ ($\Delta = 0.7$ ppm).

Compound 3.15

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 8.66 (d, J = 8.3 Hz, 1H), 8.45 (d, J = 7.2 Hz, 1H), 8.20 (d, J = 8.4 Hz, 1H), 7.68 (t, J = 7.8 Hz, 1H), 7.56 (s, 2H), 6.87 (d, J = 8.4 Hz, 1H), 5.21 (d, J = 9.1 Hz, 1H), 4.20 – 4.02 (m, 2H), 3.28 – 3.20 (m, J = 5.0 Hz, 2H), 3.02 (s, 3H), 2.73 – 2.63 (m, 1H), 1.19 (d, J = 6.4 Hz, 3H), 0.67 (d, J = 6.8 Hz, 3H).

Compound 3.16

^1H NMR (500 MHz, CD_3OD) δ (ppm): 8.57 – 8.47 (m, 2H), 8.30 (d, J = 8.4 Hz, 1H), 7.66 (dd, J = 8.4, 7.4 Hz, 1H), 6.90 (d, J = 8.4 Hz, 1H), 5.27 (d, J = 9.6 Hz, 1H), 3.65 – 3.48 (m, 2H), 3.29 (t, J = 6.0 Hz, 2H), 2.98 – 2.82 (m, 1H), 1.23 (d, J = 6.5 Hz, 3H), 0.74 (d, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 169.6, 164.6, 163.6, 153.2, 134.6, 131.6, 130.5, 129.7, 124.5, 122.6, 119.8, 108.6, 108.2, 60.2, 58.7, 42.1, 26.70, 23.3, 19.3. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{19}\text{H}_{22}\text{N}_3\text{O}_4^+$ 359.1610; found 356.1609 $[\text{M}+\text{H}]^+$ (Δ = 0.3 ppm).

Compound 3.17

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.62 (d, J = 8.3 Hz, 1H), 8.39 (dd, J = 14.9, 7.9 Hz, 2H), 8.17 (d, J = 8.4 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.44 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.72 (AB

system, $J = 15.9$ Hz, 2H), 4.18 (dd, $J = 8.5$, 6.0 Hz, 1H), 2.06 (td, $J = 13.0$, 6.5 Hz, 1H), 0.91 (dd, $J = 6.4$, 4.0 Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 173.4, 167.7, 164.1, 163.2, 153.3, 134.5, 131.5, 130.3, 129.9, 124.4, 122.1, 119.9, 108.7, 107.9, 57.7, 42.1, 30.7, 19.6, 18.5.

Compound 3.18

^1H NMR (500 MHz, CD_3OD) δ (ppm): 8.52 (t, $J = 7.9$ Hz, 2H), 8.28 (d, $J = 8.4$ Hz, 1H), 7.72 (t, $J = 8.2$ Hz, 1H), 7.67 – 7.60 (m, 1H), 6.88 (d, $J = 8.5$ Hz, 1H), 5.24 (d, $J = 9.6$ Hz, 1H), 3.45 – 3.34 (m, 3H), 3.31 – 3.24 (m, 1H), 3.23 (s, 3H), 2.96 – 2.82 (m, 1H), 1.22 (d, $J = 6.5$ Hz, 3H), 0.73 (d, $J = 6.9$ Hz, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ (ppm): 171.2, 165.1, 164.4, 153.3, 134.5, 131.5, 130.4, 129.0, 123.9, 121.9, 119.7, 108.3, 108.00, 70.5, 59.5, 57.5, 38.8, 26.4, 21.3, 17.9. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{20}\text{H}_{23}\text{N}_3\text{O}_4\text{Na}^+$ 392.1586; found 392.1585 $[\text{M}+\text{Na}]^+$ ($\Delta = 0.3$ ppm).

Compound 3.19

^1H NMR (500 MHz, d_6 -DMSO presence of CHCl_3) δ (ppm): 8.61 (d, $J = 8.4$ Hz, 1H), 8.40 (d, $J = 7.3$ Hz, 1H), 8.17 (d, $J = 8.4$ Hz, 1H), 7.65 (t, $J = 7.8$ Hz, 1H), 7.38 (d, $J = 7.5$ Hz, 3H), 6.85 (d, $J = 8.4$ Hz, 1H), 4.97 (d, $J = 9.2$ Hz, 1H), 3.55 (ddd, $J = 15.0$, 9.5, 6.1 Hz, 1H), 2.72 (td, $J = 13.5$, 6.8 Hz, 1H), 1.58 (ddd, $J = 46.2$, 22.1, 8.6 Hz, 5H), 1.27 – 1.03 (m, 6H), 1.03 – 0.85 (m, 2H), 0.58 (d, $J = 6.9$ Hz, 3H). ^{13}C NMR (126 MHz, d_6 -DMSO presence of CHCl_3) δ (ppm): 168.3, 164.6, 163.6, 153.0, 134.4, 131.4, 130.5, 129.6, 124.4, 122.8, 119.9, 108.5, 108.4, 58.8, 48.3, 32.8, 32.7, 26.8, 25.8, 25.5, 25.3, 23.2, 19.3. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{23}\text{H}_{27}\text{N}_3\text{O}_3\text{Na}^+$ 416.1950; found 416.1944 $[\text{M}+\text{Na}]^+$ ($\Delta = 1.4$ ppm).

Compound 3.20

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.63 (d, J = 8.4 Hz, 1H), 8.42 (d, J = 7.3 Hz, 1H), 8.18 (d, J = 8.4 Hz, 1H), 7.73 – 7.56 (m, 2H), 7.41 (s, 2H), 6.86 (d, J = 8.3 Hz, 1H), 5.02 (d, J = 9.2 Hz, 1H), 3.03 – 2.83 (m, 2H), 2.73 (dd, J = 15.4, 6.8 Hz, 1H), 1.40 – 1.24 (m, 2H), 1.15 (d, J = 6.3 Hz, 3H), 0.73 (t, J = 7.3 Hz, 3H), 0.59 (d, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 169.3, 164.5, 163.5, 153.1, 134.5, 131.5, 130.5, 129.6, 124.4, 122.6, 119.8, 108.6, 108.3, 58.8, 41.0, 26.7, 23.3, 22.6, 19.3, 11.8. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{20}\text{H}_{23}\text{N}_3\text{O}_3\text{Na}^+$ 376.1637; found 376.1638 $[\text{M}+\text{H}]^+$ (Δ =0.3 ppm).

Compound 3.21

^1H NMR (500 MHz, CDCl_3) δ (ppm): 8.38 (d, J = 6.0 Hz, 1H), 8.08 (dd, J = 22.5, 7.3 Hz, 2H), 7.44 (d, J = 6.6 Hz, 1H), 6.68 (s, 1H), 6.57 (d, J = 7.3 Hz, 1H), 5.73 (s, 2H), 5.37 (d, J = 10.1 Hz, 1H), 3.52 – 3.26 (m, 2H), 3.05 – 2.84 (m, 1H), 1.60 (s, J = 46.5 Hz, 2H), 1.48 – 1.01 (m, 18H), 1.01 – 0.85 (m, 6H), 0.80 (d, J = 5.6 Hz, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ (ppm): 170.2, 165.1, 164.3, 150.8, 134.2, 131.6, 129.6, 128.1, 124.4, 122.1, 119.6, 109.7, 109.1, 61.8, 39.9, 31.9, 29.6, 29.6, 29.6, 29.5, 29.5, 29.3, 29.3, 27.0, 26.99, 22.7, 21.5, 19.2, 14.1. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{29}\text{H}_{41}\text{N}_3\text{O}_3\text{Na}^+$ 502.3046; found 502.3049 $[\text{M}+\text{Na}]^+$ (Δ =0.6 ppm).

Compound 3.22

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.62 (dt, J = 15.8, 7.9 Hz, 1H), 8.41 (dd, J = 7.3, 1.0 Hz, 1H), 8.19 (t, J = 9.4 Hz, 2H), 8.02 (t, J = 5.6 Hz, 1H), 7.66 (dd, J = 8.3, 7.4 Hz, 1H), 7.46 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.76 – 4.63 (m, 2H), 4.14 (dd, J = 9.0, 6.9 Hz, 1H), 3.24 – 3.21 (m, 3H), 1.94 (dq, J = 13.7, 6.9 Hz, 1H), 0.85 (dd, J = 6.8, 3.0 Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 171.4, 167.5, 164.2, 163.2, 153.3, 134.5, 131.6, 130.3, 129.9, 124.5, 122.2, 119.9, 108.7, 107.9, 70.9, 58.4, 58.2, 42.3, 40.9, 38.8, 31.3, 19.6, 18.6. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{22}\text{H}_{26}\text{N}_4\text{O}_5\text{Na}^+$ 449.1801; found 449.1797 [$\text{M}+\text{Na}$] $^+$ (Δ = 0.6 ppm).

Compound 3.23

HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{22}\text{H}_{25}\text{N}_3\text{O}_6\text{Na}^+$ 450.1641; found 450.1639 [$\text{M}+\text{Na}$] $^+$ (Δ = 0.4 ppm).

Compound 3.24

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 8.63 (d, J = 8.4 Hz, 1H), 8.41 (d, J = 7.3 Hz, 1H), 8.21 – 8.13 (m, 2H), 7.76 (d, J = 7.9 Hz, 1H), 7.66 (t, J = 7.7 Hz, 1H), 7.45 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.69 (s, 2H), 4.12 (dd, J = 8.9, 7.0 Hz, 1H), 3.60 – 3.49 (m, 1H), 1.97 – 1.89 (m, 1H), 1.77 –

1.60 (m, 4H), 1.59 – 1.51 (m, 1H), 1.31 – 1.08 (m, 5H), 0.85 (d, $J = 6.8$ Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 170.1, 167.5, 164.2, 163.2, 153.4, 134.5, 131.6, 130.3, 130.1, 124.4, 122.1, 119.9, 108.7, 107.8, 58.2, 47.9, 42.4, 32.9, 32.6, 31.5, 25.7, 25.0, 24.9, 19.6, 18.7. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{25}\text{H}_{30}\text{N}_4\text{O}_4\text{Na}^+$ 473.2165; found 473.2168 $[\text{M}+\text{Na}]^+$ ($\Delta = 0.6$ ppm).

Compound 3.25

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.59 (d, $J = 8.3$ Hz, 1H), 8.37 (d, $J = 7.2$ Hz, 1H), 8.13 (d, $J = 8.4$ Hz, 2H), 7.82 (t, $J = 9.9$ Hz, 1H), 7.61 (t, $J = 7.7$ Hz, 1H), 7.40 (s, 2H), 6.81 (d, $J = 8.4$ Hz, 1H), 4.67 (s, 2H), 4.07 (t, $J = 7.8$ Hz, 1H), 3.03 (dtd, $J = 18.5, 12.5, 6.0$ Hz, 2H), 1.92 (td, $J = 12.7, 6.1$ Hz, 1H), 1.35 (s, 2H), 1.18 (s, 18H), 0.79 (dd, $J = 18.0, 10.5$ Hz, 9H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 170.9, 167.5, 164.1, 163.2, 153.3, 134.4, 131.5, 130.3, 129.9, 124.4, 122.2, 119.9, 108.6, 107.9, 58.3, 42.3, 40.9, 31.7, 31.2, 29.5, 29.4, 29.4, 29.2, 29.1, 26.8, 22.5, 19.6, 18.6, 14.4. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{31}\text{H}_{44}\text{N}_4\text{O}_4\text{Na}^+$ 559.3260; found 559.3268 $[\text{M}+\text{Na}]^+$ ($\Delta = 1.4$ ppm).

Compound 3.26

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.62 (d, $J = 8.3$ Hz, 1H), 8.17 (d, $J = 8.4$ Hz, 1H), 7.65 (t, $J = 7.8$ Hz, 1H), 7.44 (s, 2H), 6.85 (d, $J = 8.4$ Hz, 1H), 4.72 (s AB system, $J = 15.9$ Hz, 2H), 4.18 (dd, $J = 8.5, 6.0$ Hz, 1H), 2.06 (td, $J = 13.0, 6.5$ Hz, 1H), 0.91 (dd, $J = 6.4, 4.0$ Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 173.4, 167.7, 164.1, 163.2, 153.3, 134.5, 131.5, 130.3, 129.9, 124.4,

122.1, 119.9, 108.7, 107.9, 57.7, 42.1, 30.7, 19.6, 18.5. HRMS (ESI-TOF, positive mode): m/z calcd. For $C_{19}H_{20}N_3O_5^+$ 370.1403; found 370.1407 $[M+Na]^+$ (Δ = 1.1 ppm).

Compound 3.9

NMR spectra were consistent with those described in the literature.¹⁰³

1H NMR (500 MHz, d_6 -DMSO) δ (ppm) 7.89 (t, J = 4.9 Hz, 1H), 7.45 – 7.26 (m, 5H), 7.22 (d, J = 8.7 Hz, 1H), 5.02 (s, 2H), 3.77 (dd, J = 7.9 Hz, 1H), 3.18 – 2.96 (m, 2H), 1.99 – 1.84 (m, 1H), 1.59 – 1.46 (m, 1H), 0.90 – 0.74 (m, 6H). ^{13}C NMR (75 MHz, d_6 -DMSO, 30 °C) δ (ppm): 18.4, 19.4, 29.3, 30.3, 36.4, 60.5, 65.5, 127.6, 128.3, 137.1, 156.0, 171.0.

Compound 3.10

NMR spectra were consistent with those described in the literature.¹⁰³

1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 7.83 (t, J = 5.1 Hz, 2H), 3.14 – 3.01 (m, 4H), 2.89 (d, J = 5.1 Hz, 2H), 1.93 – 1.77 (m, 2H), 1.52 (p, J = 6.7 Hz, 2H), 0.82 (dd, J = 40.9, 6.8 Hz, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 15.8, 19.2, 29.3, 30.6, 35.1, 59.8, 174.3; ESI-MS (m/z) 273.1 ($M + H^+$), 295.1 ($M + Na^+$).

Compound 3.2

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 9.18 (d, J = 7.8 Hz, 2H, H10), 8.29 (m, 2H, H15), 8.19 (m, 6H, H3, H6, H6'), 7.99 (d, J = 5.9 Hz, 2H, H3'), 7.86 (m, 4H, H5, H5'), 7.67 (m, 4H, H4, H4'), 4.57 (t, J = 7.8 Hz, 2H, H11), 3.44-3.24 (m, 4H, H16, H16'), 2.15 (m, 2H, H12), 1.79 (m, 2H, H17), 1.04 (d, J = 6.4 Hz, 6H, H13), 1.01 (d, J = 6.4 Hz, 6H, H13'). ^{13}C NMR (126 MHz, d_6 -DMSO) δ (ppm): 170.2, 166.3, 148.2, 142.3, 130.5, 129.3, 129.1, 126.5, 126.1, 125.5, 122.2, 121.9, 59.4, 36.7, 29.7, 29.5, 19.4, 18.5. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{41}\text{H}_{43}\text{N}_6\text{O}_4^+$ 683.3346; found 683.3347 $[\text{M}+\text{H}]^+$ (Δ = 0.1 ppm).

Compound 3.27

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 9.12 (d, J = 8.3 Hz, 1H), 8.29 – 8.08 (m, 4H), 7.97 (d, J = 7.2 Hz, 1H), 7.92 – 7.83 (m, 2H), 7.72 – 7.55 (m, 2H), 4.53 (t, J = 7.9 Hz, 1H), 3.28 – 3.04 (m, 3H), 2.17 – 2.02 (m, J = 13.7, 6.7 Hz, 1H), 1.59 – 1.44 (m, 2H), 1.44 – 1.28 (m, 4H), 0.99 (dd, J = 11.0, 6.8 Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 171.0(x2), 166.7(x2), 148.7(x4), 142.9(x2), 131.0(x4), 129.7(x4), 127.1(x4), 126.0(x4), 122.61(x4), 59.7(x2), 39.02(x2), 30.2(x2), 29.6(x2), 29.2(x2), 26.8(x2), 19.9(x2), 19.0(x2). HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{46}\text{H}_{52}\text{N}_6\text{O}_4^+$ 753.4050; found 753.4136 $[\text{M}+\text{H}]^+$ (Δ = 1.1 ppm)

Compound 3.28

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 9.14 (d, J = 8.2 Hz, 2H), 8.25 – 8.15 (m, 6H), 8.13 (d, J = 7.9 Hz, 2H), 7.95 (d, J = 7.9 Hz, 2H), 7.87 (t, J = 7.2 Hz, 4H), 7.72 – 7.58 (m, 4H), 4.51 (t, J = 7.8 Hz, 2H), 3.29 – 3.10 (m, 4H), 2.15 – 2.00 (m, 2H), 1.61 – 1.45 (m, 4H), 1.46 – 1.33 (m, 2H), 0.98 (dd, J = 11.0, 6.8 Hz, 11H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 171.0, 166.8, 148.7, 142.9, 131.0, 131.0, 129.8, 129.6, 127.1, 126.8, 126.6, 126.0, 122.7, 122.4, 59.8, 39.0, 30.2, 29.7,

26.7, 19.7, 19.0. HRMS (ESI-TOF, positive mode): m/z calcd. For $C_{44}H_{49}N_6O_4^+$ 725.3815; found 725.3820 $[M+H]^+$ (Δ = 0.7 ppm).

3.5.4 Gel preparation

The required amount of gelator(s) and 1 mL of CH_3CN were heated (hot air at 240 °C from a heat-gun) in a screw-capped vial (8 mL, 1.5 cm diameter) until complete solubilization. The vial was cooled down at ambient temperature and gel formation was observed after several minutes (upon vial inversion, all the solvent remains entrapped). The standard waiting time before manipulation or measurement of the gel is 30 min. For fluorescence measurements of the gels in quartz cuvette, the mixture is heated until complete solubilisation inside the previously mentioned screw capped vial and after this, the vial is half submerged in water at 25 °C for 3 seconds. Then the vial is carefully opened (CH_3CN vapours could come out) and the hot solution transferred to the preheated quartz cuvette (75 °C) with a preheated glass Pasteur pipette (75 °C). Finally the quartz cuvette is capped and left for 30 minutes for gel formation.

For xerogel preparation the gel was extended on a black glass slide, and then it was dried off by simple evaporation waiting for 30 minutes.

3.5.5 UV-Vis spectroscopy

UV-Vis measurements were carried out in JASCO V-630 spectrophotometer equipped with a Peltier accessory ETCS-761 at 25 °C unless otherwise is indicated. The samples were placed inside 1 cm suprasil quartz cuvettes for diluted solutions and 1 mm path length for optically dense solutions and gels. Data interval acquisition was set to $\Delta\lambda = 1$ nm with UV-Vis bandwidth 1.5 nm with scan speed set at 400 nm/min.

3.5.6 Fluorescence spectroscopy

Samples were measured in a JASCO FP-8300 spectrofluorometer using 10 mm path length and 3 mL quartz cuvettes for solutions or triangular quartz cuvettes in the case of gels or optically dense solutions. For xerogels samples were measured in a SpexFluorolog 3-11 spectrofluorometer with a "front face" setup. In order to get reliable results ten

measurements were carried out for each sample in different areas of the xerogel and the results averaged. Excitation wavelength was set to the maximum of absorption of the species under study as well as the most convenient excitation and emission slits opening.

3.5.7 X-ray diffraction

Wide-angle X-ray diffraction of the xerogels was performed at room temperature with a Bruker D4 Endeavor X-ray powder diffractometer by using Cu-K α -radiation. A sample of the respective freeze dried powders was placed on a sample holder and data were collected for 2 θ values between 2 ° and 40 ° with a step size of 0.03 ° and time step of 10 s.

3.5.8 Scanning electron microscopy (SEM)

Field-emission scanning electron micrographs were taken on a JEOL 7001F microscope equipped with a digital camera. The corresponding freeze-dried xerogels were placed on top of an aluminium specimen mount stub and sputtered with Pt (Baltec SCD500).

3.5.9 Transmission electron microscopy (TEM)

Transmission electron micrographs were taken on JEOL 2100 microscope with thermionic gun LaB₆ 200 KV equipped with Gatan Orius high resolution CCD camera. TEM samples were prepared over 200 mesh Formvar carbon copper grids by putting a drop of nano/microgel suspension and incubated for 5 minutes. The suspension excess was removed with filter paper. The grids were stained with a drop of phosphotungstic acid 1% for 1 minute that was subsequently removed by capillary action of filter paper.

3.5.10 Confocal Scanning microscopy (CLSM)

Experiments were performed on inverted confocal microscope Leica TCS SP8. Images were obtained with HC PL APO CS2 63x/1.40 oil immersion objective. Excitation of samples were performed with a diode laser (excitation 405 nm) and were acquired with PMT detector. Microspectroscopy was performed with $\Delta\lambda = 6$ nm. Samples were prepared on a glass slide and covered with 0.17 mm #1.5 coverslip. Fresh gels were measured as prepared, xerogels were covered and measured after evaporation of solvent. When experiments in which acid or base additions were needed, a peristaltic pump with 0.17 mm tubing thickness was used. To

acidify the sample, 10 μL of aqueous HCl 0.01 M were added. For basification, 5 μL of NaOH 0.1 M were added.

3.5.11 Raman spectroscopy

Spectra were recorded on a JASCO-NRS-3100 device with resolution of 1 cm^{-1} . Wet gel samples were extended over a glass slide and the spectra were recorded without drying them. Solvent spectra blank was recorded and subtracted from the original spectra.

3.5.12 Rheology

Measurements were carried out on a TA AR-G2 rheometer equipped with a Peltier temperature control accessory, using steel parallel plate-plate geometry (40 mm diameter). The gap distance was fixed at 1000 nm. The gels were prepared under the desired conditions and aged for 24h. A homogeneous layer of gel was placed between the two plates. Frequency and stress sweep steps were performed at 25°C . Viscoelastic properties were studied under oscillatory experiments. All the measurements were carried out within the linear viscoelastic regime. For this purpose the experimental conditions to achieve a linear viscoelastic regime (LVR) were determined by running a stress sweep step (oscillatory Stress 0.01-1000 Pa at 1 Hz) and a frequency sweep step (0.1-10 Hz at 0.1 Pa). The storage and loss modulus independence with frequency and oscillatory stress applied defined the linear viscoelastic regime.

3.5.13 NMR spectroscopy for determination of thermodynamic parameters

For the determination of solubility, the gels in CD_3CN were prepared inside of a NMR tube, using hydroquinone as internal standard for integration. ^1H NMR spectra (500 MHz) were recorded with a previous stabilization of 10 minutes at the desired temperature.

3.6 References

- (1) Darwin, C. M. A. *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life.*, 1st ed.; Murray, Ed.; London, 1859.
- (2) Hohmann-Marriott, M. F.; Blankenship, R. E. *Annu. Rev. Plant Biol.* **2011**, 62 (1), 515.
- (3) Blankenship, R. E. *Molecular Mechanisms of Photosynthesis*; 2008.
- (4) Jordan, P.; Fromme, P.; Witt, H. T.; Klukas, O.; Saenger, W.; Krauß, N. *Nature* **2001**, 411 (6840), 909.
- (5) Chitnis, P. R. *Annu. Rev. Plant Physiol. Plant Mol. Biol* **2001**, 52, 593.
- (6) Radboud Universiteit Nijmegen Science faculty. Chloroplast TEM image <http://www.vcbio.science.ru.nl/image-gallery/show/print/PL0337/> (accessed Feb 13, 2017).
- (7) Mirkovic, T.; Ostroumov, E. E.; Anna, J. M.; Van Grondelle, R.; Govindjee, II; Scholes, G. D. *Chem. Rev.* **2017**, 117, 249.
- (8) Duysens, L. N. M. *Transfer of Excitation Energy in Photosynthesis*, State University, Utrecht, The Netherlands, 1952.
- (9) Croce, R.; van Amerongen, H. *Nat. Chem. Biol.* **2014**, 10 (7), 492.
- (10) Colbow, K.; Danyluk, R. P. *Biochim. Biophys. Acta - Bioenerg.* **1976**, 440 (1), 107.
- (11) Niyogi, K. K.; Truong, T. B. *Curr. Opin. Plant Biol.* **2013**, 16 (3), 307.
- (12) Gwizdala, M.; Berera, R.; Kirilovsky, D.; van Grondelle, R.; Krüger, T. P. J. *J. Am. Chem. Soc.* **2016**, 138 (36), 11616.
- (13) Goodsell, D. S. Photosystem I <http://pdb101.rcsb.org/motm/22> (accessed Feb 13, 2017).
- (14) Scheuring, S.; Boudier, T.; Sturgis, J. N. *J. Struct. Biol.* **2007**, 159, 268.
- (15) Springer, J. W.; Parkes-Loach, P. S.; Reddy, K. R.; Krayner, M.; Jiao, J.; Lee, G. M.; Niedzwiedzki, D. M.; Harris, M. A.; Kirmaier, C.; Bocian, D. F.; Lindsey, J. S.; Holten, D.; Loach, P. A. *J. Am. Chem. Soc.* **2012**, 134 (10), 4589.
- (16) Zeng, X.-L.; Tang, K.; Zhou, N.; Zhou, M.; Hou, H. J. M.; Scheer, H.; Zhao, K.-H.; Noy, D. *J. Am. Chem. Soc.* **2013**, 135 (36), 13479.
- (17) Liu, C.; Brendan, C. C.; Ziesack, M.; Silver, P. A.; Nocera, D. G. *Science*. **2016**, 352

- (6290), 1210.
- (18) Li, Y. *Acc. Chem. Res.* **2012**, *45* (5), 723.
- (19) Scholes, G. D.; Fleming, G. R.; Olaya-Castro, A.; Van Grondelle, R. *Nat. Chem.* **2011**, *3* (10), 763.
- (20) Fukuzumi, S. *Phys. Chem. Chem. Phys.* **2008**, *10* (17), 2283.
- (21) Gilbert, M.; Albinsson, B. *Chem. Soc. Rev.* **2014**, *44* (4), 845.
- (22) Peng, H.-Q.; Niu, L.-Y.; Chen, Y.-Z.; Wu, L.-Z.; Tung, C.-H.; Yang, Q.-Z. *Chem. Rev.* **2015**, *115* (15), 7502.
- (23) Calzaferri, G.; Huber, S.; Maas, H.; Minkowski, C. *Angew. Chem. Int. Ed.* **2003**, *42* (32), 3732.
- (24) Cheng, Y.-J.; Yang, S.-H.; Hsu, C.-S. *Chem. Rev.* **2009**, *109* (11), 5868.
- (25) Semonin, O. E.; Luther, J. M.; Choi, S.; Chen, H.-Y.; Gao, J.; Nozik, A. J.; Beard, M. C. *Science*. **2011**, *334* (6062), 1530.
- (26) Wong, K.-T.; Bassani, D. M. *NPG Asia Mater.* **2014**, *6* (7), 1.
- (27) Wang, J.-Y.; Hsu, F.-C.; Huang, J.-Y.; Wang, L.; Chen, Y.-F. *ACS Appl. Mater. Interfaces* **2015**, *7* (50), 27676.
- (28) Lee, H.; Jeong, Y.-H.; Kim, J.-H.; Kim, I.; Lee, E.; Jang, W.-D. *J. Am. Chem. Soc.* **2015**, *137* (38).
- (29) Harriman, A. *Chem. Commun.* **2015**, *51* (59), 11745.
- (30) Peng, H.; Niu, L.; Chen, Y.; Wu, L.; Tung, C.; Yang, Q. *Chem. Rev.* **2015**, *115* (15), 7502.
- (31) Förster, T. *Discuss. Faraday Soc.* **1959**, *27* (0), 7.
- (32) Förster, T. *Zeitschrift für Naturforsch. - Sect. A J. Phys. Sci.* **1949**, *4* (5), 321.
- (33) Förster, T. *Ann. Phys.* **1948**, *437* (1–2), 55.
- (34) *Reviews in fluorescence*; Geddes, C. D., Ed.; Springer, 2016.
- (35) Clegg, R. M. In *FRET and FLIM Techniques*; Gadella, T. W. J., Ed.; 2009; pp 1–57.
- (36) Andrews, D. L.; Demidov, A. A. *Resonance energy transfer.*; Wiley, 1999.
- (37) Dexter, D. L. *J. Chem. Phys.* **1953**, *21* (5), 836.
- (38) Buerkle, L. E.; Rowan, S. J. *Chem. Soc. Rev.* **2012**, *41*, 6089.
- (39) Morris, K. L.; Chen, L.; Raeburn, J.; Sellick, O. R.; Cotanda, P.; Paul, A.; Griffiths, P. C.; King, S. M.; O'Reilly, R. K.; Serpell, L. C.; Adams, D. J. *Nat. Commun.* **2013**, *4*.
- (40) Raeburn, J.; Adams, D. J. *Chem. Commun.* **2015**, *51* (25), 5170.

- (41) Draper, E. R.; Lee, J. R.; Wallace, M.; Frank, J.; Cowan, A. J.; Adams, D. J. *Chem. Sci.* **2016**, 7 (10), 6499.
- (42) Moffat, J. R.; Smith, D. K. *Chem. Commun.* **2009**, 0 (3), 316.
- (43) Smith, M. M.; Smith, D. K. *Soft Matter* **2011**, 7 (10), 4856.
- (44) Velázquez, D. G.; Luque, R. *Chem. - A Eur. J.* **2011**, 17 (14), 3847.
- (45) Colquhoun, C.; Draper, E. R.; Eden, E. G. B.; Cattoz, B. N.; Morris, K. L.; Chen, L.; McDonald, T. O.; Terry, A. E.; Griffiths, P. C.; Serpell, L. C.; Adams, D. J. *Nanoscale* **2014**, 6 (22), 13719.
- (46) Fleming, S.; Debnath, S.; Frederix, P. W. J. M.; Hunt, N. T.; Ulijn, R. V. *Biomacromolecules* **2014**, 15 (4), 1171–1184.
- (47) Heeres, A.; Van Der Pol, C.; Stuart, M.; Friggeri, A.; Feringa, B. L.; Van Esch, J. J. *Am. Chem. Soc.* **2003**, 125 (47), 14252.
- (48) Brizard, A.; Stuart, M.; Van Bommel, K.; Friggeri, A.; De Jong, M.; Van Esch, J. *Angew. Chemie - Int. Ed.* **2008**, 47 (11), 2063.
- (49) Draper, E. R.; Eden, E. G. B.; McDonald, T. O.; Adams, D. J. *Nat. Chem.* **2015**, 7 (10), 848.
- (50) Cornwell, D. J.; Daubney, O. J.; Smith, D. K. *J. Am. Chem. Soc.* **2015**, 137 (49), 15486–15492.
- (51) Singh, N.; Zhang, K.; Angulo-Pachón, C. A.; Mendes, E.; van Esch, J. H.; Escuder, B. *Chem. Sci.* **2016**, 7 (8), 5568.
- (52) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. *Chem. Rev.* **2014**, 114 (4), 1973.
- (53) Weingarten, A. S.; Kazantsev, R. V.; Palmer, L. C.; McClendon, M.; Koltonow, A. R.; Samuel, A. P.; Kiebal, D. J.; Wasielewski, M. R.; Stupp, S. I. *Nat. Chem.* **2014**, 6 (11), 964.
- (54) Weingarten, A. S.; Kazantsev, R. V.; Palmer, L. C.; Fairfield, D. J.; Koltonow, A. R.; Stupp, S. I. *J. Am. Chem. Soc.* **2015**, 137 (48), 15241.
- (55) Haedler, A. T.; Kreger, K.; Issac, A.; Wittmann, B.; Kivala, M.; Hammer, N.; Köhler, J.; Schmidt, H.-W.; Hildner, R. *Nature* **2015**, 523 (7559), 196.
- (56) Praveen, V. K.; Ranjith, C.; Armaroli, N. *Angew. Chemie - Int. Ed.* **2014**, 53 (2), 365.
- (57) Desvergne, J.-P.; Olive, A. G. L.; Sangeetha, N. M.; Reichwagen, J.; Hopf, H.; Del Guerzo, A. *Pure Appl. Chem.* **2006**, 78 (12), 2333.

- (58) Del Guerzo, A.; Olive, A. G. L.; Reichwagen, J.; Hopf, H.; Desvergne, J.-P. *J. Am. Chem. Soc.* **2005**, *127* (51), 17984.
- (59) Giansante, C.; Raffy, G.; Schäfer, C.; Rahma, H.; Kao, M.-T.; Olive, A. G. L.; Del Guerzo, A. *J. Am. Chem. Soc.* **2011**, *133* (2), 316.
- (60) Ajayaghosh, A.; Praveen, V. K. *Acc. Chem. Res.* **2007**, *40* (8), 644.
- (61) Ajayaghosh, A.; Praveen, V. K.; Vijayakumar, C.; George, S. J. *Angew. Chemie - Int. Ed.* **2007**, *46* (33), 6376.
- (62) Vijayakumar, C.; Praveen, V. K.; Ajayaghosh, A. *Adv. Mater.* **2009**, *21* (20), 2059.
- (63) Praveen, V. K.; Ranjith, C.; Bandini, E.; Ajayaghosh, A.; Armaroli, N. *Chem. Soc. Rev.* **2014**, *43* (12), 4222.
- (64) Duan, P.; Yanai, N.; Nagatomi, H.; Kimizuka, N. *J. Am. Chem. Soc.* **2015**, *137* (5), 1887.
- (65) Shu, T.; Wu, J.; Lu, M.; Chen, L.; Yi, T.; Li, F.; Huang, C. *J. Mater. Chem.* **2008**, *18* (8), 886.
- (66) Sugiyasu, K.; Fujita, N.; Shinkai, S. *Angew. Chemie - Int. Ed.* **2004**, *43* (10), 1229.
- (67) Ardoña, H. A. M.; Tovar, J. D. *Chem. Sci.* **2015**, *6* (2), 1474.
- (68) Channon, K. J.; Devlin, G. L.; MacPhee, C. E. *J. Am. Chem. Soc.* **2009**, *131* (35), 12520.
- (69) van Herrikhuyzen, J.; Syamakumari, A.; Schenning, A. P. H. J.; Meijer, E. W. *J. Am. Chem. Soc.* **2004**, *126* (32), 10021.
- (70) Xue, P.; Wang, P.; Yao, B.; Sun, J.; Gong, P.; Zhang, Z.; Qian, C.; Lu, R. *ACS Appl. Mater. Interfaces* **2014**, *6* (23), 21426–21434.
- (71) Prasanthkumar, S.; Ghosh, S.; Nair, V. C.; Saeki, A.; Seki, S.; Ajayaghosh, A. *Angew. Chemie - Int. Ed.* **2015**, *54* (3), 946.
- (72) López-Andarias, J.; Rodriguez, M. J.; Atienza, C.; López, J. L.; Mikie, T.; Casado, S.; Seki, S.; Carrascosa, J. L.; Martín, N. *J. Am. Chem. Soc.* **2015**, *137* (2), 893.
- (73) Marty, R.; Szilluweit, R.; Sánchez-Ferrer, A.; Bolisetty, S.; Adamcik, J.; Mezzenga, R.; Spitzner, E.-C.; Feifer, M.; Steinmann, S. N.; Corminboeuf, C.; Frauenrath, H. *ACS Nano* **2013**, *7* (10), 8498.
- (74) Che, Y.; Huang, H.; Xu, M.; Zhang, C.; Bunes, B. R.; Yang, X.; Zang, L. *J. Am. Chem. Soc.* **2011**, *133* (4), 1087.
- (75) Draper, E. R.; Lee, J. R.; Wallace, M.; Jäckel, F.; Cowan, A. J.; Adams, D. J. *Chem. Sci.* **2016**, *7* (10), 6499.

- (76) Zhou, M.; Smith, A. M.; Das, A. K.; Hodson, N. W.; Collins, R. F.; Ulijn, R. V.; Gough, J. E. *Biomaterials* **2009**, *30* (13), 2523.
- (77) Felip-León, C.; Díaz-Oltra, S.; Galindo, F.; Miravet, J. F. *Chem. mater.* **2016**, *28* (21), 7964.
- (78) Escuder, B.; Martí, S.; Miravet, J. F. *Langmuir* **2005**, *21* (15), 6776.
- (79) Segarra-Maset, M. D.; Nebot, V. J.; Miravet, J. F.; Escuder, B. *Chem. Soc. Rev.* **2013**, *42* (17), 7086.
- (80) Escuder, B.; Rodríguez-Llansola, F.; Miravet, J. F. *New J. Chem.* **2010**, *34* (6), 1044.
- (81) Nebot, V. J.; Ojeda-Flores, J. J.; Smets, J.; Fernández-Prieto, S.; Escuder, B.; Miravet, J. F. *Chem. - A Eur. J.* **2014**, *20* (44), 14465.
- (82) Felip-León, C.; Segarra-Maset, M. D.; Escuder, B.; Miravet, J. F. *Tetrahedron Lett.* **2015**, *56* (9), 1132.
- (83) Nebot, V. J. Design, study and applications of supramolecular hydrogels based on amino acids, Universitat Jaume I, 2012.
- (84) Duke, R. M.; Veale, E. B.; Pfeffer, F. M.; Kruger, P. E.; Gunnlaugsson, T. *Chem. Soc. Rev.* **2010**, *39* (10), 3936.
- (85) Jelley, E. E. *Nature* **1936**, *138* (3502), 1009.
- (86) Einfeld, A.; Briggs, J. S. *Chem. Phys.* **2006**, *324* (2–3), 376.
- (87) Mahler, H. R.; Kline, B.; Mehrotra, B. D. *J. Mol. Biol.* **1964**, *9* (3), 801.
- (88) Ma, X.; Sun, R.; Cheng, J.; Liu, J.; Gou, F.; Xiang, H.; Zhou, X. *J. Chem. Educ.* **2016**, *93* (2), 345.
- (89) Zhong, C.; Mu, T.; Wang, L.; Fu, E.; Qin, J. *Chem. Commun.* **2009**, *0* (27), 4091.
- (90) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Springer US: Boston, MA, 2006.
- (91) Hanabusa, K.; Tanaka, R.; Suzuki, M.; Kimura, M.; Shirai, H. *Adv. Mater.* **1997**, *9* (14), 1095.
- (92) Doi, M.; Asano, A.; Yoshida, H.; Inouguchi, M.; Iwanaga, K.; Sasaki, M.; Katsuya, Y.; Taniguchi, T.; Yamamoto, D. *J. Pept. Res.* **2005**, *66* (4), 181.
- (93) Wilson, S. M.; Bacic, A. *Nat. Protoc.* **2012**, *7* (9), 1716.
- (94) Nebot, V. J.; Ojeda-Flores, J. J.; Smets, J.; Fernández-Prieto, S.; Escuder, B.; Miravet, J. F. *Chem. - A Eur. J.* **2014**, *20* (44), 14465.
- (95) Martí-Centelles, V.; Izquierdo, M. A.; Burguete, M. I.; Galindo, F.; Luis, S. V. *Chem. - A*

- Eur. J.* **2014**, 20 (24), 7465.
- (96) Braslavsky, S. E.; Fron, E.; Rodríguez, H. B.; Román, E. S.; Scholes, G. D.; Schweitzer, G.; Valeur, B.; Wirz, J. *Photochem. Photobiol. Sci.* **2008**, 7 (12), 1444.
- (97) Yuan, L.; Lin, W.; Zheng, K.; Zhu, S. *Acc. Chem. Res.* **2013**, 46 (7), 1462.
- (98) Forster distance calculator - PyMOLWiki
https://pymolwiki.org/index.php/Forster_distance_calculator (accessed May 22, 2017).
- (99) Kaljurand, I.; Kütt, A.; Sooväli, L.; Rodima, T.; Mäemets, V.; Leito, I.; Koppel, I. A. *J. Org. Chem* **2005**, 70 (3), 1019.
- (100) Kütt, A.; Leito, I.; Kaljurand, I.; Sooväli, L.; Vlasov, V. M.; Yagupolskii, L. M.; Koppel, I. A. *J. Org. Chem* **2006**, 71 (7), 2829.
- (101) Akiyama, Y.; Wakisaka, A.; Mizukami, F.; Sakaguchi, K.; Benedict, H.; Limbach, H.; Sakaguchi, K.; Jones, H. *J. Chem. Soc. Perkin Trans. 2* **1998**, 109 (1), 95.
- (102) Burguete, M. I.; Galindo, F.; Izquierdo, M. A.; Luis, S. V.; Vígara, L. *Tetrahedron* **2007**, 63 (38), 9493.
- (103) Becerril, J.; Bolte, M.; Burguete, M. I.; Galindo, F.; García-España, E.; Luis, S. V.; Miravet, J. F. *J. Am. Chem. Soc* **2003**, 125 (22), 6677.

Chapter 4

Aggregation Induced Emission

4 Aggregation Induced Emission

4.1 Motivation

The development of new chromophores and luminescent materials has meant a big advance. New light-emitters have allowed us to develop high-tech innovations that benefit the whole world. A good proof of it are the recent Nobel laureates in Physics "for the invention of efficient blue light-emitting diodes which have enabled bright and energy-saving white light sources" and in Chemistry "for the development of super-resolved fluorescence microscopy" in 2014 and for the development of green fluorescent proteins to gain extraordinary insights into biological pathways in 2015.^{1-3,4-6}

Almost all the practical applications of luminescent materials arise when they are presented on solid or aggregated physical state. For example, developments on the optoelectronics field include organic light emitting diodes (OLEDs), organic field-effect transistors (OFETs), organic photovoltaic cells and optical waveguides. Luminescence is also of high interest in Biology and Chemistry with applications related to sensing probes, bioassaying, imaging, chemosensors and new therapeutic and theranostic applications in Medicine.^{7,8}

The aim of this forth chapter is to report on the construction of a novel, versatile, stimuli-responsive gelator which undergoes aggregation induced emission phenomena upon fibrillization. Fluorescence emission enhancement, ability to form films and strong gels, thixotropic behaviour and self-healing features of naphthalimide-derived molecular gels are investigated.

4.2 Introduction

Aggregation induced emission (AIE) concept was coined in 2001 by Tang and co-workers. They serendipitously found out that dissolved 1-methyl-1,2,3,4,5-pentaphenylsilole was non emissive under UV-lamp, but the compound was highly fluorescent when it was dried out (TLC chromatography) the compound was highly fluorescent.⁹ This process appeared in contraposition of the well-studied aggregation-caused quenching (ACQ), present in almost all

the luminophores known up to that date (Figure 1). They realized of the potential applications of a phenomenon that takes advantage of aggregate formation, which was classically undesirable and avoided.

Figure 1. Fluorescence photographs of solutions or suspensions of (left) perylene (20 μ M) and (right) hexaphenylsilole (HPS; 20 μ M) in THF/water mixtures with different fractions of water (f_w), with perylene and HPS showing typical ACQ and AIE effects, respectively.⁸

4.2.1 AIE vs ACQ

Aggregation caused quenching (ACQ) is a photophysical phenomenon very well known since mid-20th century.^{10,11} Classical luminophores strongly emit fluorescence in very diluted solutions and they have helped us grasp fundamental understanding of radiative processes.^{12,13} However, fluorescence usually became quenched upon aggregation or clustering, reducing the scope of practical applications. For example, ACQ is a problem for efficient optoelectronic devices fabrication because luminophores are used mainly in the aggregate state or in films.^{14,15} This phenomenon is mainly observed in aromatic luminophores, whose intrinsic hydrophobicity and planarity trigger the aggregation process due to strong π - π stacking. When aromatic molecules are in close proximity, the excited states decay back to the ground state via non-radiative pathways, resulting on fluorescence emission quenching.

As mentioned above, AIE represents the opposite photophysical phenomenon to ACQ. Molecules presenting this phenomenon have a very weak fluorescence in solution and they become highly fluorescent when aggregated. AIE is a cooperative effect which uses in an active, constructive way the thermodynamically favourable aggregation process.

Emission in aggregated states has been much less studied when compared with systems in solution. However, many research groups have been focusing its efforts in understanding and

developing new molecules with AIE features. Through these efforts, insights into the mechanistic process of aggregation, structure-property relationship have been gained, allowing the development of practical and high-tech applications.^{16–21} Since 2001 and because of the extensive investigation in this field, the number of publications grown exponentially to more than 700 in 2017.

Figure 2. Right: Fluorescence photographs of solutions and suspensions of fluorescein (15 μM) in water/acetone mixtures with different fractions of acetone. Left: hexaphenylsilole (HPS; 20 μM) in THF/water mixtures with different fractions of water.²²

4.2.2 Mechanistic explanation

Understanding the mechanistic process by which AIE takes place is a critical issue for designing new AIE luminophores, developing practical applications and, in the end, producing technological innovations. Several mechanisms have been proposed although in this chapter only the most accepted mechanisms will be presented: restriction of molecular rotations and restriction of molecular vibrations.

4.2.2.1 Restriction of intramolecular rotations (RIR)

Since the discovery of the AIE phenomena, several complex mechanistic explanations have been proposed to describe the fluorescence enhancement upon aggregation. Tetraphenylethene (TPE) is one of the first luminophores described to undergo AIE and represents a good example to introduce one of the mechanistic explanations of AIE.^{18,23–26} TPE presents four phenyl rings linked by single bonds to a central ethene moiety (Figure 3). The molecules of TPE in solution have free-rotating intramolecular motions by which energy of

excited states can be dissipated, constituting a non-radiative relaxation pathway to decay to the ground state. When TPE and similar molecules with rotationally free bonds aggregate, the molecular rotations become constrained and the relaxation pathway through the external group rotation is cancelled, forcing the molecule to relax by emitting photons.^{27–29} Experimental data confirmed this behaviour by applying variations of solvent viscosity, pressure and the temperature to favour or disfavour the AIE effect.³⁰

Figure 3. Propeller-shaped luminogen of tetraphenylethene (TPE) is non-luminescent in a dilute solution but becomes emissive when its molecules are aggregated, due to the restriction of intramolecular rotation (RIR) of its phenyl rotors against its ethylene stator in the aggregate state.²²

It is important to remark that extensive crystallographic studies performed with other well-studied molecule, 1,1,2,3,4,5-Hexaphenylsilole (HPS), have determined that the steric repulsion between the phenyl groups prevents face-face packing of aromatic moieties when forming aggregates. Crystal structure of HPS shows that the aggregation packing is due to non-covalent C-H $\cdots$ π (2.6–3.6 Å) interactions restricting the rotational motions of their phenyl rings.³⁰ The lack of aromatic face to face packing is very important in the context of AIE because, in contrast to luminophores presenting ACQ, the lack of strong interactions between the chromophores, that lead to bathochromic shifts and radiationless pathways via excimer and excitons, enables the chromophore unit to shine efficiently when aggregated.

4.2.2.2 Restriction of intramolecular vibrations (RIV)

Another well-known mechanism that constricts the intramolecular motions resulting in the AIE effect is the restriction of intermolecular vibrations. Although AIE can be explained by RIR for many luminophores, there are several cases in which rigid molecules that cannot rotate show AIE phenomena. A representative case is tetrahydro-5,5'-bidibenzo[a, d][7]annulenyldiene (THBA, Figure 4), which is not planar and has no rotatory elements but has a bendable flexure

that can undergo dynamic vibration in solution similarly to a bellows. The aggregation of THBA or similar molecules provokes vibration constriction and, therefore, opening of radiative relaxation pathways. There are cases in which both mechanisms, RIR and RIV, are operative and a more generalist definition of the phenomena is commonly used: restriction of intramolecular motions (RIM).³¹

Figure 4. Shell-like luminogen of tetrahydro-5,5',-bidibenzo[*a, d*][7]annulenyldiene (THBA; **1**) shows AIE due to the restriction of intramolecular vibration (RIV) of its bendable vibrators in the aggregate state.²²

4.2.3 Systems and structures with aggregation induced emission

Research groups all over the world have done fervent synthetic efforts to synthesize molecules that present AIE, known as AIEgens. A wide variety of molecules presenting AIE have been described including hydrocarbons, metal complexes, heterocycles and macromolecules (figures 5 and 6).

Figure 5. Hydrocarbon AIEgens: **a)** Cyclopentadiene derivatives,³² **b)** Arylene vinylene derivatives,³³ **c)** Hexaphenylbenzene derivatives,³⁴ **d)** Naphthalene derivatives,³⁵ **e)** Anthracene derivatives³⁶ and Heteroatom containing AIEgens: **f)** Pyrrole derivatives³⁷ **g)** Boron derivatives³⁸ **h)** Schiff base derivatives³⁹ and **i)** Nitrile derivatives.⁴⁰

Figure 6. Macromolecular AIEgens: **j**) AIEgen on side chain of branched polymers;⁴¹ **k**) Dendrimers containing AIEgens;⁴² **l**) AIEgen on backbone of linear polymers;⁴³ **m**) Hyperbranched polymers;⁴⁴ **n**) AIE in metal organic frameworks.⁴⁵ Metal AIEgen complexes: **o**) Au derived AIEgens;⁴⁶ **p**) Cu derived AIEgens;⁴⁷ **q**) Pt derived AIEgens;⁴⁸ **r**) Ru derived AIEgens;⁴⁹ **s**) Ir derived AIEgens.⁵⁰

4.2.4 Naphthalimide-based gels and aggregates

1,8-Naphthalimide is a well known chromophore, widely used as chemosensor, fluorescent label, light harvesting antenna, DNA photocleaver, component of OLEDs and as part of creative photo-switches.^{51–59} This chromophore shows absorption in the near UV region and shows weak blue fluorescence ($\phi_f = 0.016$) and short fluorescence lifetime ($\tau = 0.145$ ns) in acetonitrile.⁵¹ Several examples of naphthalimide-based organogelators have been reported and studied recently from a structural point of view. For example, Nghi Pham and co-workers studied low molecular weight gelators derived from Cbz-amino acids bearing a 1,8-naphthalimide unit (Compound A and B in figure 7), very similar to those studied in this chapter. They reported the importance of the side chains of the amino acid residue in the formation of J-type and H-type aggregates and the solvent-induced chirality inversion and its

effect in fluorescence. However, they did not report AIE phenomena upon gelation in toluene.⁶⁰ Same naphthalimide derived gelators were also prepared by Bouguet-Bonnet and co-workers, who used NMR spectroscopy methods to unravel the behaviour of fast-exchanging molecules between the gel matrix and the so-called isotropic compartment.⁶¹

Figure 7. Example of 1,8-naphthalimide derived organogelators (A and B^{60–62}, C⁶³)

In a previous work also based on NMR, Allix and co-workers, shed some light on the initial steps of organogelation of that compounds (A and B, Figure 7). These steps consisted on the formation of hydrogen-bonded “head-to-tail” stacking-up of gelator molecules, followed by the assembly of those columns into incipient precursors (small aggregates) via intercolumnar π - π stacking interactions. They concluded that free rotation of the N-N bond is relevant for the intercolumnar π - π stacking interactions, responsible for the gelation process (Figure 8).⁶²

Figure 8. 2D columnar assembly of gelators. Red dotted lines represent hydrogen bonds and blue ones represent π - π stacking interactions. Double black arrows represent the correlations observed in 2D-NMR.⁶²

Interesting mechanical properties of a naphthalimide-derived organogelator were described by Yu *et. al.* They designed an umbrella-shaped molecule containing a lysine group as linker between naphthalimide moiety and two cholesterol units (compound C in Figure 7). This molecule self-assembles in ring superstructures and forms thixotropic organogels at room temperature. The organogel in toluene possesses pore sizes ranging from the nanoscale to the microscale and has interesting self-healing features.⁶³

Practical applications of organogels based on naphthalimide moieties are scarce. In one case, Meher and co-workers designed naphthalimide derived V-shaped molecules. They were constituted by an aliphatic chain, a naphthalimide moiety and a quinoline unit, whose N atom was demonstrated to be the responsible for the head-to-tail $\pi-\pi$ interactions and, therefore, the activation of AIE. This interactions result in the formation of highly reproducible nanoribbon like structures. They proposed, using these species as a bioprobe for detecting ferritin in biological media. (Figure 9).⁶⁴

Figure 9. Illustration of the mechanism of protein unfolding and nanoribbons disruption using a fluorescent probe at physiological condition with aggregation induced emission enhancement (AIEE) characteristics.⁶⁴

Molecular flexibility has been pointed out as a key parameter to have an effective AIE in naphthalimide derived molecules. For instance, in a series of studies, Mukherjee and Thilagar manipulated the molecular flexibility to control fluorescence quantum yields of V-shaped naphthalimides, which showed moderately emissive behaviour in aggregated states (THF/ H_2O mixtures). Moreover, the flexibility-emission correlation was effectively used to devise a molecule acting as a $Hg(II)$ selective chemodosimeter (Figure 10).⁶⁵

Figure 10. Emission of compound 4 in different THF/H₂O mixture compositions (**top left**, 100 mM, λ_{ex} = 355 nm), TEM image of fluorescent organic nanoaggregates of 4 (**top right**), photographs of 4 under 265 nm UV-lamp in THF/H₂O mixtures (100 mM) with different THF contents (**bottom**).

Additionally, AIE from a naphthalimide derived molecule was used by Sun and co-workers to design a very sensitive biosensor for the detection of casein (Figure 11). The AIE probe has a hydrophobic and planar conjugated ring (naphthalimide) and polar head with two carboxylic acids, which can dock in a hydrophobic cavity of casein, binding with Tyr and Trp residues. At the same time, the same hydrophobic cavity provides binding sites to the apolar part of the bioprobe, resulting in aggregation on the casein surface and emission enhancement around 420 nm.⁶⁶ AIEgens derived from naphthalimide have been also used in bioimaging taking advantage of the formation of fluorescent organic nanoparticles for cell imaging and cell recognition.⁶⁷

Figure 11. (A) Mechanism of aggregated induced emission (AIE) of naphthalimide-derived bioprobe with casein; (B) Fluorescence spectra with different casein concentration, (0 to 22 µg/ml); **Inset:** linear range of casein assay. λ_{ex} = 300 nm.⁶⁶

4.2.5 Molecular self-assembled gels with AIE

A vast amount of gelators containing fluorescent units, have been designed mainly focused on optoelectronic applications.⁶⁸ However, most of them present ACQ because they are formed from π -conjugated chromophores, which undergo strong intermolecular interactions that prevents the excited state to decay in a radiative way. As discussed above, luminescence quenching or enhancement upon aggregation depends on the packing of the molecules in the aggregates rather than being an intrinsic property of individual molecules. Unfortunately, the packing and organization behaviour is very difficult to predict and a trial and error approach is the usual way followed to achieve the desired properties. A common strategy to construct effective AIE gelators is to combine and conjugate classical AIE luminogens with classical suitable moieties for gel formation such as urea, amide and triazol ones (which possess very directional intermolecular hydrogen bonding), or hydrocarbonated residues, such as aliphatic chains or cholesterol, to promote hydrophobic interactions.⁶⁹

4.2.5.1 Hydrogels presenting AIE

Self-assembled luminescent hydrogels have received considerable attention due to their advanced applications as luminescent probes and biosensors. Fabrication of luminescent hydrogels with traditional luminophores is a difficult task and usually a dilemma due to the ACQ effect suffered by the self-assembled structures. For this reason, only punctual investigations have been reported on hydrogels. For example, Tang and co-workers nicely described the formation of an hydrogel with AIE from polymeric chitosan functionalized with TPE⁷⁰ (Figure 12). Another example are found in some salt-responsive peptide-derived hydrogels described by Liang et al.⁷¹

Figure 12. Chitosan-based TPE derivative and its hydrogels (2% w/w) under visible UV light (365 nm).⁷¹ 1 cm scale

4.2.5.2 Organogels presenting AIE

Organogels presenting AIE are more common than hydrogels.⁷² Examples include those species shown at Figures 13-15, which are AIEgens derivatives such as TPE,^{73,74} silole^{75,76} and cyanostilbene.^{77,78} Other organogels were designed by combination of conventional chromophores but only a few luminophores in solution became AIE active when aggregated, as in the case of dendritic⁷⁹ or carbazole⁸⁰ derived gelators.

Figure 13. Left: Cholesterol-based TPE derivative gelator presenting AIE upon gelation: **a)** Photoluminescence spectra of hot methanol solution and gel. **b)** Gel photograph under visible light and **c)** Under UV light. Right: Biphenyl triazol-based TPE derivative **d)** Emission spectra of in THF/water mixtures. **e)** Plot of emission peak intensity at 480 nm vs. water fraction. **Inset:** Photograph of TPE derivative under UV-light containing different water fractions.^{73,74}

Figure 14. Silole derived gelators presenting AIE upon gelation.^{75,76}

Figure 15. Cyanostilbene derived gelators presenting AIE upon gelation with SEM micrographs of xerogels.^{77,78}

4.2.6 Principal applications of AIE luminogens

Since 2001, extensive investigation on AIE has been carried out, leading to the appearance of very different technological applications for solid/aggregated state fluorophores. The AIEgens vanished the borders of concentration imposed by quenching in fluorescence measurements and expanded the versatility and practical applications of luminescent materials. High-tech applications of AIE luminophores are summarized in Figure 16. Classically, this kind of materials have been used as optoelectronic devices (OLEDs, OFETs, waveguides, CPLs, liquid crystals, photovoltaic components),^{81–87} but more recently they have been used as luminescent sensors (ions, pH, gases, explosives, peroxides, hazardous species, fingerprints, chirality, viscosity, conformation, self-assembly and morphology)^{88–90} or as biological probes (biomolecular sensing and biological imaging).^{91–95}

Figure 16. Typical examples of structural motifs of AIEgens and their technological applications.⁸

4.3 Novel 1,8-naphthalimide derived gels with AIE features

Synthesis, gelation abilities and photophysical properties of a series of 1,8-naphthalimide derived compounds are investigated and a new family of AIEgen gelators are presented.

4.3.1 Design and synthesis of 1,8-naphthalimide derived gelators

A set of derivatives with a 1,8-naphthalimine moiety were prepared to evaluate their possible gel forming capabilities. Compounds **1-3** present the naphthalimide unit linked to the dipeptide Gly-Val and compound **4** to valine. A priori and considering literature precedents, these compounds are good candidates as hydrogelators. Simple amino acid or dipeptide derivatives containing a hydrophobic aromatic unit such as, for example, Fmoc or naphthyl are described to be good supramolecular hydrogelators.⁹⁶ Additionally, compounds **5** and **6** are

bolaamphiphilic molecules derived from valine and related to gelators widely studied in our group.^{97–102} The preparation of these compounds included conventional peptide synthesis using benzyloxycarbonyl N-protection. The 1,8-naphthalimide units were introduced by reaction with 1,8-naphthalic anhydride. Further synthetic details are available at the experimental section of Chapter 3 and 4. Compound **7** represents a simple analogue that was prepared for comparison purposes and obtained by direct imide formation between 1,8-naphthalic anhydride and n-hexylamine (Figure 17).

Figure 17. Chemical structure of compounds used in the present chapter. TPE = Triphenylethene for comparison purposes.

The synthesis of the studied compounds **1–7** is outlined at Scheme 2. Peptide coupling methodology was used and compounds were obtained in general in gram scale with high yields without further chromatographic purification steps (see experimental section for details in Chapter 3 and 4). The synthetic pathway for gelators **1–6** starts with the activation of Cbz-N-protected L-valine with N-hydroxysuccinimide (NHS) in presence of dicyclohexylcarbodiimide (DCC). The activated ester is coupled to mono- or diamines in THF. Deprotection of Cbz group

was performed in CH_3OH by catalytic hydrogenolysis using Pd on activated carbon in H_2 atmosphere to yield the corresponding amines. In the case of compounds **1-3** the glycine unit was introduced by activation of the C-terminal moiety with ethyl chloroformate. Finally, the naphthalimide moiety was introduced coupling the corresponding amines 1,8-naphthalic anhydride in CH_3OH . For the preparation of gelator **3**, with a terminal carboxylic acid unit, esterification of C-terminal moiety of N-protected valine was performed in basic conditions (DMAP) using CH_3OH to obtain the intermediate ester which was hydrolysed at the final step. Compound **7** was synthesized by direct imide formation between 1,8-naphthalic anhydride and n-hexylamine.

Scheme 2. Synthesis of peptide-naphthalimide. *i)* NHS, DCC, THF, 0 °C, 4 h. *ii)* DMAP, THF, reflux 60 °C, 12 h. *iii_a)* THF, 4 h, 25 °C. *iii_b)* Et_3N , $\text{C}_3\text{H}_5\text{ClO}_2$, THF, 12 h. *iv)* H_2 , Pd/C, MeOH, 25 °C, 4 h. *v)* 4-nitro-1,8-naphthalic anhydride, CH_3OH , 75 °C, 8 h. *vii)* LiOH, $\text{H}_2\text{O}/\text{THF}$ 25 °C, 8 h.

4.3.2 Physical characterization of 1,8-naphthalimide derived supramolecular gels

4.3.2.1 Minimum gel concentration

Hydrogelation ability of all **1-7** compounds was tested by the tube inversion test. The gelation procedure consists on heating the gelator in the presence of water inside a screw capped vial (8 mL, diameter 1.5 cm) until complete solution and cooling down to room temperature. In the case of DMSO/H₂O mixtures, an alternative gelation method was assayed, consisting on sudden addition of water to solubilised gelators in DMSO. Compound **3** afforded a stable gel in water presenting a minimum gelator concentration (mgc) of 28 mM. Most of the compounds formed gels in H₂O/DMSO mixtures. Results of the aggregation capabilities are summarized in Table 1, revealing that subtle structural differences influence dramatically the gelation /precipitation/crystallization/solubilisation behaviour. Analysis of data in Table 1 shows that the presence of the dipeptidic unit GlyVal is a requisite for gelation (compounds **1**, **2** and **5**). Compounds **4** and **6**, which are respectively analogues of **1** and **5**, presenting a Val amino acid unit instead the dipeptidic moiety GlyVal, were found not to form gels and showed swollen amorphous aggregates. Compound **4** underwent fibrillar precipitate formation after *ca.* 5 hours. Compound **7** formed turbid suspensions.

Table 1. Mgc and morphology of studied compounds in H₂O and H₂O/DMSO (8:2) mixtures.

Compound	H ₂ O	H ₂ O/DMSO*	Morphology [¥]
1	Insoluble	3 mM	F
2	Insoluble	3.4 mM	F
3	28 mM	Soluble	F
4	Insoluble	C	FP
5	Insoluble	10 mM	F
6	Insoluble	AP	AP
7	Insoluble	NP	NP
TPE	Insoluble	C	AP

FP = Fibrillar precipitate ; F = Fibrillar; AP= Amorphous precipitate; NP = Nanoparticles; C = Crystals; *20% DMSO,

[¥] From TEM microscopy

Additionally, **1**, which is the most efficient hydrogelator, was also tested in several organic solvents revealing to be a versatile organogelator which forms translucent colorless gels in a wide range of solvents (table 2).

Table 2. Mgc of **1** in different solvents

Solvent	Mgc / mg·ml ⁻¹ (mM)
CH ₃ CN	1.54 ± 0.02 (3.9)
CH ₃ OH	1.82 ± 0.06 (4.6)
THF	2.3 ± 0.2 (5.9)
DMSO	6 (15.2)
DMSO/H ₂ O (2:8)	1.18 (3)
<i>n</i> -BuOH	1.8 ± 0.1 (4.5)
CHCl ₃	4.0 ± 0.3 (10.9)
CH ₂ Cl ₂	5.5 ± 0.5 (13.9)
AcOEt	2.3 ± 0.3 (5.8)

4.3.2.2 Electron microscopy

Morphological features of different aggregates were studied by means of electron microscopy. Gels presented fibrillar nano-structures, as can be seen in Figures 18 and 19. Compound **1** showed fibrillar bundles with high aspect ratio and a remarkable homogeneous width of *ca.* 20 ± 3 nm. Similarly, **2** also showed highly crosslinked fibrillar structures but more randomly ordered and thinner than those of compound **1**. Finally, an entanglement of short elongated objects was observed for compound **5** (Figure 20). The observed morphologies can be somewhat correlated with the minimum gel concentration values reported. The compounds with highest aspect ratio, gels from **1** and **2**, present a lower mgc value than that of **5** (*ca.* 3 mM vs 10 mM respectively). This agrees with what has been reported: the highest aspect ratio, the lowest the mgc. The reason is that crosslinking of long thin fibres leads to effective solvent pool trapping as a result of solvent-gelator interactions and surface tension forces.¹⁰³

Figure 18. TEM images of the macroscopic gel network of **1** (2 mM) in a DMSO/H₂O (2:8) mixture.

Figure 19. TEM images of the macroscopic gel network of **2** (2 mM) in a DMSO/H₂O (2:8) mixture.

Figure 20. TEM images of the macroscopic gel network of **5** 2mM in DMSO/H₂O (2:8) mixture.

On the other hand, amorphous structures were detected for compounds **3**, **4** and **6** upon examination with TEM (Figure 21). Interestingly, after several days, the wet aggregates of compound **4** evolved to needle-like crystalline structures and compound **1** formed crystals from a solution in a 1,1,1,3,3,3-hexafluoro-2-propanol/CHCl₃ mixture (see XRD section 4.3.2.4).

Figure 21. TEM images of amorphous precipitates of compounds **3** (A), **4** (B), and **6** (C) at 2 mM in DMSO/H₂O (2:8).

Electron microscopy images at Figure 22A shows how TPE forms mixed phases of crystalline aggregates and amorphous precipitate. Figure 22B interestingly illustrates how compound **7** assembles in relatively monodisperse molecular nanoparticles of a diameter of $d = 30 \pm 5$ nm.

Figure 22. TEM images of crystals of TPE (A) and nanoparticles **7** (B) at 2 mM in a DMSO/H₂O (2:8) mixture.

4.3.2.3 Confocal laser scanning microscopy

The observation of the fibrillar structure of a supramolecular assembly *in situ* (without drying the solvent) is a challenging task. Hamachi et al. recorded for the first time confocal laser scanning images of a supramolecular gel doped with a fluorescent probe.¹⁰⁴ Due to fluorescent properties of gelator **1**, the fibrillary structure could be observed by fluorescence optical microscopy (Figure 23a) and confocal laser scanning microscopy (Figure 23b left). When TPE and **1** are compared in the same sample, similar emission is recorded but very different morphologies can be observed (Figure 23b right).

Figure 23a. Fluorescence optical images of the macroscopic gel network of **1**; [**1**] = 2 mM in DMSO/H₂O mixture (20/80). **Inset:** Images of gel of **1** (3.8 mM) under ambient light (**left**) and under UV lamp excitation ($\lambda_{\text{ex}} = 365 \text{ nm}$) (**right**).

Figure 23b. CLSM images of the macroscopic gel network of **1** 2 mM (**left**) and of **1** in presence of TPE (**right**) in DMSO/H₂O (20:80) mixture $\lambda_{\text{ex}} = 405 \text{ nm}$ $\lambda_{\text{em}} = 410\text{-}500 \text{ nm}$.

4.3.2.4 Rheology

Gels of **1** presented interesting mechanical properties, being strong gels which can undergo syneresis and be extracted easily from the vial. These gels showed remarkable self-healing thixotropic properties. As can be seen in Figure 25, when two gels individually formed (one of them stained with methylene blue dye) are placed one on top of the other, perfect fusion was

observed after several minutes. Rheological studies showed differences between gels of **1** formed in different DMSO/H₂O mixtures. A stress sweep was carried out at a frequency of 1 Hz (linear viscoelastic regime, Figure 24C). It can be seen how the gels formed with less amount of DMSO are stronger, showing $\tan(\delta) = 14.6$ for 20% DMSO versus $\tan(\delta) = 4.3$ for 50% DMSO (Figure 24A-B). The thixotropic behavior of gels of **1** was studied by monitoring the elastic and viscous modules (G' and G''). After applying a strong stress (25 Pa creep) for 5 seconds, the recovery of G' and G'' was again monitored. It can be observed how in the first few seconds after the gel breakage ($t = 150$ s), G'' is above G' . Therefore, after 10 s ($t = 160$ s), the viscoelastic behavior of the sample was recovered. It has to be noted that the gel does not restore its initial rheological features, probably because more time is required and possible centrifugation of sample out of the rheometer when applying the creep experiment occurs (Figure 24D).

Figure 24. A) Stress sweep (1 Hz) showing elastic modulus G' for gels of **1** in different proportions of DMSO/H₂O mixtures; B) Stress sweep (1 Hz) for a gel of **1** in aqueous solvent mixture containing 20 % DMSO; C) Frequency sweep (1 Pa) showing elastic modulus G' for gels of **1** in different proportions of DMSO/H₂O; D) Evolution of elastic and viscous modules with the time of a gel of **1** in DMSO/H₂O (50% DMSO) by applying constant stress of 1 Pa and 1 Hz. Creep of sample was applied when $t = 140$ s for 5 s at 25 Pa to observe the recovery in 5 minutes. [**1**] = 3.8 mM in all experiments.

Figure 25. Gels of **1** at 3mM presenting self healing features. **Top row:** under visible light; **bottom row:** under UV light.

4.3.2.5 X-Ray Diffraction (XRD)

XRD is a very powerful tool to assess the structural characteristics of crystalline structures. Considering that compound **1** provides gels in aqueous media and crystallizes in chloroform, XRD analysis was performed to assess if there is similar molecular arrangement in both cases. On the one hand, xerogels of **1** from methanol and H₂O/DMSO gels showed similar diffractograms with broad peaks at periodic distances around 12 Å and 7.7-6.1 Å (Figure 26). On the other hand, the diffractogram of crystals obtained from chloroform present sharp peaks with repeating distances of 17.1, 11.1, 8.7, 7.3, 6.8, 5.8, and 4.6 Å. Therefore the gel network presents a rather amorphous structure and seems to present a rather different packing to that found in the crystals. This result is not surprising considering that polymorphism is a well known issue in crystal engineering and also has been described,¹⁰⁵ although quite less studied in supramolecular gels.^{106,107}

Figure 26. XRD of **A)** xerogels of **1** in CH₃OH (blue) and DMSO/H₂O mixture (red); **B)** crystalline phase of compound **1**.

4.3.3 Photophysical characterization

4.3.3.1 Solvent-promoted aggregation induced emission

The solvent plays a crucial role in any aggregation process. For example, by altering solvent polarity is possible to induce aggregation or fibrillization processes. In the case of compound **1**, aggregation can be promoted by addition of water (poor solvent) to a DMSO solution (good solvent), giving rise to an AIE process. In the case of compound **1**, the aggregation process could be followed recording absorption spectra, which show a shift in the maximum and a shape change (figure 27). Also, fluorescence studies reveal significant changes, resulting in emission intensity enhancement and a shift of the maximum emission wavelength (λ_{max}) (Figure 28).

Figure 27. Absorption spectra of **1** ([**1**]=0.25 mg/mL) at different ratios of DMSO/H₂O (from 0% to 80% water content), measured in suprasil quartz 1 cm path length cuvette.

Figure 28. Fluorescence emission spectra of **1** ([**1**] = 0.51 mg/mL) at different ratios of DMSO/H₂O (from 0 % to 50 % water content), λ_{ex} =330 nm. Measured in suprasil quartz triangular cuvette. 85 μ L of **1** stock solution in DMSO is diluted with the correspondent amount of DMSO and then with water (V_{tot} = 500 μ L). **Inset:** vials at different water content under UV light (λ_{ex} = 365 nm).

4.3.3.2 Aggregation induced emission with concentration

The formation of aggregates of **1** with increasing concentration was studied by means of fluorescence emission spectroscopy. Figure 29 represents the observed variations of λ_{max} and fluorescence intensity upon changing the concentration of **1**. It can be clearly seen that when the concentration exceeds the solubility product (K_s) of the compound in the aqueous mixture (DMSO 10%), which is around $1 \cdot 10^{-5}$ M, the fluorescence intensity undergoes notable trend change and experiments a high emission enhancement until $1 \cdot 10^{-4}$ M. At this point the slope is softened because the AIE effect balances with the inner filter effect commonly observed in optically dense solutions/dispersions.¹⁰⁸ The value of λ_{max} is also shifted upon increasing concentration ($\Delta\lambda = 14$ nm), following a similar sigmoidal variation. The close analysis of the graphics at figure 29 shows that the shift of λ_{max} reaches a maximum at the same concentration where the onset of emission enhancement takes place, around $1 \cdot 10^{-5}$ M. This result points out that although nano-aggregates are formed at this concentration (the species show a shift in λ_{max}) they do not experiment an AIE process. It seems that only the sorting in a higher aggregation level (fibrillar) is able to block efficiently the intramolecular motions leading to a radiative relaxation pathway.

Figure 29. Left: Emission maximum shift of **1** in H₂O/DMSO mixture (9:1) ($\lambda_{\text{ex}} = 342$ nm). Right: Fluorescence intensity enhancement of compound **1** (AIE) ranging from 0.5 μ M to 100 μ M.

Taking this phenomenon in consideration, the variation of compound concentration can control the critical amount of water content necessary to induce the aggregation, as can be seen on figure 30. Changing the concentration of **1** from 0.2 mM to 6.5 mM, progressively shifts the onset of aggregation (and appearance of AIE) from 45 % to 20 % of water in DMSO.

This effect could be practically used to solve a common problem in bioassays optimization as is the determination whether the amount of water present in DMSO solutions used for biological testing is above a given threshold limit.¹⁰⁹

Figure 30. Left: Normalized fluorescence intensity vs % DMSO in DMSO/H₂O mixtures with different concentrations of **1** ([**1**] = 0.2, 0.5, 1.3, 2.6 and 5.1 mg/mL). Measurements done with triangular cuvette, λ_{ex} =330; $\lambda_{\text{em max}}$ = 403 nm. Starting with 500 μL of DMSO stored with molecular sieves and adding 10 μL of H₂O. **Right:** Critical % of water in DMSO to trigger the AIE vs the variation of concentration of **1** (Calculated from the inflection point of the represented sigmoidal curves in A).

4.3.3.3 Comparison of aggregation induced emission in a small library of 1,8-naphthalimide derivatives

A comparison of the spectra obtained for soluble species and for the aggregates formed at a concentration of 3 mM in DMSO:H₂O 80:20 was carried out for all the synthesized compounds (Figure 31 and table 3)

Figure 31. Left: Evolution of fluorescence emission spectra of studied compounds 3.16 mM in DMSO when adding H₂O, fluorescence raises as H₂O content increases in all cases. **Right:** Absorption spectra of studied compounds in DMSO (black line) and DMSO/H₂O (blue line).

In this solvent mixture, all the compounds except **3** and **4** show aggregation. Aggregation induced hypochromism as well as a bathochromic shift was detected in all cases (see Table 3). Regarding fluorescence, remarkable AIE was observed (see discussion below) and, noticeably, rather different emission spectra (Figure 32) were obtained respectively for studied compounds: those soluble (**3** and **4**), those forming amorphous aggregates (**6** and **7**) and those giving place to fibrillar objects (gelators **1**, **2** and **5**). A more resolved vibrational structure, attributed to free, non-aggregated species was recorded for **3** and **4**, presenting $\lambda_{\text{em max}} = 392$ and 395 nm, respectively. On the other hand, non-fibrillar aggregates formed by **6** and **7** emitted with $\lambda_{\text{em max}} = 458$ and 462 nm, respectively, which was attributed to excimer formation.

Figure 32. Summary of different aggregation modes of 1,8-naphthalimides observed by means of fluorescence spectroscopy ($\lambda_{\text{ex}} = 340$ nm). Dashed line: S = Soluble species ; straight line: F = Fibrillar aggregates; dotted line AP = Amorphous precipitate excimer like emission. From left to right: **3**, **4**, **1**, **2**, **5**, **6** and **7**.

The formation of excimers by the aggregation of 1,8-naphthalimide aggregation in organic solvents has been reported previously.^{53,110} Finally, no excimer emission was detected for the fibrillar aggregates formed by the gelators **1**, **2** and **5**. In these compounds, $\lambda_{\text{em max}}$ were respectively 405, 405 and 409 nm, presenting a moderate Stokes shift of around 10 nm when compared to soluble fluorophores **3** and **4**.

Table 3: Spectroscopic characterization of studied compounds and packing behaviour observed by TEM microscopy.

Compound	DMSO		DMSO/H ₂ O			Solid
	$\lambda_{\text{abs}} / \text{nm}$	$\lambda_{\text{em}} / \text{nm}$	$\lambda_{\text{abs}} / \text{nm}$	$\lambda_{\text{em}} / \text{nm}$	Emitting species	$\lambda_{\text{em}} / \text{nm}$
1	335	391	342	405	A	415
2	335	391	341	405	A	412
3	335	391	341	392	M	453
4	336	391	341	395	M	442
5	338	402	339	409	A	432
6	336	391	343	458	E	469
7	335	391	341	461	E	471
TPE	307	452	335	462	E	453

M= Monomer, E= Excimer, A= Fibrillar aggregates

To study quantitatively the AIE behaviour, relative fluorescence quantum yields were evaluated taking 9,10-diphenylanthracene in cyclohexane as emission standard ($\Phi_f = 0.97$).¹¹¹ Measurements were carried out in DMSO and DMSO/H₂O mixtures (5 % DMSO), at a concentration of 50 μM . Absolute quantum yields were also determined in solid state for all compounds (Table 4). Additionally, results were compared with those from TPE, which has been extensively studied in the literature as a molecule experiencing AIE.^{20,112}

In DMSO solution, all the compounds but **5** show poor emission, having $\Phi_f < 0.003$. It has to be noted that compound **5** ($\Phi_f = 0.06$) forms aggregates in DMSO even in the absence of water. In general the amount of water is raised to 95 %, Φ_f experiences a notable increase, reaching values between 0.10 and 0.30, except for compound **3** which does not aggregate. The highest quantum yields were observed for compounds **1**, **2** and **4**. The first two are good gelators and the latter forms fibrillar aggregates (Figure 33). The ratio between Φ_f in aggregated state over Φ_f in solution is defined as α factor.⁸³ The α factor values for **1**, **2** and **4** are 132, 115 and 96 respectively, that means that these compounds present a superior AIE behaviour than the widely studied AIEgen TPE ($\alpha = 61$).⁸³ Gelation capabilities seem to play a crucial role in the enhancement of the emission since α values of non-gelating molecules **3**, **6** and **7** (4, 34 and 61, respectively) are far way below the previous ones. Compound **5** represents an exception,

and despite being a hydrogelator shows poor AIE (Table 4). Seemingly, the arrangement of the fluorophores in the aggregates formed by bolaamphiphilic compound **5** is such that non radiative decay is predominant.

Figure 33. Relative fluorescence quantum yield in mixtures DMSO/H₂O dispersion (stripped blue bars); Absolute fluorescence quantum yields in solid samples (green solid bars).

In another set of experiments, λ_{max} of emission was determined for systems with a variable ratio of DMSO/water, using a constant concentration of 3.16 mM for the studied compounds **1-7** and TPE. Firstly, Figure 34 shows results for a given solvent composition of 50 % water. The emission of compound **1** is shown to be several times higher than that of **3-7** and TPE and almost twice than that of **2**. The critical water content that promotes AIE was calculated from the inflection point of sigmoid fits (Figure 35). Compounds **1** and **2** showed AIE for a water content above 20 % while compounds **6** and **7** required higher water percentages. Opposite extreme behaviour was shown by non-aggregating compounds **3-4** and compound **5**, which already formed aggregates in 100 % DMSO (Figure 35).

Figure 34. Fluorescence intensity of the compounds at the same concentration (3.16 mM) in DMSO/H₂O (1/1) mixture. Measurements done with triangular cuvette, λ_{ex} on the absorption maxima of each compound (the naphthalimide-

based compounds at 330 nm and the tetraphenylethene at 350 nm) λ_{em} recorded at the emission maxima of each compound. Inset: Picture of vials containing studied compounds at 1,54 mM under UV-light (365 nm).

Figure 35. Fluorescence intensity vs % DMSO in for compounds at the same concentration (3.16 mM) in different DMSO/H₂O mixtures). Measurements done with triangular cuvette; λ_{ex} on the absorption maxima of each compound (the naphthalimide-based compounds at 330 nm and the tetraphenylethene at 350 nm); λ_{em} recorded at the emission maxima of each compound. For each compound, process is started with 100 % DMSO previously dried with molecular sieves and add $\Delta Vol = 40 \mu L$ of H₂O.

Fluorescence lifetimes also showed notable increase when passing from solution ($\tau_f < 0.1$ ns) to aggregated state. All observed decays could be fitted to biexponential decay and showed different behaviours depending on the aggregation mode (Figure 36 and table 4). For fibrillating compounds **1**, **2**, **4** and **5**, decays are dominated by the shorter component of the biexponential fit, with values ranging from to $\tau_f = 0.327$ to 1.97 ns. On the other hand, non-fibrillating compounds **6** and **7**, showed much longer lifetimes ($\tau = 18.7$ and 23.5 ns), dominated by the second component of the biexponential decay lifetimes. The existence of longer emission lifetimes confirms the excimeric nature of the emitting species at $\lambda > 450$ nm. Thus, Majima and co-workers have described excimeric species of 1,8-naphthalimides with emission

lifetimes in the range between 8-16 ns.^{110,113–115} Analogously, Pischel, Barberan-Santos and co-workers have reported long wavelength emissive species in a 1,8-naphthalimide dyad with averaged emission lifetime > 20 ns, identified as an excimer.⁵³

Figure 36. Fluorescence lifetimes of studied 1,8-naphthalimide derivatives in DMSO/H₂O mixtures (5/95). Inset: Extension of short lifetime region showing decays of gelating compounds. λ_{ex} = 303 nm; λ_{em} = emission maxima for each compound shown on table 4.

Compound **4** has been identified as a special case. Interestingly, the initial amorphous aggregates displaying excimer emission at 460 nm evolve with time into a fibrillar crystalline solid. This process was followed by steady state and time resolved fluorescence spectroscopy (Figure 37). On the first stage of aggregation process, when water addition to a solution of **4** in DMSO is performed, the emission profile shows the presence of excimeric species (amorphous aggregates) showing λ_{em} = 460 nm and predominantly long lifetimes (τ_f = 19.8 ns). After a few hours (or several seconds by sonication), the system rearranges to fibrillar aggregates, showing λ_{em} = 410 nm and shorter lifetimes (τ = 3.35 ± 0.09) ns, analogous to those found in the fibrillar aggregates of **1**, **2**, and **5** (Table 4).

Figure 37. Fluorescence lifetime analysis (**left**) and fluorescence emission spectra (**right**) of compound **4** (3 mM) in DMSO/H₂O mixture (5:95) at different times, showing typical excimer emission maximum and lifetimes at $t = 0$. As time passes, increase of short lifetime contribution and a change in emission maximum is observed (fibrillary) as rearrangement takes place ($t_{\text{final}} = 5$ h).

Table 4: Fluorescence quantum yield and lifetimes of studied compounds in aggregated and in solution state.

Comp.	τ_f (DMSO/H ₂ O) / ns	λ_{em} / nm	Φ_f^* (DMSO/H ₂ O)	Φ_f^* (DMSO)	Φ_f^{**} (Solid)	α
1	1.97 ± 0.04 (80%) 5.24 ± 0.16 (20%)	405	0.304	0.0023	0.285	132
2	1.47 ± 0.02 (92%) 6.8 ± 0.4 (7%)	405	0.301	0.0026	0.058	115
3	> 0.1	392	0.013	0.0030	0.089	4
4	0.716 ± 0.04 (64%) 3.35 ± 0.09 (35%)	410	0.250	0.0026	0.092	96
5	0.327 ± 0.02 (45%) 1.31 ± 0.05 (55%)	409	0.126	0.0554	0.060	2
6	3.23 ± 0.17 (23%) 18.7 ± 0.2 (76%)	458	0.082	0.0030	0.057	27
7	0.38 ± 0.1 (3%) 23.5 ± 0.1 (97%)	462	0.199	0.0027	0.114	73
TPE	3.9 [‡]	462	0.195	0.0032	0.137	61

α = ratio between Φ_f in aggregated state over Φ_f in solution. *Relative quantum yield in aqueous suspension (5% DMSO) and in pure DMSO calculated as: $\Phi_f = \Phi_{\text{st}} \cdot (A_{\text{st}}/A_{\text{d}}) \cdot (F_{\text{d}}/F_{\text{st}}) \cdot (n_{\text{d}}^2/n_{\text{st}}^2) \cdot (D_{\text{d}}/D_{\text{st}})$, Taking 9,10-Diphenylanthracene (DPA) in cyclohexane as reference $\Phi_{\text{st}} = 0.97$; A = Absorbance at Ex wavelength; F = Area of emission spectrum; n = Refractive index value of solvent; D = Dilution ratio. Absorption spectra measured at 50 μM for all compounds and fluorescence spectra measured at 10 μM $\lambda_{\text{ex}} = 340$ nm. **Absolute quantum yield of solids using integrating sphere calculated as $\Phi_f = S_2/(S_0 - S_1)$, where S_2 = Area emitted from sample; S_0 = Area from incident light; S_1 = Area scattered from sample. [‡]Lifetimes of TPE in crystal solid state from reference.²²

Absolute fluorescence quantum yield in dry solids was also investigated and showed a reduction of the emission ability compared to aggregates in the presence of solvent (Table 4 and Figure 33). This behaviour could be attributed to the collapse of aggregates, leading to specific interactions responsible of an enhancement of non radiative pathways. An exception is compound **3**, which experiences fluorescence emission enhancement upon drying since it is soluble in 100 % water content and does not aggregate in the solvent mixture used.

All these results highlight the importance in AIE of weak intermolecular interactions, molecular flexibility and molecular relative disposition in aggregated states. Unfortunately, further insight in the structure of the aggregates could not be obtained by X-ray diffraction studies of the dry samples, as they showed poor crystallinity.

4.3.3.4 Stimuli-responsiveness

Molecular gels are known to be a versatile stimuli-responsive materials.¹¹⁶ Considering the gels studied here, it was hypothesized that fluorescence emission could be modulated in response to different salt species and temperature.

Salt content

The effect of different salts in the increase or decrease of the solubility of proteins was studied in the late XIX century by Frank Hofmeister.¹¹⁷ The so-called Hoffmeister effect has shown to be applicable to different systems such as self-assembled molecular gels or polymers.^{101,118} Classification was made by studying the ability to promote protein aggregation and precipitation of different salts. Generally, soft ions with a low density charge that are weakly hydrated promote solubilisation and are called chaotropes. On the other hand, hard ions with a high density charge that are highly hydrated possess a kosmotropic character and favour precipitation. Recent theories explain these phenomena in terms of accumulation/exclusion of ions at the solute–water hydrophobic–hydrophilic interfaces, which suggests direct ion–solute interactions for chaotropes.^{119–121}

An experiment to evaluate the effect of a strong kosmotropic anion, SO_4^{2-} , in the aggregation of **1** was devised. This effect was compared to the results obtained in the presence of Cl^- ,

which is an intermediate anion in the Hofmeister series. In both cases, the counter-cation used was sodium. Contrary to the expectations, experimental results show only a small shift in the amount of water required for AIE to take place when a high concentration of SO_4^{2-} was used (ca. 1M). In the case of Cl^- , the critical concentration of water in DMSO to promote AIE was unaffected (Figure 38). The relative insensitivity against a strong precipitating anion such as SO_4^{2-} can be rationalized considering the high amount of DMSO present in the system, which disturbs the hydrogen bonding network present in pure water. Nevertheless, the invariability of the AIE effect to the presence of salts could be considered a positive fact, as the analysis of water content by AIE with this system would not depend on the salinity of the water present in the medium.

Figure 38. Fluorescence intensity vs % DMSO for compound **1** at 0.531 mg/mL in DMSO/salty H_2O mixtures containing different concentrations of Na_2SO_4 and NaCl ($[\text{Na}_2\text{SO}_4]$ = 2.24, 22.4 and 224 mg/mL; $[\text{NaCl}]$ = 0.2, 10, 20, 35 and 100 mg/mL). Measurements done with triangular cuvette; λ_{exc} = 330 nm; $\lambda_{\text{em max}}$ = 403 nm. Starting with 460 μL of DMSO stored with molecular sieves and adding increasing amounts of H_2O (ΔVol = 10 μL).

Temperature

Temperature is an external stimulus able to switch on/off the aggregate formation and to turn on its fluorescence emission. T_{gel} of **1** was determined to be 70 °C by the tube inversion method in a thermostatic bath. Figure 39 shows how the fluorescence intensity correlates well with the aggregation/solution processes. Experiments were carried out in *n*-butanol and in a DMSO/H₂O mixture (20% DMSO). In both cases, heating conduces to a progressive quenching of the fluorescence emission due to gel disassembly. It has to be noted that temperature regulation of the fluorescence in this system brings the opportunity of uses related with optoelectronic applications as well as sensing approaches.

Figure 39. **A)** Fluorescence emission spectra of **1** ($[1] = 0.25$ mg/mL) with 80% H₂O at different temperatures ($\lambda_{\text{ex}} = 330$ nm). Measured in suprasil quartz 1 cm pathlength cuvette. 85 μ L of **1** stock solution in DMSO is diluted with the correspondent amount of DMSO and, then, with water. **B)** Fluorescence emission spectra of **1** ($[1] = 2.4$ mg/mL) in butanol at different temperatures ($\lambda_{\text{ex}} = 335$ nm). Measured in suprasil quartz 1 cm pathlength cuvette. 85 μ L of stock **1** solution in DMSO is diluted with the correspondent amount of DMSO and, then, with water.

4.4 Conclusions

In summary, AIE shown by several derivatives of 1,8-naphthalimide has been studied. Some of the derivatives form molecular hydrogels constructing self-assembled sample spanning networks visualized by TEM. Formation of self-assembled gels of 1,8-naphthalimide in aqueous media is unprecedented and paves the way for biomedical related applications. It is observed that the fibrillar assembly is associated to AIE, with emission at *ca.* 405 nm. Notably, compounds showing non-fibrillar aggregation also experience AIE, but their emission is shifted to *ca.* 460 nm, which can be ascribed to excimer formation. Therefore, the aggregation mode modulates the wavelength of the emitted light. The different aggregation patterns of the 1,8-naphthalimide derivatives are also reflected in notable changes of the excited states lifetimes. Additionally, quantification of the emission measuring quantum yields of the different colloids indicates that fibrillar aggregation affords stronger AIE effect in comparison to amorphous aggregates. Most likely, two different spatial arrangements of the 1,8-naphthalimide units in the aggregates produce the observed results. Moreover, it has been found that aggregation can be triggered by water addition to DMSO solutions. In this way, AIE is achieved at critical humidity content in DMSO. The humidity threshold that produces AIE can be modulated by variation of the concentration of the fluorophore. The capabilities of these systems to act as humidity threshold sensors in DMSO might be used as a simple tool to detect a possible excess of water in samples used for bioassays. Finally, aggregation is found to be thermoreversible in the range 25–80°C, affording temperature regulated fluorescence based on the AIE effect. The stimuli responsiveness (low temperature gel-sol transition, and high sensibility for water presence), very interesting viscoelastic behaviour (capacity to construct self-healing and thixotropic thin films or bulky gels) and highly tuneable emissive features make this new family of materials very attractive for future applications in optoelectronics or sensing.

4.5 Experimental section

4.5 Experimental section

4.5.1 Synthesis

4.5.2 Gel preparation

4.5.3 UV-Vis spectroscopy

4.5.4 Fluorescence spectroscopy

4.5.5 X-ray diffraction

4.5.6 Transmission electron microscopy (TEM)

4.5.7 Confocal Laser Scanning microscopy (CLSM)

4.5.1 Synthesis

Scheme 3. Scheme 2. Synthesis of peptide-naphthalimide. *i)* NHS, DCC, THF, 0 °C, 4 h. *ii)* DMAP, THF, reflux 60 °C, 12 h. *iii_a)* THF, 4 h, 25 °C. *iii_b)* Et₃N, C₃H₅ClO₂, THF, 12h. *iv)* H₂, Pd/C, MeOH, 25 °C, 4 h. *v)* 4-nitro-1,8-naphthalic anhydride, CH₃OH, 75 °C, 8 h. *vii)* LiOH, H₂O/THF 25 °C, 8 h.

Experimental details of synthetic procedures were described in the experimental section of Chapter 3 (Section 3.5.1).

Compound 4.9

NMR spectra were consistent with those described in the literature.¹²²

^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.46 – 7.21 (m, 5H), 5.42 (d, J = 8.3 Hz, 1H), 5.10 (s, 2H), 4.36 – 4.18 (m, 1H), 3.71 (s, 3H), 2.14 (td, J = 12.8, 6.3 Hz, 1H), 0.95 (d, J = 6.8 Hz, 3H), 0.88 (d, J = 6.9 Hz, 3H).

Compound 4.10

NMR spectra were consistent with those described in the literature.¹²³

^1H NMR (500 MHz, CDCl_3) δ (ppm): 3.73 (s, 3H), 3.38 (d, J = 4.9 Hz, 1H), 2.11 – 2.01 (m, 1H), 0.98 (d, J = 6.9 Hz, 3H), 0.92 (d, J = 6.9 Hz, 3H).

Compound 4.11

NMR spectra were consistent with those described in the literature.¹²⁴

^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.32 – 7.22 (m, 4H), 7.08 (d, J = 7.6 Hz, 1H), 6.04 (t, J = 5.4 Hz, 1H), 5.08 (s, 2H), 4.50 (dd, J = 8.8, 5.3 Hz, 1H), 3.89 (d, J = 4.9 Hz, 2H), 3.65 (s, 3H), 2.09 (dq, J = 13.4, 6.7 Hz, 1H), 0.85 (dd, J = 10.9, 6.9 Hz, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 172.4, 169.4, 156.7, 136.3, 128.4, 128.1, 127.9, 67.0, 57.2, 52.1, 44.4, 31.1, 18.9, 17.8.

Compound 4.12

NMR spectra were consistent with those described in the literature.¹²⁵

^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.79 – 7.60 (m, 1H), 4.52 (dd, $J = 9.0, 5.1$ Hz, 1H), 3.71 (s, 2H), 3.37 (s, 2H), 2.17 (td, $J = 13.5, 6.8$ Hz, 1H), 0.92 (t, $J = 6.9$ Hz, 6H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 172.8, 172.5, 56.7, 52.0, 44.7, 31.1, 19.0, 17.8.

Compound 4.13

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 8.59 (d, $J = 8.4$ Hz, 1H), 8.49 (t, $J = 7.3$ Hz, 4H), 7.89 (t, $J = 7.7$ Hz, 2H), 4.77 (s AB system, $J = 16.1$ Hz, 2H), 4.23 (dd, $J = 8.0, 6.8$ Hz, 1H), 3.66 (s, 2H), 2.05 (td, $J = 13.4, 6.7$ Hz, 1H), 0.91 (dd, $J = 12.1, 6.8$ Hz, 6H). ^{13}C NMR (126 MHz, d_6 -DMSO) δ (ppm): 172.4, 167.4, 163.7, 135.0, 131.8, 131.3, 127.9, 127.7, 122.3, 57.9, 52.2, 42.5, 30.7, 19.4, 18.7. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{20}\text{H}_{20}\text{N}_2\text{O}_5\text{Na}^+$ 391.1270; found 391.1268 $[\text{M}+\text{Na}]^+$ ($\Delta = 0.5$ ppm).

Compound 4.3

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.50 (ddd, $J = 6.2, 4.6, 1.0$ Hz, 4H), 8.46 (d, $J = 8.8$ Hz, 1H), 7.89 (dd, $J = 8.2, 7.3$ Hz, 2H), 4.77 (s AB system, 2H), 4.19 (dd, $J = 8.7, 5.9$ Hz, 1H), 2.06 (dq, $J = 13.5, 6.8$ Hz, 1H), 0.91 (dd, $J = 6.8, 2.7$ Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 173.34, 167.27, 163.72, 135.04, 131.84, 131.35, 128.05, 127.77, 122.35, 57.76, 42.53, 30.64, 19.62, 18.46. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_5\text{Na}^+$ 377.1113; found 377.1113 $[\text{M}+\text{Na}]^+$ ($\Delta = 0.0$ ppm).

Compound 4.14

NMR spectra were consistent with those described in the literature.¹²⁶

¹H NMR (300 MHz, *d*₆-DMSO) δ (ppm): 7.43 (t, *J* = 7.2 Hz, 1H), 7.39 – 7.27 (m, 5H), 6.73 (s, 1H), 5.05 (s, 2H), 3.85 (t, *J* = 17.2, 9.0 Hz, 1H), 3.63 – 3.48 (m, 1H), 1.95 (sext, *J* = 6.0, 9.0, 12.0, 21.0 Hz, 1H), 1.70 (t, *J* = 12.1 Hz, 4H), 1.60 – 1.45 (m, 1H), 1.38 – 1.05 (m, 5H), 0.87 (d, *J* = 6.1 Hz, 3H), 0.84 (d, *J* = 6.1 Hz, 3H). ¹³C NMR (75 MHz, *d*₆-DMSO) δ (ppm): 169.6, 136.8 (C=O), 127.9 (x3) (CH), 127.3 (C), 127.1 (x2) (CH), 65.1 (CH₂), 60.0, 47.2 (CH), 32.0, 31.8 (CH₂), 30.2 (CH), 24.9, 24.0 (x2) (CH₂), 18.7, 17.7 (CH₃). HR ESMS: *m/z*: calcd. for C₁₉H₂₈N₂O₃: 333.2178; found: 333.2173 [M + H⁺].

Compound 4.15

NMR spectra were consistent with those described in the literature.¹²⁶

¹H NMR (500 MHz, *d*₆-DMSO) δ (ppm): 7.59 (d, *J* = 7.7 Hz, 1H), 3.63 – 3.48 (m, δ 1H), 3.48 (br, 2H), 2.87 (d, *J* = 5.3 Hz, 1H), 1.86 – 1.75 (m, 1H), 1.74 – 1.60 (m, 4H), 1.58 – 1.50 (m, 1H), 1.32 – 1.21 (m, 2H), 1.21 – 1.09 (m, 3H), 0.85 (d, *J* = 6.8 Hz, 3H), 0.77 (d, *J* = 6.8 Hz, 3H). ¹³C NMR (126 MHz, *d*₆-DMSO) δ (ppm): 173.5 (C=O), 59.9 (CH), 48.6 (CH₃OH), 47.1 (CH), 32.5, 32.4 (CH₂), 31.8 (x2) (CH, CH₂), 25.2, 24.5 (CH₂), 19.5, 17.2 (CH₃). HR ESMS: *m/z*: calcd. for C₁₁H₂₂N₂O: 199.1810; found: 199.1813 [M + H⁺].

Compound 4.4

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.50 (ddd, $J = 7.3, 5.6, 1.1$ Hz, 1H), 7.89 (dd, $J = 8.3, 7.3$ Hz, 1H), 7.79 (t, $J = 5.6$ Hz, 1H), 5.03 (d, $J = 9.2$ Hz, 1H), 3.06 – 2.84 (m, 1H), 2.80 – 2.65 (m, 1H), 1.39 – 1.24 (m, 1H), 1.19 (t, $J = 7.7$ Hz, 1H), 0.72 (t, $J = 7.4$ Hz, 1H), 0.62 (d, $J = 7.0$ Hz, 1H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 168.7, 164.1, 134.8, 131.8, 131.3, 128.2, 127.8, 122.8, 59.0, 41.0, 26.8, 23.3, 22.6, 19.3, 11.8.

Compound 4.16

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 7.83 (d, $J = 7.6$ Hz, 1H), 7.69 (d, $J = 8.9$ Hz, 1H), 7.45 (t, $J = 5.8$ Hz, 1H), 7.39 – 7.28 (m, 5H), 5.03 (s, 2H), 4.16 – 4.08 (m, 1H), 3.66 (d, $J = 6.0$ Hz, 2H), 3.57 – 3.47 (m, 1H), 1.94 – 1.83 (m, 1H), 1.76 – 1.59 (m, 5H), 1.53 (d, $J = 12.4$ Hz, 1H), 1.31 – 1.04 (m, 5H), 0.81 (dd, $J = 11.3, 6.7$ Hz, 6H). ^{13}C NMR (126 MHz, d_6 -DMSO) δ (ppm): 170.1, 169.3, 156.9, 137.5, 128.8, 128.2, 128.1, 65.9, 57.9, 47.9, 43.9, 32.9, 32.6, 31.4, 25.7, 25.0, 24.9, 19.5, 18.6.

Compound 4.17

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 7.89 (d, $J = 7.8$ Hz, 1H), 4.15 (t, $J = 7.7$ Hz, 1H), 3.60 – 3.43 (m, 1H), 3.10 (s, 2H), 1.87 (td, $J = 13.6, 6.9$ Hz, 1H), 1.67 (s, 4H), 1.54 (d, $J = 12.3$ Hz, 1H),

1.33 – 1.02 (m, 5H), 0.82 (t, $J = 6.6$ Hz, 6H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 172.9, 170.1, 57.2, 47.8, 45.0, 32.9, 32.6, 31.9, 25.7, 25.0, 24.9, 19.5, 18.6. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{13}\text{H}_{25}\text{N}_3\text{O}_2^+$ 256.2025; found 256.2020 $[\text{M}+\text{H}]^+$ ($\Delta = 2.0$ ppm).

Compound 4.1

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.50 (ddd, $J = 6.8, 5.2, 1.0$ Hz, 1H), 8.30 (d, $J = 9.0$ Hz, 1H), 7.94 (t, $J = 5.6$ Hz, 1H), 7.89 (dd, $J = 8.2, 7.3$ Hz, 1H), 4.76 (s, 1H), 4.14 (dd, $J = 9.0, 7.2$ Hz, 1H), 3.11 (td, $J = 13.0, 6.9$ Hz, 1H), 2.97 (td, $J = 12.4, 7.0$ Hz, 1H), 2.04 – 1.88 (m, 1H), 1.42 (h, $J = 7.3$ Hz, 1H), 0.86 (dt, $J = 14.6, 5.4$ Hz, 2H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 171.0, 167.0, 163.8, 135.0, 131.9, 131.3, 128.0, 127.8, 122.4, 58.4, 42.7, 40.8, 40.7, 31.3, 22.7, 19.7, 18.7, 11.9. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{22}\text{H}_{25}\text{N}_3\text{O}_4\text{Na}^+$ 418.1743; found 418.1736 $[\text{M}+\text{Na}]^+$ ($\Delta = 1.7$ ppm).

Compound 4.2

^1H NMR (400 MHz, $\text{CDCl}_3/\text{HFIPA}$) δ (ppm): 8.58 (dd, $J = 7.4, 1.0$ Hz, 2H), 8.33 (d, $J = 7.8$ Hz, 2H), 7.85 – 7.78 (m, 2H), 6.71 (d, $J = 8.6$ Hz, 1H), 6.20 (d, $J = 8.2$ Hz, 1H), 4.86 (s AB system, $J = 41.0, 15.6$ Hz, 2H), 2.05 (dq, $J = 13.7, 6.8$ Hz, 1H), 1.91 – 1.79 (m, 2H), 1.79 – 1.67 (m, 2H), 1.67 – 1.59 (m, 1H), 1.43 – 1.26 (m, 2H), 1.26 – 1.08 (m, 3H), 0.95 (t, $J = 6.3$ Hz, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ (ppm): 170.6, 169.0, 165.3, 135.7, 132.4, 131.7, 128.2, 127.2, 121.1, 77.1, 59.9, 49.4, 42.8, 32.5, 32.1, 30.8, 25.1, 24.4, 18.5, 17.7. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{25}\text{H}_{29}\text{N}_3\text{O}_4\text{Na}^+$ 458.2063; found 458.2063 $[\text{M}+\text{Na}]^+$ ($\Delta = 0.0$ ppm).

Compound 4.6

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.46 (d, J = 6.6 Hz, 8H), 7.86 (t, J = 7.7 Hz, 4H), 7.70 (t, J = 5.5 Hz, 2H), 4.97 (d, J = 9.1 Hz, 2H), 2.92 (tt, J = 12.1, 5.9 Hz, 4H), 2.63 – 2.51 (m, 2H), 1.40 – 1.28 (m, 2H), 1.06 (d, J = 6.3 Hz, 6H), 0.54 (d, J = 6.8 Hz, 6H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 168.8, 164.1, 134.8, 131.8, 131.3, 128.1, 127.7, 122.7, 58.9, 36.72, 29.4, 26.7, 23.2, 19.2. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{37}\text{H}_{36}\text{N}_4\text{O}_6\text{Na}^+$ 655.2533; found 655.2537 $[\text{M}+\text{Na}]^+$ (Δ = 0.6 ppm).

Compound 4.18

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 7.90 (s, 2H), 7.73 (d, J = 8.8 Hz, 2H), 7.50 – 7.16 (m, 10H), 5.15 – 4.92 (m, 4H), 4.22 – 3.99 (m, 2H), 3.77 – 3.56 (m, 4H), 3.18 – 2.88 (m, 6H), 1.92 (dd, J = 13.3, 6.7 Hz, 2H), 1.62 – 1.40 (m, 2H), 0.81 (t, J = 5.5 Hz, 12H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 171.1, 169.4, 156.9, 137.5, 128.8, 128.2, 128.1, 65.9, 58.1, 46.0, 43.9, 36.8, 31.1, 29.5, 19.6, 18.5. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{33}\text{H}_{47}\text{N}_6\text{O}_8^+$ 655.3455; found 655.3460 $[\text{M}+\text{H}]^+$ (Δ = 0.8 ppm).

Compound 4.19

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.05 (s, 2H), 7.98 (s, 2H), 4.11 (s, 2H), 3.16 (s, 4H), 3.12 – 2.92 (m, 4H), 1.91 (dt, J = 12.9, 6.3 Hz, 2H), 1.58 – 1.42 (m, 2H), 0.82 (t, J = 5.6 Hz, 12H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 172.40, 171.16, 57.73, 44.52, 36.66, 31.31, 29.49, 19.62, 18.49.

Compound 4.5

^1H NMR (400 MHz, $\text{CDCl}_3/\text{HFIPA}$) δ (ppm): 8.47 (d, J = 7.2 Hz, 3H), 8.19 (d, J = 8.1 Hz, 3H), 7.72 (t, J = 7.8 Hz, 3H), 7.04 (s, 2H), 6.97 (d, J = 8.2 Hz, 2H), 4.90 (dd, J = 50.3, 15.9 Hz, 4H), 3.40 – 3.15 (m, 4H), 2.11 (dq, J = 13.5, 6.8 Hz, 2H), 1.76 – 1.62 (m, 3H), 0.94 (t, J = 6.9 Hz, 14H). ^{13}C NMR (101 MHz, $\text{CDCl}_3/\text{HFIPA}$) δ (ppm): 172.7, 169.3, 165.3, 135.6, 132.2, 131.6, 128.0, 127.2, 120.9, 59.9, 42.7, 36.5, 30.3, 28.1, 18.4, 17.1. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{41}\text{H}_{42}\text{N}_6\text{O}_8\text{Na}^+$ 769.2962; found 769.2974 $[\text{M}+\text{Na}]^+$ (Δ = 1.6 ppm).

Compound 4.7

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm) 8.39 (dd, J = 15.0, 7.8 Hz, 4H), 7.79 (t, J = 7.7 Hz, 2H), 4.03 – 3.89 (m, 2H), 1.64 – 1.51 (m, 2H), 1.36 – 1.19 (m, 6H), 0.83 (t, J = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 163.7, 134.6, 131.7, 131.1, 127.7, 127.6, 122.4, 40.1, 31.4, 27.9, 26.7, 22.4, 14.3. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{23}\text{H}_{27}\text{N}_3\text{O}_3\text{Na}^+$ 304.1313; found 304.1313 $[\text{M}+\text{Na}]^+$ (Δ = 0.0 ppm).

4.5.2 Gel preparation

Heating cooling method: The required amount of compounds were dissolved in the desired amount of solvent and then, heated until complete solution in a screw-capped vial (8 mL, diameter 1.5 cm). The vial was left to rest at ambient temperature for 10 minutes, gel formation was assessed by vial inversion method.

Solvent shift method: The required amount of compounds was dissolved in the desired amount of DMSO and then, water is added quickly in a screw-capped vial (8 mL, diameter 1.5 cm). The vial was left to rest at ambient temperature overnight. Gel formation was assessed by vial inversion method.

4.5.3 UV-Vis spectroscopy

UV-Vis measurements were carried out in a JASCO V-630 spectrophotometer equipped with a Peltier accessory ETCS-761 at 25 °C unless otherwise is indicated. The samples were placed inside 1 cm suprasil quartz cuvettes for diluted solutions and 1 mm path length for optically dense solutions and gels. Data interval acquisition was set to $\Delta\lambda = 1$ nm with UV-Vis bandwidth 1.5 nm with scan speed set at 400 nm/min.

4.5.4 Fluorescence spectroscopy

Samples were measured in JASCO FP-8300 spectrofluorometer with peltier temperature control (ETC-815) using 10 mm path length and 3 mL quartz cuvettes for solutions or triangular quartz cuvettes in the case of gels or optically dense solutions.

4.5.4.1 Quantum yield determination in solution/dispersion

For quantum yield determination in solution or dispersion, 9,10-diphenylanthracene in cyclohexane was used as standard ($\Phi_f = 0.95$)¹¹¹ in accordance with the following equation:

$$\Phi_x = \Phi_{st} \cdot \frac{A_{st}}{A_x} \cdot \frac{F_x}{F_{st}} \cdot \frac{n_x^2}{n_{st}^2}$$

Where Φ_x is the fluorescence quantum yield of the sample to be determined, Φ_{st} is the fluorescence quantum yield of DPA; F is the area of the corrected fluorescence spectrum when

excited at certain wavelength; A is the absorbance of the sample at certain wavelength (same wavelength as F) and n is the refraction index of solvents. Example of quantum yield calculation is summarized in Table 5.

Table 5: Representative spectra of quantum yield table for its calculation

	Standard (st)	Unknown (x)
Yield (Φ)	0.9500	0.2711
Area (F)	122965	37769
Absorbance (340 nm) (A)	0.1043	0.1062
Refraction (n)	(cyclohexane) 1.4254	(H ₂ O) 1.3325

4.5.4.2 Quantum yield determination in solids

Solid absolute quantum yield is calculated in a JASCO FP-8300 spectrofluorometer with an integrating sphere ISF-834 60 mm in accordance with the following: because of the use of an integrating sphere, the photon absorption of the material can be calculated directly by the difference between the incident light (measurement performed with a 100 % dispersive material) and the sample light scattering (S_0 and S_1). The fluorescence emission efficiency is calculated from the area of S_2 . Internal quantum yield % = $\frac{S_2}{(S_2 - S_1)} \cdot 100$ (Figures 39 and 40).

Figure 40. Summary of peaks used for absolute quantum yield calculation

Figure 41. Summary of peaks used for absolute quantum yield calculation (in compound **1**, solid (black line) and gel (green line) spectra are shown).

4.5.5 X-ray diffraction

Wide-angle X-ray diffraction of the xerogels was performed at room temperature with a Bruker D4 Endeavor X-ray powder diffractometer by using Cu-K α -radiation. A sample of the respective freeze dried powders was placed on a sample holder and data was collected for 20 values between 2 ° and 40 ° with a step size of 0.03 ° and time step of 10 s. XRD patterns of xerogels of compound **1** formed in different solvents (CH₃OH and DMSO/H₂O) have been investigated, as well as in a more crystalline phase when evaporation method was carried out upon solving the compound in 1,1,1,3,3,3-Hexafluoro-2-propanol/CHCl₃ mixtures.

4.5.6 Transmission electron microscopy (TEM)

Transmission electron micrographs were taken on a JEOL 2100 microscope with thermionic gun LaB6 200 KV equipped with Gatan Orius high resolution CCD camera. TEM samples were prepared over 200 mesh Formvar carbon copper grids by putting a drop of supernatant suspension and incubating for 5 minutes. The suspension excess was removed with filter paper. Grids were stained with a drop of phosphotungstic acid 1 % for 1 minute, subsequently removed by capillary action of filter paper.

4.5.7 Confocal Scanning microscopy (CLSM)

Experiments were performed on an inverted confocal microscope Leica TCS SP8. Images were obtained with HC PL APO CS2 63x/1.40 oil immersion objective. Excitation of samples was performed with diode laser (excitation 405 nm) and was acquired with PMT detector. Samples were prepared on a glass slide and covered with 0.17 mm #1.5 coverslip. Fresh gels were measured as prepared.

4.6 References and notes

- (1) Betzig, E. *Rev. Mod. Phys.* **2015**, 87 (4), 1153.
- (2) Hell, S. W. *Rev. Mod. Phys.* **2015**, 87 (4), 1169.
- (3) Moerner, W. E. *Rev. Mod. Phys.* **2015**, 87 (4), 1183.
- (4) Sancar, A. *Angew. Chemie Int. Ed.* **2016**, 55 (30), 8502.
- (5) Modrich, P. *Angew. Chemie Int. Ed.* **2016**, 55 (30), 8490.
- (6) Lindahl, T. *Angew. Chemie Int. Ed.* **2016**, 55 (30), 8528.
- (7) Busseron, E.; Ruff, Y.; Moulin, E.; Giuseppone, N. *Nanoscale* **2013**, 5 (16), 7098.
- (8) Mei, J.; Leung, N. L. C.; Kwok, R. T. K.; Lam, J. W. Y.; Tang, B. Z. *Chem. Rev.* **2015**, 115 (21), 11718.
- (9) Luo, J.; Xie, Z.; Lam, J. W. Y.; Cheng, L.; Tang, B. Z.; Chen, H.; Qiu, C.; Kwok, H. S.; Zhan, X.; Liu, Y.; Zhu, D. *Chem. Commun.* **2001**, 103 (18), 1740.
- (10) Förster, T.; Kasper, K. *Berichte der Bunsengesellschaft für Phys. Chemie* **1955**, 59 (10), 976.
- (11) Birks, J. B. *Photophysics of aromatic molecules*; Wiley-Interscience, 1970.
- (12) Valeur, B.; Berberan-Santos, M. N. *Molecular Fluorescence : principles and applications*; Wiley-VCH, 2013.
- (13) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Springer US: Boston, MA, 2006.
- (14) Tang, C. W.; VanSlyke, S. A. *Appl. Phys. Lett.* **1987**, 51 (12), 913.
- (15) Miyata, S.; Nalwa, H. S. *Organic electroluminescent materials and devices*; Gordon and Breach Publishers, 1997.
- (16) Chen, J.; Xu, B.; Ouyang, X.; Tang, B. Z.; Cao, Y. *J. Phys. Chem. A* **2004**, 108 (37), 7522.
- (17) Tong, H.; Hong, Y.; Dong, Y.; Ren, Y.; Ha, M.; Lam, J. W. Y.; Wong, K. S.; Zhong Tang, B. *J. Phys. Chem. B* **2007**, 111, 2000.
- (18) Tong, H.; Hong, Y.; Dong, Y. Y.; Häussler, M.; Li, Z.; Lam, J. W. Y.; Dong, Y. Y.; Sung, H. H.-Y.; Williams, I. D.; Tang, B. Z. *J. Phys. Chem. B* **2007**, 111 (40), 11817.
- (19) Wang, M.; Zhang, G.; Zhang, D.; Zhu, D.; Tang, B. Z. *J. Mater. Chem.* **2010**, 20, 1858.
- (20) Hong, Y.; Lam, J. W. Y.; Tang, B. Z. *Chem. Commun.* **2009**, 0, 4662.
- (21) Qin, A.; Tang, B. Z. *Aggregation-induced emission : applications*; Wiley, 2013.
- (22) Mei, J.; Hong, Y.; Lam, J. W. Y.; Qin, A.; Tang, Y.; Tang, B. Z. *Adv. Mater.* **2014**, 26 (31),

- 5429.
- (23) Shi, H.; Kwok, R. T.; Liu, J.; Xing, B.; Tang, B. Z.; Liu, B. *J. Am. Chem. Soc.* **2012**, *134* (43), 17972.
- (24) Wang, E.; Lam, J. W. Y.; Hu, R.; Zhang, C.; Zhao, Y. S.; Tang, B. Z. *J. Mater. Chem. C* **2014**, *2* (10), 1801.
- (25) Shen, X. Y.; Wang, Y. J.; Zhao, E.; Yuan, W. Z.; Liu, Y.; Lu, P.; Qin, A.; Ma, Y.; Sun, J. Z.; Tang, B. Z. *J. Phys. Chem. C* **2013**, *117*, 7334.
- (26) Zhu, C.; Pang, S.; Xu, J.; Jia, L.; Xu, F.; Mei, J.; Qin, A.; Sun, J.; Ji, J.; Tang, B. *Analyst* **2011**, *136*, 3343.
- (27) Deng, S.-L.; Chen, T.-L.; Chien, W.-L.; Hong, J.-L. *J. Mater. Chem. C* **2014**, *2* (4), 651.
- (28) Parrott, E. P. J.; Tan, N. Y.; Hu, R.; Zeitler, J. A.; Tang, B. Z.; Pickwell-MacPherson, E. *Mater. Horiz.* **2014**, *1* (2), 251.
- (29) Zeng, Q.; Li, Z.; Dong, Y.; Chong 'an, D.; Qin, A.; Hong, Y.; Ji, L.; Zhu, Z.; Jim, C. K. W.; Yu, G.; Li, Q.; Li, Z.; Liu, Y.; Qin, J.; Zhong Tang, B. *Chem. Commun.* **2007**, 70.
- (30) Chen, J.; Law, C. C. W.; Lam, J. W. Y.; Dong, Y.; Lo, S. M. F.; Williams, I. D.; Zhu, D.; Tang, B. Z. *Chem. Mater.* **2003**, *15*, 1535.
- (31) Leung, N. L. C.; Xie, N.; Yuan, W.; Liu, Y.; Wu, Q.; Peng, Q.; Miao, Q.; Lam, J. W. Y.; Tang, B. Z. *Chem. - A Eur. J.* **2014**, *20* (47), 15349.
- (32) Yang, L.; Ye, J.; Xu, L.; Yang, X.; Gong, W.; Lin, Y.; Ning, G. *RSC Adv.* **2012**, *2* (30), 11529.
- (33) Shimizu, M.; Tatsumi, H.; Mochida, K.; Shimono, K.; Hiyama, T. *Chem. - An Asian J.* **2009**, *4* (8), 1289.
- (34) Hu, R.; Lam, J. W. Y.; Liu, Y.; Zhang, X.; Tang, B. Z. *Chem. - A Eur. J.* **2013**, *19* (18), 5617.
- (35) Feng, J.; Chen, X.; Han, Q.; Wang, H.; Lu, P.; Wang, Y. *J. Lumin.* **2011**, *131* (12), 2775.
- (36) He, J.; Xu, B.; Chen, F.; Xia, H.; Li, K.; Ye, L.; Tian, W. *J. Phys. Chem. C* **2009**, *113* (22), 9892.
- (37) Feng, X.; Tong, B.; Shen, J.; Shi, J.; Han, T.; Chen, L.; Zhi, J.; Lu, P.; Ma, Y.; Dong, Y. *J. Phys. Chem. B* **2010**, *114* (50), 16731.
- (38) Pan, H.; Fu, G.-L.; Zhao, Y.-H.; Zhao, C.-H. *Org. Lett.* **2011**, *13* (18), 4830.
- (39) Liu, G.; Yang, M.; Wang, L.; Zheng, J.; Zhou, H.; Wu, J.; Tian, Y. *J. Mater. Chem. C* **2014**, *2* (15), 2684.
- (40) Zhao, X.; Xue, P.; Wang, K.; Chen, P.; Zhang, P.; Lu, R. *New J. Chem. New J. Chem* **2014**,

- 38 (38), 1045.
- (41) Wang, Z.; Chen, S.; Lam, J. W. Y.; Qin, W.; Kwok, R. T. K.; Xie, N.; Hu, Q.; Tang, B. Z. *J. Am. Chem. Soc.* **2013**, *135* (22), 8238.
- (42) Zeng, Y.; Li, P.; Liu, X.; Yu, T.; Chen, J.; Yang, G.; Li, Y. *Polym. Chem.* **2014**, *5* (20), 5978.
- (43) Hu, R.; Maldonado, J. L.; Rodriguez, M.; Deng, C.; Jim, C. K. W.; Lam, J. W. Y.; Yuen, M. M. F.; Ramos-Ortiz, G.; Tang, B. Z. *J. Mater. Chem.* **2012**, *22* (1), 232.
- (44) Xu, Y.; Chen, L.; Guo, Z.; Nagai, A.; Jiang, D. *J. Am. Chem. Soc.* **2011**, *133* (44), 17622.
- (45) Sun, H.-L.; Jiang, R.; Li, Z.; Dong, Y. Q.; Du, M. *CrystEngComm* **2013**, *15* (9), 1669.
- (46) Liang, J.; Chen, Z.; Yin, J.; Yu, G.-A.; Liu, S. H. *Chem. Commun.* **2013**, *49* (34), 3567.
- (47) Xin, X.-L.; Chen, M.; Ai, Y.; Yang, F.; Li, X.-L.; Li, F. *Inorg. Chem.* **2014**, *53* (6), 2922.
- (48) Li, Y.; Tsang, D. P.-K.; Chan, C. K.-M.; Wong, K. M.-C.; Chan, M.-Y.; Yam, V. W.-W. *Chem. - A Eur. J.* **2014**, *20* (42), 13710.
- (49) Chen, Y.; Xu, W.-C.; Kou, J.-F.; Yu, B.-L.; Wei, X.-H.; Chao, H.; Ji, L.-N. *Inorg. Chem. Commun.* **2010**, *13* (10), 1140.
- (50) You, Y.; Huh, H. S.; Kim, K. S.; Lee, S. W.; Kim, D.; Park, S. Y.; Metz, T.; Bolte, M.; Schumidt, M. U.; Liu, S.; Ma, D.; Ma, Y.; Liu, Y.; Qin, J.; Tang, B. Z. *Chem. Commun.* **2008**, *44* (34), 3998.
- (51) Abad, S.; Kluciar, M.; Miranda, M. A.; Pischel, U. *J. Org. Chem.* **2005**, *70* (25), 10565.
- (52) Remón, P.; Parente Carvalho, C.; Baleizão, C.; Berberan-Santos, M. N.; Pischel, U. *ChemPhysChem* **2013**, *14* (12), 2717.
- (53) Ferreira, R.; Baleizão, C.; Muñoz-Molina, J. M.; Berberan-Santos, M. N.; Pischel, U. *J. Phys. Chem. A* **2011**, *115* (6), 1092.
- (54) Daffy, L. M.; de Silva, A. P.; Gunaratne, H. Q. N.; Huber, C.; Lynch, P. L. M.; Werner, T.; Wolfbeis, O. S. *Chem. - A Eur. J.* **1998**, *4* (9), 1810.
- (55) Ramachandram, B.; Saroja, G.; Sankaran, N. B.; Samanta, A. *J. Phys. Chem. B* **2000**, *104*, 11824.
- (56) Gunnlaugsson, T.; Lee, T. C.; Parkesh, R.; Kennedy, R.; Pfeffer, F. M.; Hussey, G. M.; Rice, T. E.; Fierke, C.; Herman, P. *Org. Biomol. Chem.* **2003**, *1* (19), 3265.
- (57) Licchelli, M.; Biroli, A. O.; Poggi, A.; Sacchi, D.; Sangermani, C.; Zema, M.; Rice, T. E.; Chang, S. C.; Matthews, J. L.; Qian, X. D.; Keilhauer, G.; Romerdahl, C. A. *Dalt. Trans.* **2003**, *30* (23), 4537.

- (58) Kolosov, D.; Adamovich, V.; Djurovich, P.; Thompson, M. E.; Adachi, C. *J. Am. Chem. Soc.* **2002**, *124* (33), 9945.
- (59) Rogers, J. E.; Abraham, B.; Rostkowski, A.; Kelly, L. A. *Photochem. Photobiol.* **2001**, *74* (4), 521.
- (60) Pham, Q. N.; Brosse, N.; Frochot, C.; Dumas, D.; Hocquet, A.; Jamart-Grégoire, B. *New J. Chem.* **2008**, *32* (7), 1131.
- (61) Bouguet-Bonnet, S.; Yemloul, M.; Canet, D. *J. Am. Chem. Soc.* **2012**, *134* (25), 10621.
- (62) Allix, F.; Curcio, P.; Pham, Q. N.; Pickaert, G.; Jamart-Grégoire, B. *Langmuir* **2010**, *26* (22), 16818.
- (63) Yu, X.; Cao, X.; Chen, L.; Lan, H.; Liu, B.; Yi, T. *Soft Matter* **2012**, *8* (12), 3329.
- (64) Meher, N.; Chowdhury, S. R.; Iyer, P. K. *J. Mater. Chem. B* **2016**, *4*, 6023.
- (65) Mukherjee, S.; Thilagar, P. *Chem. Commun.* **2013**, *49* (66), 7292.
- (66) Sun, Y.; Liu, Z.; Liang, X.; Fan, J.; Han, Q. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* **2013**, *108*, 8.
- (67) Lin, H.-H.; Chan, Y.-C.; Chen, J.-W.; Chang, C.-C. *J. Mater. Chem.* **2011**, *21* (9), 3170.
- (68) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. *Chem. Rev.* **2014**, *114* (4), 1973.
- (69) Chandler, D. *Nature* **2005**, *437* (7059), 640.
- (70) Wang, Z.; Nie, J.; Qin, W.; Hu, Q.; Tang, B. Z. *Nat. Commun.* **2016**, *7*, 12033.
- (71) Zhang, C.; Liu, C.; Xue, X.; Zhang, X.; Huo, S.; Jiang, Y.; Chen, W.-Q.; Zou, G.; Liang, X.-J. *Appl. Mater. Interfaces* **2014**, *6*, 757.
- (72) Babu, S. S.; Kartha, K. K.; Ajayaghosh, A. *J. Phys. Chem. Lett* **2010**, *1*, 3413.
- (73) Liu, Y.; Lam, J. W. Y.; Mahtab, F.; Kwok, R. T. K.; Tang, B. Z. *Front. Chem. China* **2010**, *5* (3), 325.
- (74) Yuan, W. Z.; Mahtab, F.; Gong, Y.; Yu, Z.-Q.; Lu, P.; Tang, Y.; Lam, J. W. Y.; Zhu, C.; Tang, B. Z. *J. Mater. Chem.* **2012**, *22* (21), 10472.
- (75) Wan, J.-H.; Mao, L.-Y.; Li, Y.-B.; Li, Z.-F.; Qiu, H.-Y.; Wang, C.; Lai, G.-Q. *Soft Matter* **2010**, *6*, 3195.
- (76) Wang, M.; Zhang, D.; Zhang, G.; Zhu, D. *Chem. Phys. Lett.* **2009**, *475* (1–3), 64.
- (77) Yoon, S.-J.; Kim, J. H.; Chung, J. W.; Park, S. Y. *J. Mater. Chem.* **2011**, *21*, 18971.
- (78) An, B.-K.; Lee, D.-S.; Lee, J.-S.; Park, Y.-S.; Song, H.-S.; Park, S. Y. *J. Am. Chem. Soc.* **2004**, *126*, 10232.

- (79) Ryu, S. Y.; Kim, S.; Seo, J.; Kim, Y.-W.; Kwon, O.-H.; Jang, D.-J.; Park, S. Y. *Chem. Commun.* **2004**, 106 (1), 70.
- (80) Yang, X.; Lu, R.; Xu, T.; Xue, P.; Liu, X.; Zhao, Y. *Chem. Commun.* **2007**, 0, 453.
- (81) Tang, C. W.; VanSlyke, S. A. *Appl. Phys. Lett.* **1987**, 51 (12), 913.
- (82) Chen, B.; Jiang, Y.; He, B.; Zhou, J.; Sung, H. H. Y.; Williams, I. D.; Lu, P.; Kwok, H. S.; Qiu, H.; Zhao, Z.; Tang, B. Z. *Chem. Asian J.* **2014**, 9 (10), 2937.
- (83) Zhao, Z.; Chen, S.; Shen, X.; Mahtab, F.; Yu, Y.; Lu, P.; Lam, J. W. Y.; Kwok, H. S.; Tang, B. Z. *Chem. Commun.* **2010**, 46, 686.
- (84) Forrest, S. R.; Baldo, M. A.; Thompson, M. E. *Nature* **2000**, 403 (6771), 750.
- (85) Luo, H.; Chen, S.; Liu, Z.; Zhang, C.; Cai, Z.; Chen, X.; Zhang, G.; Zhao, Y.; Decurtins, S.; Liu, S.-X.; Zhang, D. *Adv. Funct. Mater.* **2014**, 24 (27), 4250.
- (86) Zhao, N.; Zhang, C.; Lam, J. W. Y.; Zhao, Y. S.; Tang, B. Z. *Asian J. Org. Chem.* **2014**, 3 (2), 118.
- (87) Li, Y.; Li, Z.; Wang, Y.; Compaan, A.; Ren, T.; Dong, W.-J. *Energy Environ. Sci.* **2013**, 6 (10), 2907.
- (88) Cui, K.; Lu, X.; Cui, W.; Wu, J.; Chen, X.; Lu, Q. *Chem. Commun.* **2011**, 47, 920.
- (89) Chen, S.; Liu, J.; Liu, Y.; Su, H.; Hong, Y.; Jim, C. K. W.; Kwok, R. T. K.; Zhao, N.; Qin, W.; Lam, J. W. Y.; Wong, K. S.; Tang, B. Z. *Chem. Sci.* **2012**, 3 (6), 1804.
- (90) Pramanik, S.; Bhalla, V.; Kumar, M. *Anal. Chim. Acta* **2013**, 793, 99.
- (91) Hong, Y.; Chen, S.; Leung, C. W. T.; Lam, J. W. Y.; Tang, B. Z. *Chem. - An Asian J.* **2013**, 8 (8), 1806.
- (92) Li, Y.; Wu, Y.; Chang, J.; Chen, M.; Liu, R.; Li, F. *Chem. Commun.* **2013**, 49 (49), 11335.
- (93) Hu, F.; Huang, Y.; Zhang, G.; Zhao, R.; Zhang, D. *Tetrahedron Lett.* **2014**, 55 (8), 1471.
- (94) Shen, X.; Shi, Y.; Peng, B.; Li, K.; Xiang, J.; Zhang, G.; Liu, Z.; Chen, Y.; Zhang, D. *Macromol. Biosci.* **2012**, 12 (11), 1583.
- (95) Leung, C. W. T.; Hong, Y.; Chen, S.; Zhao, E.; Lam, J. W. Y.; Tang, B. Z. *J. Am. Chem. Soc.* **2013**, 135 (1), 62.
- (96) Zhou, M.; Smith, A. M.; Das, A. K.; Hodson, N. W.; Collins, R. F.; Ulijn, R. V.; Gough, J. E. *Biomaterials* **2009**, 30 (13), 2523.
- (97) Felip-León, C.; Díaz-Oltra, S.; Galindo, F.; Miravet, J. F. *Chem. mater.* **2016**, 28 (21), 7964.

- (98) Escuder, B.; Martí, S.; Miravet, J. F. *Langmuir* **2005**, *21* (15), 6776.
- (99) Segarra-Maset, M. D.; Nebot, V. J.; Miravet, J. F.; Escuder, B. *Chem. Soc. Rev.* **2013**, *42* (17), 7086.
- (100) Escuder, B.; Rodríguez-Llansola, F.; Miravet, J. F. *New J. Chem.* **2010**, *34* (6), 1044.
- (101) Nebot, V. J.; Ojeda-Flores, J. J.; Smets, J.; Fernández-Prieto, S.; Escuder, B.; Miravet, J. F. *Chem. - A Eur. J.* **2014**, *20* (44), 14465.
- (102) Felip-León, C.; Segarra-Maset, M. D.; Escuder, B.; Miravet, J. F. *Tetrahedron Lett.* **2015**, *56* (9), 1132.
- (103) Hardy, J. G.; Hirst, A. R.; Smith, D. K. *Soft Matter* **2012**, *8* (12), 3399.
- (104) Kiyonaka, S.; Sugiyasu, K.; Shinkai, S.; Itaru Hamachi. *J. Am. Chem. Soc.* **2002**, *124* (37), 10954.
- (105) Bernstein, J. *Polymorphism in molecular crystals*; Oxford University Press, 2002.
- (106) Díaz-Oltra, S.; Berdugo, C.; Miravet, J. F.; Escuder, B. *New J. Chem.* **2015**, *39* (5), 3785.
- (107) Nebot, V. J.; Díaz-Oltra, S.; Smets, J.; Fernández Prieto, S.; Miravet, J. F.; Escuder, B. *Chem. - A Eur. J.* **2014**, *20* (19), 5762.
- (108) Parker, C. A.; Rees, W. T. *Analyst* **1962**, *87* (1031), 83.
- (109) Di, L.; Kerns, E. H. *Drug Discov. Today* **2006**, *11* (9–10), 446.
- (110) Cho, D. W.; Fujitsuka, M.; Choi, K. H.; Park, M. J.; Yoon, U. C.; Majima, T. *J. Phys. Chem. B* **2006**, *110* (10), 4576.
- (111) Morris, J. V.; Mahaney, M. A.; Huberr, J. R. *J. Phys. Chem.* **1976**, *80* (9), 969.
- (112) Zhao, Z.; Lam, J. W. Y.; Tang, B. Z. *J. Mater. Chem.* **2012**, *22* (45), 23726.
- (113) D'Agostino, S.; Grepioni, F.; Braga, D.; Moreschi, D.; Fattori, V.; Delchiaro, F.; Motta, S. Di; Negri, F. *CrystEngComm* **2013**, *15*, 1040.
- (114) Barros, T. C.; Filho, P. B.; Toscano, V. G.; Politi, M. J. *J. Photochem. Photobiol. A Chem.* **1995**, *89* (2), 141.
- (115) Cho, D. W.; Cho, D. W. *New J. Chem.* **2014**, *38* (6), 2233.
- (116) Hirst, A. R.; Escuder, B.; Miravet, J. F.; Smith, D. K. *Angew. Chemie - Int. Ed.* **2008**, *47* (42), 8002.
- (117) Kunz, W.; Henle, J.; Ninham, B. W. *Curr. Opin. Colloid Interface Sci.* **2004**, *9* (1), 19.
- (118) Lo Nostro, P.; Ninham, B. W. *Chem. Rev.* **2012**, *112* (4), 2286.
- (119) Pegram, L. M.; Record, M. T. *J. Phys. Chem. B* **2008**, *112* (31), 9428.

- (120) Boström, M.; Williams, D. R. M.; Ninham, B. W. *Biophys. J.* **2003**, 85 (2), 686.
- (121) Gibb, C. L. D.; Gibb, B. C. J. *Am. Chem. Soc.* **2011**, 133 (19), 7344.
- (122) Sakaitani, M.; Ohfuné, Y. *Tetrahedron Lett.* **1985**, 26 (45), 5543.
- (123) Kuroda, Y.; Kato, Y.; Higashioji, T.; Ogoshi, H. *Angew. Chemie* **1993**, 105 (5), 774.
- (124) Acher, F.; Wakselman, M. J. *Org. Chem.* **1984**, 49 (22), 4133.
- (125) Waldmann, H. *Tetrahedron Lett.* **1988**, 29 (10), 1131.
- (126) Angulo-Pachón, C. A.; Gascó-Catalán, C.; Ojeda-Flores, J. J.; Miravet, J. F. *ChemPhysChem* **2016**, 17 (13), 2008.

Chapter 5

**Self-assembled
molecular micro- and
nanogels as new
carriers**

Chapter 5: Self-assembled molecular micro- and nanogels as new carriers

5.1 Motivation

Illness is inherent to the fact of being alive. Survival instinct of humanity has led to the development of Medicine since the beginning of time and current advances in Medicine are due to millennia of history evolution, tests, scientific research and, unfortunately, lots of victims of erroneous diagnosis and trial and error treatments. Life expectancy has boosted in the 20th century, from around 50 years in 1900 to 81 years nowadays in developed countries, with data on life expectancies between 1840 and 2007 showing a steady increase averaging about three months of life per year.¹ This raise has its basis on the critical discovery and development of antibiotics,² which shifted the leading causes of death from infectious and parasitic diseases to chronic diseases that last a long time and require ongoing care. However, ageing of biological systems implies the appearance of new diseases that require further research or critical discoveries to heal contemporary disorders. Cancer is still the second leading cause of death, globally causing 8.8 million deaths in 2015.³ Our inability to fight cancer is mainly due to the fact that: 1) we are unable to deliver therapeutic agents selectively to the tumour sites without damaging healthy cells; and 2) cancer multidrug resistance boost for secondary tumours.⁴

In 1966, science fiction movie “Fantastic Voyage” (Richard Fleischer) and subsequent novel (Isaac Asimov) presented the adventure of a submarine shrunk to nanometric size that entered on a researcher bloodstream and was able to destroy a blood clot. The concept of engineer and manipulate nano-sized objects inside bloodstream, as usual in Asimov’s predictions, were not far from actual scientific concept known as Nanomedicine. Many of the technical things science fiction dreamed about have become a reality in daily life.

Promising results encouraged scientists all over the world to develop increasingly sophisticated nanosized structures. In this chapter, up to our knowledge, miniaturization of

molecular gels up to nanometric level is achieved for the first time and their possible uses as biomedical nano-carriers are studied just as the Proteus submarine did.

5.2 Introduction

5.2.1 Nanotechnology

Nanotechnology is the understanding and control of matter at the nanoscale dimensions (from 1 nm, corresponding to size of the molecules, to 100 nm, where quantum effects dominate properties of materials). To have a comparative idea of the dimensions of a nanometer, if a marble diameter were 1 nm, then 1m would be the earth diameter.⁵ Size of matter (Figure 1) governs its features and enables very interesting novel applications. Bulk materials behave very different from the nanosized ones, even with the same chemical composition. The basis of nanotechnology was set on a talk imparted by physicist Richard Feynman in 1959 entitled “There is plenty of room at the bottom”, where he envisaged a future in which scientists could control single atoms or molecules. Nanotechnology concept grew behind the back of van der Waals statements about weak interaction forces postulated on 1873.⁶

Figure 1. Nanosize scale.⁷

The birth of nanotechnology went hand in hand of the development and construction of scanning electron microscopes (by Ernst Ruska and Max Knoll in early 1931 at the Berlin Technische Hochschule) and transmission electron microscope (Manfred Von Ardenne in 1937). Possibility of manipulation of single atoms or molecules came after the invention of the scanning tunnelling microscope in 1981 by Heinrich Rohrer and Gerd Binning in Zürich (Nobel laureates in Physics 1986)⁸ and atomic force microscope by Quate and Gerber.⁹ Microscopy available up to this date, allow to visualize and start to manipulate and play with these kind of materials.

5.2.2 Nanomedicine

Nanomedicine plays at the same size scale as much biology occurs and has emerged as a very promising interdisciplinary area which makes use of nanotechnology and nanostructures for different therapeutic goals. The development of nanotechnology has provided outstanding and versatile tools to complement or improve classical therapies.^{10,11} The extremely small size of nanostructures allows direct interaction with proteins (haemoglobin is *ca.* 5.5 nm diameter), DNA (2 nm diameter) and other biomolecules, opening great possibilities for drug delivery, gene therapy, and medical diagnostics. The use of nanostructures for biomedical applications is aimed to overcome some drawbacks of conventional drugs in aspects such as formulation, administration and biodistribution. Nowadays, 95% of all new potential therapeutics has poor pharmacokinetics and biopharmaceutical properties.¹² Incorporation of actives (drugs, bioimaging agents or sensors) into nanocarriers used as delivery systems^{13,14} has addressed important issues: 1) low biodisponibility, by protecting the cargo from hostile environments, avoiding rapid clearance and being able to reduce doses improving its pharmacokinetics; 2) low solubility, by providing hydrophobic pockets to effectively load high amounts of active species and dispend high drug doses; 3) low selectivity (high toxicity), by increasing targeting of specific recognition groups to avoid side effects and providing controlled drug release; 4) low stability, by providing suitable environments to host fragile therapeutic agents such as oligonucleotides and peptides.¹⁵⁻¹⁷

5.2.3 Physicochemical properties of nanomaterials for medical uses

As discussed before, nanomedicine works in the sub-cellular level and its action is mostly dependent on the cell internalization.¹⁸ For this reason, before starting the adventure of synthesizing new nanomaterials for medical applications, is important to know the cellular uptake mechanisms (Figure 2) and their tissue distribution, which will depend on the well understanding of the most desirable physicochemical features that our system must have if we do not want to fail before we start. The particle uptake and subsequent influence on cells are largely dependent on the physiochemical properties of the particles: size, shape, charge, hydrophobicity and rigidity are key features that must be taken into account for designing a suitable nanomaterial and to adapt their properties to the function we aim for them. It is important to understand those *a priori* effects that these properties have on the distribution, metabolism, excretion, and toxicity of new nanomaterials.

Figure 2. Summary of nanoparticles that have been explored as carriers for actives together with illustrations of biophysicochemical properties.¹⁹

5.2.3.1 Size

Size is a determinant feature to be controlled: depending on particle size, different tissues could be preferentially reached. For example, particles smaller than 100 nm have a high

circulating time and reduced opsonisation. Lungs, liver, spleen and kidneys act as a series of filter organs that ultimately determine the body persistence and biodistribution. Generally, big particles become passively entrapped on the spleen, while kidneys and liver perform clearance of smaller particles (Figure 3).¹⁹

Figure 3. Biodistribution and clearance of nanoparticles from the human body.¹⁹

Passive tumour targeting through Enhanced Permeation and Retention Effect (EPR)

Size is an intrinsic allied of nanomaterials for cancer treatment, since by adjusting the size, different bioaccumulation could be achieved.²⁰ Upon nanoengineered material injection into the circulatory system, nanostructured systems need to extravasate through the vascular walls and reach the site of interest. In healthy tissues, endothelial cells are very tightly disposed and particles larger than 10 nm cannot perform the extravasation phenomenon. However, it is well known that fast tumour angiogenesis produces leaky walls with relatively big holes that permit the nanoparticles to extravasate efficiently.²¹ Additionally, the tumour mass has no mechanisms of drainage, producing nanoparticle accumulation on tumour tissue (Figure 4). Nanoparticles in a size range from 30 to 200 nm are believed to be the best option to target solid tumours by EPR effect. However, EPR strategy has lots of limitations, such as

fighting against small tumours with weak angiogenesis and tumour permeability issues. For this reason, researchers' efforts have been focusing on active targeting in last decades.

Figure 4. Transport of small molecules and nanoparticles with different sizes and through normal (left) and cancerous (right) tissues. The enhanced permeability and retention (EPR) effect is a unique feature of most tumours, allowing nanoparticles of appropriate sizes to accumulate more in cancerous tissues than in normal tissues.¹⁹

Cellular uptake

Once nanoparticles have reached the cell, they are firstly attached to the cell membrane and then wrapped by the cell membrane to form membrane-bounded vesicles; size also plays a crucial role in the way they enter and the final localization inside the cell. In a very simple and illustrative way, two extremely different pathways for cellular uptake can be described: phagocytosis (conducted primarily by macrophages, monocytes and neutrophils) and pinocytosis (clathrin-mediated, caveolin-mediated, macropinocytosis and clathrin/caveolin-independent endocytosis). These mechanisms involve different types of receptor–ligand interactions, which are mainly dependent on particle size and surface chemistry (Figure 5). Generally, ingestion of microparticles ($>0.5\ \mu\text{m}$) usually happens through a phagocytosis route and that of smaller ones ($<0.2\ \mu\text{m}$) usually takes place through pinocytosis, while very small nanoparticles undergo direct cell penetration via membrane diffusion.¹⁹

Figure 5. Phagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and clathrin- and caveolin-independent endocytosis.¹⁸

5.2.3.2 Shape

Particle shape also governs the way nanoparticles biodistribute and the cell. The shape of the particles have been demonstrated to affect the extravasation phenomena and, in consequence, their bioaccumulation on tumour tissue. Spherical particles tend to follow laminar flow pattern and, only those particles that move near the surface of the capillary, extravasate. On the other hand, rod-like shapes and other shapes with high aspect ratios are more unstable hydrodynamically and the failure of following the flow pattern enhances extravasation ability.²²

Endocytosis is also affected by the particle shape; nanoparticles with moderate aspect ratios (between 1 and 4) tend to be easily engulfed by macrophages and are more readily to be phagocytised effectively than spherical objects. Contrarily, when the cell internalization is

mediated by pynocytosis mechanism, spherical shapes are more likely to have faster internalization rates (Figure 6)²³

Figure 6. (A) Gold nanorods with different aspect ratios and (B) its cell internalization within human breast adenocarcinoma (MCF-7) cells.²³

Particle surface texture also affects opsonisation and uptake.²⁴ However, the shape effect has not been extensively tackled due to the existence of so many variations depending on particle surface and cell type.

5.2.3.3 Charge and hydrophobicity

Probably, nanoparticles surface features are the most important factors regarding biodistribution, cell uptake and protein adsorption. Surfaces determine the cell internalization mechanism, as they provide the driving forces necessary to produce the internalization: mainly by electrostatic interactions,²⁵ but also hydrogen bonding, hydrophobic interactions and van der Waals' forces are important. Regarding the polarity of nanoparticle surface, hydrophilic surfaces are generally not favourable for uptake due the lipidic nature of cell membranes.

Regarding nanoparticles surface charge, it has to be noted that cellular membranes are, in general, negatively charged. For this reason, positively charged particles are more likely to have high nonspecific internalization rates and shorter circulation times.^{26,27} This is also related to the potential highest toxicity of positively charged nanoparticles, as their internalization is

more effective. However, high positively charged particles tend to aggregate and this aggregation inhibits the cellular uptake.

Of course, active targeting is based on the functionalization of nanoparticles surfaces with various ligands (such as antibodies, proteins, aptamers, peptides, cell growth factors and other small molecules) that facilitate the cellular uptake by receptor-mediated endocytosis and reduce side effects by directing the particles to the desired site of action.^{28,29}

5.2.3.4 Rigidity

The structural flexibility of the nanoparticles could also affect the biodistribution. More flexible particles showed an increase in pore penetration due to the advantage of EPR effect.³⁰ Cell internalization is also affected by rigidity; soft particles tend to enter the cell via macropinocytosis and stiffer ones via clathrin dependent mechanism.³¹ Recent calculations showed that stiffer particles can achieve full wrapping easily than the softer ones.³²

5.2.4 Nano-engineered structures for medical applications

Nano-sized systems with medical applications have been extensively developed since nanotechnology emergence. Classical carriers of different chemical nature are used for nanomedicinal purposes: drug nanoparticles, liposomes, solid-lipid hybrid particles, polymeric hydrogels, protein-drug conjugates, dendrimers and inorganic nanoparticles are examples of structures explored as nanoengineered materials for application in medicine (Figure 7). Ideal features for these nano-engineered systems are depicted below:

- Encapsulation stability: Actives should not leak during circulation to avoid side effects and ensure maximal efficacy.
- Response to stimuli: Actives should be released selectively at the target of interest in response to a differentiating stimulus.
- Passive and active targeting: Size plays a crucial role in passive targeting, as well as control clearance and circulation times. Targeting is also a key aspect to overcome side effects.
- Toxicity: Should be non-toxic and biodegradable in non-toxic degradation products.

- Ease of synthesis: Important for industrial scaling.

Figure 7. Common nanoengineered platforms: **(a)** liposome, **(b)** polymeric nanoparticle, **(c)** quantum dot (QD), **(d)** dendrimer, and **(e)** polymeric bioconjugate nanocarrier.³³

5.2.4.1 Inorganic nanomaterials

Inorganic nanoparticles can be used for therapeutic and diagnostic purposes. Particles of very different nature such as porous silicon, metal (typically gold and silver but also iron oxide) nanostructures, quantum dots or carbon-based nanostructures (graphene, fullerene and carbon nanotubes) have been used in medical applications (Figure 8). The inability of degradation of these particles limits their applications. However, they are ideal systems in terms of size and shape control, with excellent physicochemical properties and multifunctionality.

Among all the inorganic vectors, mesoporous silica showed to be the most successful inorganic system for drug delivery. Mesoporous silica consists on nanoparticles of 50-300 nm diameter, with honeycomb-shaped pores of 2-10 nm and an easily functionalizable surface. These particles have been successfully used in drug, probe, enzyme and oligonucleotide delivering tasks and present low immunogenicity and good biocompatibility.^{34,35} Quantum dots are other interesting inorganic nanomaterials. They are semiconductive nanocrystals composed of

cadmium selenide and have shown a great potential for *in vivo* imaging, sensing and diagnostic purposes.^{36,37} Carbon nanotubes also showed to be good nanocarriers³⁸ due to the extremely high specific surface area and to the potential uses in photothermal therapy and imaging as gold nanoparticles do.³⁹

Figure 8. Summary of inorganic nanomaterials with applications in nanomedicine.⁴⁰

Moreover, nanoparticles of inorganic nature are also used for tissue engineering and repair. Carbon-based nanostructures and molybdenum and tungsten disulphides are used in conjunction with polymers to create nanocomposites for bone tissue engineering, showing significant improvements in mechanical properties and healing ability.⁴¹

5.2.4.2 Organic nanomaterials

Nanocarriers based on organic building blocks (Figure 9) are nowadays the most used and effective agents for drug-delivery, imaging or sensing applications. The stimuli-responsiveness, simplicity of preparation methods, monodispersity, appropriate size, high loading rate and biodegradability provide to this kind of systems the ideal features for medical application and to successfully overcome clinical trials.^{28,42}

Figure 9. Graphical representation of the most common types of organic nanoparticles.

Bioconjugates

Proteins, peptides or nucleotides can be considered drugs when the diseases to be targeted are due to the lack of these specific biomolecules. Biomolecules are relatively unstable, moreover, the direct injection of biomolecules in the bloodstream produces fast immune system clearance (proteases). For this reason, biomolecules are linked to polymeric components for targeting therapeutic effects. The most successful bioconjugation process is PEGylation (linking of poly(ethylene glycol)), in order to improve pharmacokinetics, reduce toxicity, increase solubility, decrease systemic clearance, and reduces antigenicity and immunogenicity.⁴³ Bioconjugates advantages are their high biocompatibility and their very small size (≈ 10 nm), which enables to take advantage of EPR effect. Technological progress in the development of new protein carriers (e.g. monoclonal antibodies), drugs and especially linkers has greatly improved their capability as potent therapeutics.⁴⁴ So much so is the case that some bioconjugates are nowadays marketed for treating different diseases, for example Pegintron® (PEGylated interferon alfa-2b) and Pegasys® (PEGylated interferon alfa-2a) for hepatitis C treatment, or Kadcyla® (ado-trastuzumab emtansine), an antibody drug conjugate for the treatment of HER2-positive metastatic breast cancer. Other bioconjugate-like approved drug is Abraxane®, composed of albumin nanoparticles containing paclitaxel for breast cancer treatment.^{45,46}

Vesicles and Liposomes

Liposomes are spherical vesicles, typically on the size range of 50-500 nm, formed by a lipid bilayer that can be composed of cationic, anionic or neutral (phospho)lipids that encloses an aqueous core (Figure 10).⁴⁷ Both the lipid bilayer and the aqueous space can incorporate hydrophobic or hydrophilic compounds, respectively. Liposome characteristics and behaviour *in vivo* can be modified by addition of a hydrophilic polymer coating, polyethylene glycol (PEG), to the liposome surface to confer steric stabilization, reduce opsonisation and prolong plasma circulation time. An easy introduction of theranostic actives to liposome surface (targeting elements, imaging components, therapeutic components) and targeting elements (antibodies, peptides, and carbohydrates) confers to liposomes enormous versatility and multifunctionality.^{42,48,49} Taking advantage of these features, Doxil (liposomal doxorubicin)

was the first FDA-approved nano-drug.⁵⁰ After Doxil, several liposome-based materials have overfilled the market of nanoengineered materials for therapeutic purposes: DaunoXome® (liposomal daunorubicin),⁵¹ Caelyx® (PEGylated liposome loaded with doxorubicin),⁵² Marqibo® (liposomal vincristine sulphate),⁵³ Myocet® (unPEGylated liposomal doxorubicin) and Visudyne® (liposomal verteporfin).⁵⁴

Figure 10. Schematic representation of the different types of liposomal drug delivery systems. **(A)** Conventional liposome; **(B)** PEGylated liposome; **(C)** Ligand-targeted liposome; **(D)** Theranostic liposome.⁴⁹

Futhermore, liposomes also have been extensively used as biosensors. For example, Zhang and co-workers designed perylene bisimide amphiphiles that formed vesicles of diameter of *ca.* 40 nm, able to encapsulate bis-pyrene molecules. FRET between this two chromophoric systems (encapsulated energy-donor molecules and a bilayer dye membrane as an energy acceptor) provides ultrasensitive pH information about the aqueous environment by displaying pH-dependent fluorescence and colour changes covering the whole visible light range.⁵⁵ Vesicular systems also showed useful properties for biomolecular recognition when bioreceptors were embedded in. In a representative example, Kolusheva designed liposomal phospholipid/polydiacetylene vesicles with embedded amphiphilic catecholamine receptors, which underwent fluorescence variation upon binding of hosts to the receptor matrix.⁵⁵

Dendrimers

Dendrimers are highly branched single macromolecules, very precisely synthesized by sequential reaction of a central core and outer molecules to construct the branches. The precise synthesis of dendrimers enables a total control over the polydispersity and the degree of branching (Figure 11). The choice of the central core, branching nature and number of shells determines physicochemical features, such as molecular weight, size, branching, density, flexibility and water solubility. Numerous conjugation sites are present for selective tuning and the presence of a large volume cavity also permits passive entrapment of actives. The fine polydispersity control achieved in the preparation of dendrimers simplifies the study of toxicity and analytical evaluation, as well as gives control of stoichiometry of loaded actives and, consequently, uniform output of biological effects *in vitro* and *in vivo*.^{56–58} Nowadays DEPTm-Docetaxel®, a dendrimeric nanoengineered material, is in phase I of clinical trials for breast, prostate, lung and ovarian cancer.⁵⁹

Figure 11. Schematic representation of the different types of dendrimers.⁶⁰

5.2.4.3 Polymeric micelles

Micelles are particles formed by amphiphilic agents with a polar head and an apolar tail (Figure 12), with sizes typically within 5-100 nm. Self-assembly of micelles occurs in a narrow concentration window (critical micellar concentration, CMC). The driving force of the formation of micelles is the hydrophobic effect, desolvation of hydrophobic tails leads to a favourable entropic change. Additionally, van der Waals interactions keep cohesion of the tails and new hydrogen bonds between the hydrophilic shell and water are formed. Two well-differenced kinds of micelles are known as active vectors in medicine: amphiphilic copolymers and low-molecular-weight surfactants. Copolymer based micelles show CMC values much lower (*ca.* 10^{-6} M) than surfactant based ones (*ca.* 10^{-3} M) and, therefore, they are thermodynamically stable even at high dilutions usually used in biomedicine. For this reason, polymeric micelles have extensively been used as drug delivery systems.⁶¹

Polymeric micelles systems present a hydrophobic core stabilized with a hydrophilic shell. They have several advantages over other nanosized drug delivery systems, such as a smaller size and higher abilities to load hydrophobic actives. Easy surface functionalization can also be achieved for active targeting.^{62,63}

Figure 12. Schematic representation of micelle loaded with doxorubicin and the different typical polymeric monomers used for preparing micelles.

Polymeric micelles are extensively studied as nanocarrier systems for potential active delivery with medical applications. Hydrophobic biodegradable polymers are mainly used for creating these nanoengineered systems (polycaprolactone, poly(lactide-co-glycolide) -PLGA-, polyglycolic acid and polylactic acid -PLA-).

Most of the polymer (usually co-polymer) nanoparticles fabricated are limited to present a spherical shape and relatively high polydispersity. However, some other properties, such as size, hydrophobicity and biodegradability are tunable by just choosing the proper polymer. The preparation of nanoparticles from these biodegradable polymers is relatively easy and efficient loading of hydrophobic actives can be achieved by direct physical adsorption,⁶⁴ whereas hydrophilic ones can be loaded using multiple methods, such as double emulsions or covalent link on the surface of nanoparticles after their fabrication. The biodegradable nature of these polymers (degradation of PLA and PLGA produces lactic acid and glycolic metabolites that are naturally eliminated) leads to a much better cargo release control. PLA and PLGA have achieved FDA approval for human administration. Several polymeric nanoparticles have reached clinical trials: CALAA-01 and Docetaxel-PNP are in phase I, Genexol-PM against ovarian and lung cancer and BIND-014 for prostate cancer are in phase II and Zinostatin Stimalamer (styrene-maleic acid copolymer + neocarzinostatin) for human hepatocellular carcinoma⁶⁵ and Genexol-PM[®] for breast cancer treatment are approved.

Polymeric nanogels

The difference between polymeric micelles and nanogels is subtle but important, and, indeed, in the literature these two types of polymer-based nanocarriers are often confused. It should be noted that polymeric nanogels are formed by flexible hydrophilic polymers crosslinked physically (via hydrophobic or electrostatic interactions)⁶⁶ or chemically (via disulfide, amine, click chemistry or photo induced, for example)⁶⁷ and are unequivocally characterized of being capable of retaining high amount of water or physiological fluids, usually more than the polymers' own weight (Figure 13).

Figure 13. Schematic representation of different synthetic methods of crosslinking.⁶⁸

To accomplish this requirements, wide chemical nature of polymers has been used for nanogel synthesis (Figure 14):⁶⁹

- Natural polymers: anionic (hyaluronic acid, pectin, carrageenan, chondroitin sulphate, dextran sulphate), cationic (chitosan, polylysine) and neutral (collagen, fibrin, dextran, agarose, pullulan).
- Synthetic polymers: polyesters (PEG, PLA, PLGA, PCL, PHB, PF, PBO) and other polymers (CD, PAAm, PNIPAAm, EMA, PVA, PNVP, MMA, PGEMA-sulfate, PEO, PPO, PHPMA, HEMA, PMMA, PAN).

Where PEG, poly(ethylene glycol); PLA, poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); PCL, polycaprolactone; PHB, poly(hydroxy butyrate); PF, propylene fumarate; PBO, poly(butylene oxide); CD, cyclodextrin; PAAm, polyacrylamide, PNIPAAm, poly(N-isopropyl acrylamide); PVA, poly(vinyl alcohol); PVA, poly(vinyl amine); PVAc, poly(vinyl acetate); PNVP, poly (N-vinyl pyrrolidone); PAAc, poly(acrylic acid); HEMA, hydroxyethyl

methacrylate; PGEMA, poly(glucosylethyl methacrylate); PEO, poly(ethylene oxide); PPO, poly(propyleneoxide); PHPMA, poly(hydroxypropyl methacrylamide); PEMA, poly(ethyl methacrylate); PAN, polyacrylonitrile; PMMA, poly(methyl methacrylate).

Figure 14. Example of different kinds of monomeric species for nanogel formation.

Nanogels are very promising as drug-delivery carriers due to their high loading capacity, flexibility, versatility, high stability and unprecedented stimuli-responsiveness to environmental factors (pH, temperature and ionic strength). Nanogels present swelling-shrinking properties that make them unique nanomaterials. The swelling of nanogels is controlled by pH, ionic strength and chemical nature of low-molecular mass ions in the case of polyelectrolyte gels as well as temperature for thermoresponsive gels (Figure 15). The driving force behind this behaviour is the balance between the osmotic pressure and the polymer elasticity. The osmotic pressure of polyelectrolyte hydrogels results from the net difference in the concentration of mobile ions between the interior of the gel particle and the outer solution. The ionized groups attract hydrated counterions. This favours the swelling of the gel, while the entropy elasticity of the polymer chains opposes the expansion. The ionization of weak polyelectrolyte gels depends on the pH value. A reduction in the total charge and number of counterions as the pH changes results in compression of the gel (because of decreased osmotic pressure) until the excluded volume of the polymer chains limits further compression.⁷⁰

Figure 15. Factors that affect nanogel swelling. **a)** An increase in the amount of cross-linking decreases the swelling of nanogels formed by a hydrophilic polymer. **b)** An increase in the pH value results in the collapse of a nanogel comprised of weak polybase chains, and the swelling of nanogels comprised of a weak polyacid. **c)** An increase in the ionic strength decreases the swelling of polyelectrolyte nanogels. **d)** Nanogels collapse as the temperature increases. **e)** Drug release from nanogels: diffusion of the drug from nanogels; drug release through degradation of the biodegradable polymer chains or cross-links; a change in the pH value leads to release of the electrostatically bound drug; multivalent low-molecular-mass cations or polyions of positive or negative charge can displace drugs having the same charge sign from electrostatic complexes with an ionic nanogel; drug release can be induced by the application of external energy to the nanogels, which induces degradation or structural transition of the nanogel polymer chains.⁷⁰

In a representative example, S. Thayumanavan described a PEG-Pyridyl disulfide (PDS) polymer with disulfide crosslinking. The PEG chains gave biocompatibility, water solubility and prolonged circulation. On the other hand, PDS units provided the lipophilic functionality to attain the right hydrophilic lipophilic balance (HLB) in solution to form the assembly, in addition to allowing hydrophobic drug encapsulation and crosslinking (Figure 16). Doxorubicin (Dox) loadings up to 24 % wt/wt were achieved and the leakage could be regulated by tuning the crosslinking density.^{71,72}

Figure 16. Schematic representation for PEG-PDS nanogel assembly.⁶⁷

Polymeric self-assembled nanogels

Self-assembled nanogels are a special kind of engineered nanomaterials in which the nanogel formation occurs driven by physical interactions (mostly leaded by hydrophobic crosslinkers). First self-assembled nanogel was synthesized in 1993, formed by cholesterol-bearing pullulan (CHP).⁷³ CHPs with less than 2 cholesterol groups per 100 glucose units, formed nano-sized particles and had over 70% water by weight were (Figure 17). One of the most attractive characteristics of self-assembled nanogels is their role as molecular chaperones, namely, they can spontaneously trap proteins in the nanoscale hydrogel matrix suppressing protein irreversible aggregation, and significantly increasing their colloidal and thermal stability.⁷⁴ It has to be noted that high efficient protein and nucleic acid encapsulation is an attractive property for immunological drug delivery, particularly for cancer vaccines.

Development of cationic nanogels enhanced nanogel interaction with the cellular membrane, helped with the permeation of nasal mucosa and enhanced the encapsulation of anionic biomolecules, such as siRNA and oligo-DNA. Intranasal vaccination and intracellular delivery of proteins, siRNA, and oligo-DNA are representative examples of successful applications of cationic nanogels (several formulations are in phase I clinical trials).^{75,76} It has been clearly demonstrated that self-assembled nanogels have strong potential as advanced drug delivery systems that enhance the therapeutic efficacy of a diversity of pharmaceuticals. Success in the treatment of immunological disorders encourages researchers to look for applications of nanogels in other clinical fields, with many ongoing investigations on therapeutic applications of these promising nanomaterials.

Figure 17. Representative sequence of polymeric self-assembled nanogel formation. Adapted from ref. 66.

Low molecular weight self-assembled nanocrystals

Unlike the organic systems mentioned above, organic nanocrystals are aggregates with a relatively ordered solid structure at nanoscale. They are often made from rather water-insoluble organic compounds.⁵⁵ Organic nanocrystals commonly do not include aggregates formed by amphiphilic compounds, whose aggregation tends to produce rather amorphous structures, as highlighted in a comparison reported for crystalline vs amorphous supramolecular gels.⁷⁷ Organic nanocrystals show wide applications in photonics, optoelectronics, biosensors, diagnostics and cancer therapy. Synthetic strategies to form these self-assembled nanostructures are easy and straightforward. The simplest and most used is based on the bottom-up approach, in which the organic compound is dissolved in the minimal amount of a suitable solvent and then poured into a bulky poor solvent as for example water.

Self-assembled organic nanocrystals from small molecules can be platforms for sensing biomolecules. The most used systems are based in aggregation induced emission fluorophores (extensively discussed in chapter 2). These species have a very weak emission in solution, but, upon aggregation, the restriction of intramolecular motions blocks non-radiative relaxation pathways and enhances enormously fluorescence emission. Since 2002, lots of self-assembled nanocrystals with their biosensing capabilities based on this phenomenon were described. For instance, Tang developed TPE (tetraphenylethylene) and TPS (tetraphenylsilole) based biosensors for bioanalytes as glucose, cysteine, insulin, albumin, ssDNA and dsDNA, among others. Detection methods were mainly based on regulation of the FRET process taking place between the nanocrystal and the corresponding acceptor/donor located inside or outside of the nanomaterial.^{78,79} In a representative example, aiming to detect the presence of carboxyl

esterase, Wu and co-workers employed TPE derivative based on a quaternary amine with a highly flexible hydrophobic tail, which self-assembled in nanoparticulate crystals of around 30 nm diameter. The addition of a fluorescein diacetate to the solution did not show any FRET mechanism, as fluorescein was too far in the solution phase. However, when esterase hydrolyzed the two acetate moieties, two negative charges were released and electrostatic interactions between quaternary amine and negatively charged fluorescein approximated the fluorophores and activated FRET efficient mechanism (Figure 18).⁸⁰

Figure 18. Schematic illustration for the assay system and its ratiometric fluorescence response to carboxyl esterase. Photographs were taken under handheld UV light (365 nm).⁸⁰

Organic nanocrystals also have been extensively used as bioimaging agents. In a nice example Mandal and co-workers synthesized uniform cyanostilbene-derived nanoparticles of around 175 nm with attached folic acid. These nanoparticles were able to be excited by three photons (1300 nm excitation) and showed luminescence at 520 nm. They used these nanocrystals for the visualization of tumour cells with over-expressed folic acid receptors such as HeLa cell line.⁸¹

Finally, organic nanocrystals can serve as drug vectors to accomplish drug delivery and therapy.^{82,83} As commented before, during nanoparticle formation drugs or other bioactive agents can be embedded or encapsulated in the inner spaces of the particles and, then, released spontaneously (sustained release) or due to external stimuli (controlled release).

Other convenient approach to carry out antitumor therapy is transforming drugs directly into nanoparticles (self-carried system), via ball-milling or re-precipitation methods. As a recent example, Liang and co-workers presented *in vitro* and *in vivo* studies of curcumin self-carried nanoparticles, which showed a sustained release of over 60 % of active drug after 300 hours in a sustained manner (Figure 19).⁸⁴ However, some drawbacks of this self-carried system are a comparatively long drug-release time, low release rate (due the compactness of the nanoparticle) and the absence of stimuli-responsive sites in curcumin.

Figure 19. Schematic illustration of functionalized self-carried Curcumin NPs from nanoparticle formation, to delivery. **(a)** Preparation of pure Cur NPs with no fluorescence (OFF) via solution exchange method. **Upper-left:** chemical structure of Cur. **(b)** Surface modification of the pure Cur NPs with C₁₈PMH-PEG through hydrophobic interaction. **(c)** PEGylated Cur NPs enter into tumor cells by endocytosis and releases the Cur molecules, which recover their strong green fluorescence (ON).⁸⁴

To overcome these drawbacks, several approaches have been developed to construct stimuli-responsive self-assembled nanocrystals. In this regard, several pH-responsive drug delivery nanocrystals have been developed. For example, Cao and Yan presented a pH-responsive system that showed excellent antitumor effect *in vitro*: doxorubicin was linked to a pyridinium terminated alkyl chain to give self-assembled nanoparticles in aqueous media. The system was engineered to undergo rapid and efficient release of doxorubicin at endosomal pH (5.5) via carboxylic acid assisted hydrolysis of the hydrazone bonds.⁸⁵ Light-responsive nanocrystals have been also engineered as promising active-delivery systems. Singh and co-workers prepared nanoparticles consisting in chlorambucil linked by an ester bond to perylene-3-

ylmethane. The photocleavable nature of that molecule enables chlorambucil release upon visible light exposure.⁸⁶

Nanogels from low molecular weight gelators

Opposed to the well-known polymeric nanogels, the miniaturization to nanoscopic level of macroscopic molecular gels has not been addressed so far. From here on, the miniaturized molecular gels at nanoscopic level will be referred as “molecular nanogels”, being “molecular microgels” the analogous micrometer-sized structures. In this chapter, a study of minituarization of macroscopic molecular gels into nano-sized structures is reported. The study tries to answer questions such as if nanometer sized porous particles can be prepared from molecular gels. Thermodynamic and kinetic stability of the particles is also investigated and, very importantly, the ability of molecular nanogels to incorporate and release actives. This property would turn this nanogels into potentially useful nanocarriers in fluorescence imaging or drug delivery systems.

5.3 1,8-Naphthalimide nano(micro) carriers

Two nano-engineered 1,8-naphthalimide derivatives are studied in this section: **1** and **2** (Figure 20). The selection of these gelators was motivated by their ability to form molecular gels (self-assembled fibrillar networks) in a great variety of solvents, including aqueous media (mgc of compound **1** in different solvents shown in Chapter 4, section 4.3.2.1). This feature is very appropriate considering the potential application of nanogels in the biomedical field. Moreover, the aggregation induced emission phenomena that these two molecules have (seen in chapter 4) facilitates the task of monitoring aggregation process and, more importantly, the visualization of microgels by confocal laser scanning microscopy (CLSM).

Figure 20. 1,8-naphthalimide derived gelators.

5.3.1 Concentration-dependent self-assembled structures

Preliminary studies were performed in order to assess the concentration effect on the morphology of the aggregates of these molecular gelators by using the precipitation method. Experimentally, this method is based on the addition of gelator dissolved in DMSO over water (more details provided in experimental section 5.7.2 in the present chapter). Results showed that the final concentration of the sample determines the shape or size of the aggregates. Figure 21 compares the electron microscopy images of samples of gelator **1** prepared at final concentrations of 2 mM, 0.1 mM and 10 μ M. It can be observed that in the case of the most concentrated sample, elongated fibers of monodisperse width (*ca.* 20 ± 3 nm) were formed, corresponding to the appearance of macroscopic gel particles. Interestingly, a 20-fold reduction of the concentration affords discrete objects composed of several short elongated fibers with a sheaf-like arrangement. As discussed in more detail below, these microgel particles would result from truncated growth of the large fibers. Finally, at the lowest concentration (10 μ M), a rather monodisperse distribution of nanosized spherical objects (diameter of *ca.* 20 ± 5 nm) was observed by EM. These structures can be ascribed to nanogel particles formed in the initial stages of nucleation of the self-assembly process.

Figure 21. Electron microscopy of the evolution of nano-micro-macro gels prepared by the same method due to an increase of gelator concentration. H₂O/DMSO (9:1); [**1**]=10 μ M / 100 μ M / 2 mM.

NMR studies show how the aggregates start to be formed as the poor solvent enters the system, and they become invisible to the NMR due to the reduced relaxation time of big aggregates (Figure 22).

Figure 22. Aggregation process of **1** (15.9 mM) in DMSO/H₂O; % H₂O= 0, 11.1, 20.0, 27.3 and 38.5, using 0.5 μ l of nitromethane as internal standard (23.3 mM, singlet at 4.4 ppm). The experiment is performed by adding increasing volumes of water to the same NMR tube. The formation of aggregates as the water enters on the system can be seen by the progressive disappearing and broadening of the signals.

As discussed elsewhere (chapter 3), spectroscopic techniques are ideal for the characterization of the aggregation process of this kind of molecules. The analysis of the UV-Vis spectra of samples with increasing concentration of **1** in the same solvent reveals a change in the shape of the absorption band, together with a bathochromic shift (Figure 23). Additionally, light scattering produced by aggregates formation as the concentration raises results in a baseline intensity increase, as well as a shoulder appearance at 356 nm.

Figure 23. Normalized UV-Vis spectra of compound **1** in H₂O/DMSO (9:1), with concentration values ranging from 0.5 μ M to 100 μ M.

Figure 24. Normalized fluorescence spectra showing peak shift and shape transformation upon aggregation of compound **1** in H₂O/DMSO (9:1) ranging from 0.5 μ M to 100 μ M.

In the case of fluorescence measurements, aggregation also results in the change of the emission peak shape and shifts the maximum wavelength (Figure 24). More importantly, the aggregation is associated to a strong AIE effect. The onset of AIE effect is estimated to be around 10 μ M (Figure 25 right). However, nanogel formation is estimated to occur in an interval from 0.1 μ M to 15 μ M, according to peak shift and TEM micrographs (Figure 25 left and Figure 21), revealing that the degree of aggregation and, presumably, the morphology of the aggregates is relevant for the observed AIE.

Figure 25. Left: Emission maximum shift of **1** in a H₂O/DMSO mixture (9:1) (λ_{ex} = 342 nm). Right: Fluorescence intensity enhancement of compound **1** (AIE) ranging from 0.5 μ M to 100 μ M.

Summarizing, Table 1 collects the different type of aggregates and morphologies formed by **1** upon aggregation at different concentrations and how the emission and absorption maximum wavelength is affected.

Table 1. Summary of different spectroscopic and morphologic features.

Concentration range	Aggregation state	Size range	Morphology	$\lambda_{\text{abs max}}$ / nm	$\lambda_{\text{em max}}$ / nm
<0.1 μM	Solution	-	-	341	394
0.1-15 μM	Nanogels	100-200nm	Spherical	343	394-406
15 μM -500 μM	Microgels	100-200nm (core) 1-3 μm (length)	Sheaf-like	343	406 intense emission
>500 μM	Macroscopic Gels	>5 μm	Regular fibres	343	406 intense emission

5.3.2 1,8-naphthalimide derived molecular nanogels

Sizing down of 1,8-naphthalimide derivatives (**1** and **2**) molecular gels into nanoparticles by two different synthetic methods (precipitation and reverse emulsion) is studied in this section. Furthermore, sheaf-like fibrillar microgels is also manufactured by the precipitation method and fully characterized. Finally, loading/release ability of different actives and their behaviour in biological environment are evaluated.

5.3.2.1 Synthesis of Nanogels by precipitation method

Dropwise addition of dissolved substrates into a solvent where the species are insoluble represents a simple and straightforward approach for the preparation of micro- and nanosized aggregates.¹⁸ In our case, controlled slow addition (syringe pump) of compound **1** dissolved in DMSO to water afforded nice particles, whose size is regulated by the concentration used in the experiment (Figure 26). On a representative example, 100 μL of stock solution of **1** (1 mM, 0.4 mg/ml) in DMSO is diluted to achieve 1 mL of solution 0.1 mM in DMSO. This solution is dropwise added, with an addition pump at 0.6 ml/min rate to a glass vial containing 9mL of mili-Q water filtered over PVDF filter (polyvinylidene difluoride 0.22 μm mesh) with vigorous stirring to achieve 10 μM dispersion of **1** with a 9/1 H₂O/DMSO solvent ratio.

Figure 26. Schematic representation of precipitation method. A solution of the gelator in a good solvent (DMSO) (**left**), is added to a poor solvent (H_2O) with vigorous stirring (**right**).

5.3.2.2 Nanogel size dependence on concentration

Dynamic light scattering (DLS) analysis has been used for the following experiments. This technique permits to calculate the hydrodynamic diameter of nanoobjects, assuming that the nanoparticles being studied diffuse in the same fashion as a hypothetical hard sphere. Among all the information provided by DLS analysis, two related hydrodynamic diameter distributions were of interest: i) Intensity distribution (D_i) is naturally weighed according to the scattering intensity of each particle, which means that big particles present predominant contribution to the size distribution. For this reason small amount of big aggregates can mislead the measurement interpretation. ii) Number distribution (D_n) is calculated by applying Mie theory to transform the intensity distribution to one describing the relative proportion of multiple components. This distribution is based on their number rather than on their scattering.

Nanogels could be prepared reliably in the range of concentrations 0.1 to 25 μM . No nano-objects were detected by DLS for $[1] < 0,1 \mu\text{M}$ and microparticles were predominant above 25 μM (Table 2 and Figure 27). Studied samples (concentrations between 0.1 and 25 μM) showed to be among 100 and 200 nm in and did not present size increase with the concentration. The variability of the hydrodynamic diameter of nanogels at this concentration range is attributed to the little distortions introduced by stirring velocity or droplet size when precipitation method is carried out. For this reason, it is recommended for further experiments to perform several replicas. At concentrations over 25 μM and up to 100 μM , microgel formation was observed ($D_n > 1000 \text{ nm}$), which correlates well with the aggregation monitorization of **1** by fluorescence experiments shown in Table 1 and Figure 25. The change in the emission maxima was used to estimate a critical aggregation concentration around 0.1 - 1 μM (Figure 25).

Therefore, this value represents the bottom concentration limit of thermodynamic stability for the self-assembled nanoparticles.

Table 2. Particle size diameter variation with the concentration of gelator [1] in 10% DMSO without stabilizer.

[1] / M	Dn / nm
$1,00 \cdot 10^{-8}$	-
$1,00 \cdot 10^{-7}$	183 ± 5
$5,43 \cdot 10^{-7}$	93 ± 56
$1,00 \cdot 10^{-6}$	109 ± 3
$2,50 \cdot 10^{-6}$	117 ± 14
$5,43 \cdot 10^{-6}$	87 ± 3
$1,05 \cdot 10^{-5}$	224 ± 42
$1,57 \cdot 10^{-5}$	83 ± 20
$2,62 \cdot 10^{-5}$	90 ± 20
$5,23 \cdot 10^{-5}$	Aggregates present
$7,85 \cdot 10^{-5}$	Aggregates present
$1,05 \cdot 10^{-5}$	Aggregates present

Figure 27. Up: Representative DLS size distribution histogram by number of nanoparticle sample [1] = 5.43 μ M. Down: Particle size diameter variation with the concentration of gelator 1 in 10 % DMSO.

5.3.2.3 Nanogel stability with time

Nanogel colloidal stability was studied at three different concentrations and has shown excellent temporal stability towards further aggregation/precipitation, being the DLS analysis reproducible for several days (Figure 29 and Table 3). Even after one month, nanoparticles were still present, although with moderately higher average diameter (D_n). Z-potential is defined as the electric potential between the interfacial double layer in the slipping plane (where ions in solution are loosely attached to the particle and move with the particle when it moves in the liquid) and any ions outside the boundary (this ions will stay where they are no matters if the particle is moving) (Figure 28). It is measured using a combination of two measurement techniques: electrophoresis and laser Doppler velocimetry. This method measures how fast a particle moves in a liquid when an electrical field is applied. Z-potential is calculated from the velocity, the electrical field applied, the viscosity and the dielectric constant of the system by applying the Henry equation:

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta}$$

Figure 28. Diagram showing the ionic concentration and potential difference as a function of distance from the charged surface of a particle suspended in a dispersion medium.⁸⁷

Where z = zeta potential; U_E = Electrophoretic mobility; ε = Dielectric constant; $f(ka)$ = Henry's function (typically values of 1 for organic solvents and 1.5 for aqueous solutions); η = Viscosity.

Z-potential can be used as an indicator of colloidal stability of a nanoparticles suspension. It has been studied that high values (negative or positive) of Z-potential confers stability to the dispersion and it will prevent aggregation to occur. In general, absolute values between 0 to 5 mV indicate rapid flocculation, from 10 to 30 mV, slow flocculation, from 30 to 40 mV moderate stability and above 40 mV it is considered as good stability.^{88,89} The particles formed by **1** showed negative charge with a Z-potential of -22 ± 0.9 mV despite the absence of ionisable groups. It is important to recall that assembled apolar colloidal nanostructures often present negatively charged surfaces when entering in contact with water. For example, negative zeta potentials are measured in the literature for self-assembled monolayers with hydrocarbon functionalities.⁹⁰ The charging of non-polar surfaces when entering in contact with aqueous solutions has been investigated by several researchers but it is still a controversial issue. The most accepted hypothesis is that negative charges are due to the adsorption of hydroxide ions from the solution.⁹¹ The value of -22 mV is in good agreement with the previous studies carried out about the stability of nanoparticles of **1** with time, in which it could be seen that nanoparticles diameter increased from ≈ 100 nm to ≈ 300 nm in one month.

Table 3. Particle size (diameter) variation of nanogels of **1** with time in 10 % DMSO without stabilizer.

Figure 29. Particle size diameter evolution of fresh samples of compound **1** and after a month.

5.3.2.4 Nanogel stability with temperature

Nanogel stability with temperature was studied. The soft intermolecular forces that maintain nanogels assembled suggest that they should be relatively responsive to temperature. However, DLS measurements carried out at four different temperatures and two concentrations (Table 4 and Figure 30), showed that temperature does not significantly affect the size of the particles. The relative insensibility of supramolecular gels in water to temperature increase has been recently studied and can be rationalized taking into account that the aggregation enthalpy is almost null for this process. For this reason, the disassembly of supramolecular aggregates become an entropy-prevailing process, being the hydrophobic effect the driving force.⁹²

Table 4. Particle size (diameter) variation of nanogels of **1** with temperature (25 °C, 45 °C, 65 °C, 85 °C) in 10 % DMSO.

	5.43 μ M	2.5 μ M
Temp / °C	Size _{t=0} / nm	Size _{t=0} / nm
25	222 $\pm$ 18	194 $\pm$ 5
45	254 $\pm$ 8	218 $\pm$ 6
65	233 $\pm$ 16	194 $\pm$ 42
85	206 $\pm$ 5	208 $\pm$ 8

Figure 30. Particle size (diameter) variation of nanogels of **1** with temperature in 10 % DMSO. [1] = 2.5 μ M and 5.4 μ M.

5.3.2.5 Nanogel size variation with salt content

As we discussed in chapter 4, salt presence usually modifies the water structure in the solvation sphere of hydrophobic surfaces, generating alterations in the solvophobic interactions between the macroscopic gelators and the water, giving rise to the so-called Hoffmeister effect.⁹³ Colloidal suspensions, as polymeric nanogels, usually show swelling or shrinking⁹⁴ when salts are added to the media.^{95,96}

Studies for nanogels of **1** were carried out in the presence of increasing amounts of NaCl. It can be observed at Figure 31 and Table 5 that there is a range of NaCl concentration where the nanogels are practically insensitive to the presence of NaCl, with size of the particles

remaining around 180 nm. However, when salt concentration is similar to the concentration in blood, nanogels begin to undergo size increase up to *ca.* 256 nm. When a very high concentration of NaCl is present, 330 mM, similar to sea water, particle size undergoes a big increase up to 928 nm. This behaviour could be rationalized as when NaCl enters the system the repulsive interactions between nanoparticles can become screened by the accumulation of cations in the first hydration layer, provoking aggregation.

Table 5. Particle size (diameter) variation of nanogels of **1** with NaCl concentration in 10 % DMSO.

Figure 31. Particle size (diameter) variation of nanogels of **1** with NaCl concentration.

5.3.2.6 Electron microscopy of 1,8-naphthalimide derived nanogels.

An important aspect to consider is if the described nanogels are really gels, regarding if they constitute particles mainly composed of a solvent retained in the microstructure, or, on the contrary, are solid-like, non-porous objects. Evidence on this respect comes following the important size reduction detected in the comparison of wet samples of nanogels of **1** at 5.4 μM (87 ± 3 nm width, by DLS) and the dried ones (*ca.* 20 ± 5 nm width, by TEM and SEM), suggesting that nanoparticles are swollen in the solvent (Figure 32a-b).

Figure 32a. Tentative models of swelled and shrunk nanogels.

On the left top row of Figure 32b, individual organic nanoparticles can be observed by SEM, standing on the surface of a bronze holder. On the right top row of the figure, the agglomeration of nanoparticles of **1** due to the drying process can be observed. SEM samples were coated with Pt prior to observation. On the bottom row, TEM images are shown, presenting individual organic nanoparticles. No further staining was used for TEM observation.

Figure 32b. Nanogel particles formed by **1**. **Top row:** SEM; **Bottom row:** TEM

5.3.3 pH responsive nanogels by inverse emulsion method

Another assayed method for the synthesis of nanogels is the inverse emulsion method (Figure 33). In our case, a pH-responsive gelator, **2**, was used to create this kind of system. Compound **2** has a $pK_a \approx 5$ and, therefore for pH values below 5 the molecule aggregates, but at neutral and basic pH values, the molecule is completely soluble in water. The procedure starts with a solution of compound **2** in basic (NaOH) water. This aqueous mixture is dispersed in a solution of hexane that contains bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT). The

Figure 33. Nanogel synthesis of **2** by inverse emulsion method.

The final mixture, a transparent solution, contains 98.4 % hexane at only 1.6 % of water. Then, aqueous HCl is added, causing nanogel aggregation inside the water micellar medium. The particles are isolated from hexane and resuspended with HCl 0.01 M. Additionally, the addition of CaCl_2 , a precipitating chelating agent, and several extractions of the aqueous suspension with hexane are performed to remove the surfactant excess. The evolution of the system during the preparation of the nanoparticles was followed by fluorescence and UV-Vis spectroscopy, DLS and transmission electron microscopy. Different absorption and emission behaviour was observed depending on the environment of the gelator molecules (Figure 34).

Figure 34. UV-Vis spectra (**top**) and fluorescence spectra (**bottom**) of along nanogel **2** preparation by inverse emulsion method. Black slashed line: basic medium in water; red dotted line: basic medium, inverse emulsion in hexane; green solid line: acidic medium, inverse emulsion in hexane. $[2] = 0.25$ mM.

When **2** is deprotonated and water-soluble, its absorption and emission maxima are at 343 nm (shoulder at 335 nm) and at 390.5 nm, respectively. Introduction of the aqueous solution into hexane via inverse micelles results in a shift both in absorption and emission spectra, probably due to a more hydrophobic environment ($\lambda_{\text{abs}} = 335$ nm and shoulder at 323 nm; $\lambda_{\text{em}} = 385$ nm). Isolated nanogel particles as colloidal solutions in acidic water show further shifts of the spectra ($\lambda_{\text{abs}} = 343$ nm, $\lambda_{\text{em}} = 393$ nm).

The preparation was also followed by DLS analysis. As shown in Figure 35, the basic emulsion showed the presence of objects with a diameter of $ca. 4.4 \pm 0.2$ nm, ascribed to inverse micelles that remained after acidification. Upon particle isolation and AOT removal, a drastic size increase was observed, probably due to the collapse of the aggregated nanostructures, resulting in objects with an average diameter of 67 ± 10 nm. Finally, upon further extractions to remove remanent AOT, objects at 407 ± 30 nm were detected. Particles obtained in this

way are composed of a mixture of AOT/gelator in a proportion 0.5/1, according to ^1H NMR spectroscopy.

Table 6. Size of the objects present in the system as different synthesis steps were carried out.

Figure 35. Nanogel synthesis of **2** by inverse emulsion method followed by DLS. Acidic emulsion in red, basic emulsion in green, AOT removal in blue and final dispersion in black.

Transmission electron microscopy showed relatively polydisperse nanoobjects (Figure 36). In the bottom row of Figure 36, the fibrillary nature of the nanogels can be clearly observed. It should be noted that segregation of AOT from the fibrillar particles can be observed as a dark stain in the same images.

Figure 36. Transmission electron microscopy of nanogels suspended in water. $[\mathbf{2}] = 0.25 \mu\text{M}$, $[\text{AOT}] = 0.12 \mu\text{M}$ (calculated by NMR).

Therefore, nanogel synthesis by the microemulsion method and pH shift is found to be a feasible approach for creating nanostructured self-assembled materials. However, several drawbacks were detected:

- Removal of AOT is not complete due to close interaction with hydrophobic fibres.
- Complex experimental procedure when compared with the precipitation method.
- Purification of nanogels requires several organic extractions.

Further loading and release experiments will be required to assess the future possible applications of the nanoparticles prepared in this way. In the present thesis, more efforts and knowledge deepening has been performed on microgels as a proof of concept, because of the ease of synthesis and purification steps.

5.3.4 1,8-Naphthalimide derived molecular microgels

As discussed in section 5.3.2.1, microgels of **1** could be prepared by precipitation, with final concentrations between 15 and 100 μM and principally characterized by DLS, electron microscopy and confocal laser microscopy. The preparation of a stable dispersion of microgels required the presence of gelatine (0.01 % w/w), in order to avoid premature aging into macroscopic aggregates.⁹⁷ In this way, stable samples containing microgels could be reproducibly prepared with moderate polydispersity (polydispersity index = 0.303). Polydispersity index (Pdl) is a measure of the particle distribution.

$$Pdl = \left(\frac{\sigma}{d} \right)^2$$

Where σ is the standard deviation and d is the mean diameter.

In a representative example, 1 mL of stock solution of **1** (1 mM, 0.4 mg/mL) in DMSO is added dropwise, through a syringe pump at 0.6 mL/min, to a glass vial containing 9 mL of filtered milli-Q water with 0.01 % w/w gelatine acting as stabilizer. The addition is carried out with vigorous stirring to achieve the colloidal microparticles of **1**, with a final solvent composition of 9/1 H_2O /DMSO. The dispersion becomes turbid and is left stirring for 5 min. The microgel purification step is performed by centrifugation at 6000 rpm for 60 min (x3), adding 10 mL of fresh water each centrifugation. A white pellet of microgel particles is formed, which can be

dispersed in pure water, PBS or culture media prior to use. It can be hypothesized that the initially formed nanogels act as nucleation points for the formation of sheaf-like structures of the microgels. Indeed, DLS analysis of fresh samples before centrifugation, show a bimodal distribution with species of $D_n = 84 \pm 2$ nm, corresponding to nanogels, and larger particles of $D_i = 920 \pm 198$ nm, corresponding to the microgels (Figure 37). Noticeably, when the same sample is measured after several minutes, nanogel particles are not present, having evolved most likely into microparticles.

Figure 37. Left: Representative DLS of fresh prepared nano/microgels, $[1] = 0.1$ mM. Right: Representative DLS of “aged” microgels, $[1] = 0.1$ mM in aqueous solution containing 10 % DMSO and 0.01 % gelatine.

The sheaf-like assembly of short fibers is clearly visualized by SEM and TEM, revealing that the objects are *ca.* 2 μm long and *ca.* 1 μm width (Figure 38, Figure 39). Nicely, the morphology observed for the dry samples used for electron microscopy is in accordance with that observed by CLSM in wet samples of the microgels (Figure 38). The analysis of the electron microscopy images shows that the sheaf-like bundles coexist with spherical nanogel particles, as indicated by DLS above (Figure 39).

Figure 38. Left: Scanning electron microscopy of xero-microgels 25 μM ; Centre: Transmission electron microscopy images of microgels 25 μM ; Right: Confocal fluorescence microscopy of molecular microgels 100 μM in suspension $\text{H}_2\text{O}/\text{DMSO}$ (9:1) 100 μM stabilized with gelatine 0.01%.

Figure 39. Top: SEM images of **1** nano/micro gels coexistence. Down: TEM images of **1** nano/micro gels coexistence.

5.3.4.1 1,8-Naphthalimide microgels as molecular carriers: Loading abilities

The loading ability and transport of actives are one of the principal objectives of the nanogels to be used as nano-sized delivery systems. For this reason, once the synthesis and characterization of the nano/micro gels were described, the capability to be loaded with organic molecules was studied. The loading process is performed by a coprecipitation-entrapment method. The loading and purification step are similar to the one described for microgel formation. The active to be loaded in the particles is dissolved together with gelator **1** in DMSO and, then, added to the aqueous media. The loading of 26 chemically different

molecules (drugs, photosensitizers, sensors, stains, bioimaging actives, etc...) was assayed. Notable loading into microgels of **1** was observed for 13 of the dyes (see structures in Figure 40 and Table 7).

Figure 40. Chemical structures of assayed dyes and photosensitizers.

Loading was followed by UV-Vis spectroscopy and was calculated by difference between the absorption peak maximum of the initial free dye introduced into the system (20 μ M) and the absorption peak maximum of the loaded dye inside microgels after centrifugation and resuspension steps. As it can be seen on the bottom row of Figure 41, coloured colloidal suspensions of microgels are obtained in this way. On the top row of that figure, the centrifuged microgels situated at the bottom of the cuvettes can be observed.

Figure 41. Up: Selection of loaded microgels after centrifugation step. Down: Resuspension of loaded microgels after purification step. $[1] = 100 \mu\text{M}$. From left to right: Acridine Orange, hypericin, Oxazyn 170, Toluidin Blue, Rhodamine 6G and Protoporphyrin IX.

A summary of the loading experiments is shown in Table 7. The loading capacity is expressed as mmol of loaded dye over mol of gelator. In almost all cases, absorption and fluorescence maximum peak shifts are observed, associated to the incorporation of the dyes into the microgels. It can be noted that dyes presenting high hydrophobicity (hypericin, Protoporphyrin IX and Nile Red) show the best loading abilities. Rose Bengal (RB) also showed very good loading values (*ca.* 60 mmol/mol or 15% w/w). In general, loading values between 5 and 25 % are considered as good, based on reports for micro- and nano-carriers of all chemical nature.^{67,98,99} Besides, big shifts are observed in both absorption and fluorescence spectra, indicating high affinity between gelator and those dyes. Other dyes presented a poor loading ability, in agreement with the more hydrophilic ones (i.e Eosin Y, Methylene Blue, Rhodamine 123 and Rhodamine B). Nile Red (NR) was the best loading dye, since no free Nile Red was detected after the loading procedure.

Table 7. Spectroscopic details of selected loaded dyes in 1 microgels. [1] = 100 μ M.

DYE	Abs free 20 μ M / nm	Abs loaded / nm	Fluorescence max free / nm	Fluorescence max loaded / nm	Loading mmol/mol
Acridine Orange	493 (1,098)	489 (0,103)	532	531	12.5
Rose Bengal	552 (2,074)	579 (1,060)	571	590	60.3
Nile Red	600	516	647	607	200 (Quantitative)
Eosin Y	519 (1,525)	540 (0,0174)	544	537	1.5
Hypericin	561 (0,247) 599 (0,215)	551(0,098) 598(0,032)	591	605	52.9
Oxazyme 170	582 (0,991) 619 (1,071)	619(0,141)	651	642	17.6
Methylene Blue	630	630	686	688	2.8
Protoporphyrin IX	642,5 (0,066)	644(0,0532)	588, 623	634	107.3
Rhodamine 6G	528(1,929)	528,5(0,157)	558	551	10.9
Rhodamine 123	502(1,972)	502,5(0,0348)	534	529	2.3
Rhodamine B	554(2,436)	554(0,0330)	581	572	1.8
Toluidine blue	633(1,200)	641,5(0,108)	647	641	12
Flavilium Et₃N	565,5(0,786)	570(0,00661)	606	593	1.7

Representative UV-Vis spectra of free and loaded dyes and are shown in Figure 42. Peak shifts as well as high baselines can be observed in a microgels spectra, due to light dispersion. Dyes loaded in these microgels are of very different chemical nature, cationic (i.e. Toluidine Blue), anionic (i.e. RB) or neutral (i.e. hypericin), but all have planar fused rings, which are hypothesized to intercalate into the microgel fibres.

Figure 42. Comparison of loaded and free dyes by means of UV-Vis spectroscopy ($[1] = 100 \mu\text{M}$). **Dotted line:** Dye in solution. **Continuous line:** Loaded dye after resuspension in H_2O .

A more detailed study was carried out for microgels loaded with Nile Red. Nile Red is a well-known hydrophobic dye that undergoes a high solvatochromic effect and is extensively used as a fluorescent dye to detect hydrophobic surfaces.^{100,101} Nile Red is poorly soluble in aqueous media, forming non-fluorescent aggregates. However, when it is in a hydrophobic environment, aggregates are disassembled, producing an increase in the fluorescence intensity and a notable hypsochromic shift. In the present case, UV-Vis spectroscopy revealed that the absorption spectrum changed its maximum from 599 nm to 516 nm (Figure 43 left). Nile Red incorporation was also detected by means of fluorescence emission spectroscopy. A four-fold intensity increase and an emission maximum shift from 644 nm to 605 nm were observed (Figure 43 right).

Figure 43. Comparison of loaded (black lines) and free Nile Red (red dashed lines) by means of UV-Vis (left) and fluorescence emission spectroscopy (right); $[1] = 100 \mu\text{M}$, $[\text{Nile Red}] = 20 \mu\text{M}$.

Nile red loaded microgels could also be nicely observed by confocal laser microscopy. The large separation between fluorescence emission of the naphthalimide unit (λ_{em} = 435 nm) and Nile Red (λ_{em} = 615 nm), allows to easily visualize the colocalization of the microgel of **1** and the Nile Red dye (Figure 44).

Figure 44. Left: Confocal fluorescence microspectroscopy of loaded microgels, showing the intrinsic blue fluorescence emission (blue circles) of **1** and the red emission of loaded Nile Red dye (red squares). Right: Images of microgels of **1** (100 μM) loaded with Nile Red (10 μM) in a H₂O/DMSO (9:1) suspension stabilized with 0.01% gelatine. Blue channel ($\Delta\lambda$ = 410-560 nm), red channel ($\Delta\lambda$ = 560-700 nm) and overlap of both channels.

Similar images were obtained with some biologically interesting dyes such as acridine orange (used as drug, DNA binder and lysosomal probe¹⁰²⁻¹⁰⁴) and RB and hypericin (extensively used as photosensitizers^{105,106}) (Figure 45).

Figure 45. Confocal fluorescence images of single microgels of **1** (100 μM) loaded with different dyes.

5.3.4.2 1,8-Naphthalimide microgels as molecular carriers: Release abilities

Once loading abilities of microgels have been described, the study of the release of loaded actives was assayed. Molecular gels are ideal delivery systems because of the intrinsic stimuli-responsiveness behaviour associated to the soft intermolecular forces that keep the gels cohered. It is important to remark that no leaching phenomenon has been observed after 24 hours. The stimuli-responsiveness of loaded microgels has been investigated by temperature increase as a proof of concept that the disassembly of the microgels produces dye release. RB was the dye chosen for the experiment as his absorption maximum is very different when RB is in free solvated state and when it is entrapped in the microgel matrix (Figure 46).

Figure 46. Normalized UV-Vis absorption spectra of loaded and free RB.

As described in Chapter 4, temperature increase promotes to microgel disassembly, since gel to sol transition temperature of compound **1** is around 60 °C. Apparently, the macroscopic behaviour detected for molecular gels and microgels is different from that for nanoscopic molecular nanoparticles, since they are insensible to temperature changes (section 5.3.2.4). This apparent contradiction could be rationalized by the achievement of the most stable thermodynamic state when heating up macroscopic molecular gels, which is the formation of nanoparticles. In the case of microgels, upon progressive heating, RB is released to the medium temperatures between 60 °C and 80 °C (Figure 47). Very importantly, loading process is reversible and upon cooling down, dye is again trapped into the microgel, although with a moderate decrease in loading efficiency (60 % of the initial).

Figure 47. Top: UV-Vis absorption spectra of loaded RB at different temperatures (25°C, 45°C, 65°C and 85°C). Inset: Absorption ratio between λ_{552} (inside) and λ_{579} (outside) the microgel. Red dot indicates the ratio of peaks upon readsorption when cooling down to 25°C; [1] = 100 μ M, [RB] = 5.6 μ M in H₂O. **Bottom:** Normalized UV-Vis absorption spectra of loaded and free RB at 85°C. [1] = 100 μ M [RB] = 5.6 μ M in H₂O.

Release kinetics of RB was also studied by UV-Vis spectroscopy at different temperatures. The evolution of the absorption maximum was monitored at 552 and 579 nm, in order to estimate the amount of RB released or entrapped in the microgels of **1**. Different kinetics were observed. A very slow release was observed at 37 °C, since absorption at 552 nm showed slight increase ($\Delta\text{abs}_{550} = 0.035$) after 60 min. In contrast, when the temperature was set to 60 °C or 80 °C, a very fast release was observed in <15 min and <5 min, respectively (Figure 48).

Figure 48. Release kinetics of Rose Bengal (5 μM) loaded in microgels of **1** (100 μM) at different temperatures (green line loaded RB abs peak at 579 nm; blue line free RB, abs peak at 550 nm): (a) 25 $^{\circ}\text{C}$, (b) 37 $^{\circ}\text{C}$, (c) 60 $^{\circ}\text{C}$, (d) 80 $^{\circ}\text{C}$.

5.3.4.3 1,8-Naphthalimide microgels as molecular carriers: $^1\text{O}_2$ production and modulation

As a proof of concept, the well-known capabilities of RB as photosensitizer for singlet oxygen production ($^1\text{O}_2$) were evaluated inside and outside the microgels. RB produces singlet oxygen upon irradiation at the visible region. Once photon absorption takes place, RB undergoes electron promotion to a singlet excited state S_n . Relaxation of the S_n state yields the lowest excited singlet state of RB, S_1 . Then, intersystem crossing (ISC) generates the sensitizer triplet state T_1 . From this long lived triplet state, a collisional energy transfer between RB (T_1) and $^3\text{O}_2$ ($^1\Sigma_g^-$) to yield $^1\text{O}_2$ ($^1\Delta_g$) can take place.¹⁰⁷

Several probes can be used to detect the generation of singlet oxygen, based on their oxidation and the consequent change in the UV-Vis spectra experienced. In our present case, ABDA (9,10-anthracenediyl-bis(methylene)dimalonic acid) was used as trap for the photogenerated singlet oxygen, as illustrated in Scheme 1.

Scheme 1. ABDA reaction with photosensitized $^1\text{O}_2$. (RB = Rose Bengal; ISC = intersystem crossing).

As can be seen in Figure 49 and Figure 50, RB singlet oxygen production is modulated by its incorporation into microgel particles. The production of singlet oxygen was notably slowed down for RB trapped into microgel, as revealed by the kinetic constants of the process ($k_{\text{free}}/k_{\text{gel}} = 4.6$). The release of RB by heating to 80 °C restored the singlet oxygen photogeneration capabilities ($k_{\text{free}}/k_{\text{gel}} = 0.9$). Slight variations in absorption spectra at RB region (500-600 nm) are observed due to little temperature variations derived from the measurement procedure.

Figure 49. UV-Vis analysis of singlet oxygen production by microgels of **1** loaded with RB. ([1]=100 μM , [RB] $\approx$ 5.6 μM , [ABDA] = 50 μM) in a $\text{H}_2\text{O}/\text{DMSO}$ (9:1) mixture stabilized with gelatine 0.02 %. Irradiation $\lambda > 400$ nm.

Figure 50. Singlet oxygen production rate upon temperature variation. The reaction rate increases as the RB is released from the microgels.

5.3.4.4 1,8-Naphthalimide microgels as molecular carriers: Photoprotection

A common drawback of fluorescent dyes and, in particular, of xantene derived photosensitizers is the photobleaching process. Upon irradiation, Rose Bengal undergoes oxidative electron transfer resulting in dye destruction.¹⁰⁸ Photobleaching evaluation experiments of free and buried RB into the microgels revealed interesting results (Figure 51). Free RB suffered decomposition upon visible irradiation with a rate of $(-18.5 \pm 0.2) \cdot 10^{-3} \text{ min}^{-1}$. However, loaded RB on the microgels underwent different degradation kinetics. In the first stages of the experiment (from 0 to 2 min irradiation), high photobleaching rate was observed $(-38 \pm 7) \cdot 10^{-3} \text{ min}^{-1}$, whereas in the second stage of the experiment (from 2 to 15 min irradiation), the photobleaching rate was reduced considerably to $(-8.4 \pm 0.3) \cdot 10^{-3} \text{ min}^{-1}$. These different decomposition rates can be attributed to the position of RB in the microgels. Presumably, RB adsorbed on the surface suffers high photobleaching rates and RB buried inside microgel structure undergoes low photobleaching phenomenon. The prevention of photobleaching of RB inside the microgels is an interesting feature for the loading and delivery of dyes undergoing fast photodegradation.

Figure 51. Photodegradation of free (red dots) and loaded (blue diamonds) RB (5 μ M) inside the microgels when irradiated with a 100 W visible lamp at 10 cm.

5.3.4.5 1,8-Naphthalimide microgels as molecular carriers: *In vitro* imaging

Intracellular transport of different dye-loaded microgels was assayed in a tumour cell line from human lung (carcinoma A549). Behaviour of six different biologically interesting dyes inside microgels (RB, Acridine Orange, Rhodamine 123 (R123), Oxazine 170, Nile Red and hypericin, see structures on Figure 40) were tentatively studied as a proof of concept by confocal microscopy in cellular media.

Oxazine 170

Oxazine 170 (5,9-bis(diethylamino)-10-methylbenzo-[R]phenoxazonium perchlorate) is a small cationic molecule with four fused aromatic rings, very similar to a nucleic base triplet, which code for an amino acid or some other signal in protein synthesis. Nucleic acid triple helices (DNA and RNA strands) play key roles in many biological processes and have attracted considerable attention, due to their potential as tools in molecular biology as well as possible therapeutic agents (antigene, antiviral and diagnostic agents).^{109,110} Triplexes are usually very thermodynamically disfavoured and ligands that stabilize hybrid triplexes are highly

appreciated. Oxazine 170 is able to induce the formation of the poly(rA), 2poly(dT) triplex, as a consequence of its highly selective binding to triplex structures.¹¹¹

Loading of Oxazine 170 in microgels was successfully achieved (see section 5.3.4.1), with a loading of 17.6 $\mu\text{mol/mol}$. Nicely, the incorporation of Oxazine 170 into the microgels can be followed by fluorescence spectroscopy, undergoing a notable peak shift from 651 nm (free form) to 642 nm (buried into the microgels) (Figure 52).

Figure 52. Normalized fluorescence emission spectra of free (red dotted line) and microgel-loaded (black continuous line) Oxazine 170; $\lambda_{\text{ex}} = 620 \text{ nm}$.

The release ability of microgels was studied in cellular media and confocal laser microscopy showed very interesting results. In Figure 53 and Figure 54, it can be seen how Oxazine 170 is effectively released into the intercellular space and how it has entered the cell. Sheaf-like microgels can be beautifully seen on the images (blue fluorescence), as well as oxazine 170 (red fluorescence). Spectra of loaded microgels and intercellular space were collected and showed different fluorescence behaviour. In the microgel, two emission maxima can be observed, one at the blue region and other at the violet/red region of spectra, suggesting that the microgel has not released all the cargo yet. When fluorescence spectra of Oxazine 170 is analyzed in two different regions (inside microgels and in the intercellular zone), different emission maximum is detected, meaning that the Oxazine 170 is in two different environments, one loaded in the microgels (slight hydrophobic environment $\lambda_{\text{em max}} = 640 \text{ nm}$) and other at its free form (hydrophilic environment $\lambda_{\text{em max}} = 650 \text{ nm}$) when present in intercellular region, as confirmed by comparison of fluorescence spectra shown in Figure 52.

Moreover, control experiments by the addition of the same amount of free Oxazine 170 (0.63 μM) did not show detectable fluorescence in the same irradiation/detection conditions.

Figure 53: Confocal laser microscopy images of microgel-loaded Oxazine 170 in cellular media (A549 cell line). **Left:** bright field + blue channel + red channel. **Centre and right:** Blue channel + red channels. White circles indicate the areas where fluorescence spectra is recorded.

Figure 54. Normalized fluorescence emission microspectroscopy of free (red line, white continuous line circle in figure 53) and microgel-loaded (black line, white dashed circle in figure 53) Oxazine 170 in the areas marked in figure 53; [1] = 20 μM , [Ox 170] = 0.63 μM . λ_{ex} = 405 nm.

Nile Red

Nile Red is used in biological systems as a lipophilic specific stain for intracellular lipid droplets and membranes.¹⁰¹ Loading of Nile Red in microgels was successfully achieved (see section 5.3.4.1), with a quantitative loading. The incorporation of Nile Red inside the microgels can be followed by fluorescence spectroscopy, undergoing a notable peak shift from 644 nm (free form) and to 605 nm (buried into the microgels) (Figure 43). The release ability of Nile Red-

loaded microgels in tumour A549 cell line (from human lung carcinoma) was studied by confocal laser microscopy. At Figure 55, it can be seen that Nile Red is released from the microgels, staining effectively lipidic droplets inside cells, since this dye is extensively used as bioimaging agent to stain this concrete intracellular structures.¹¹² Sheaf-like microgels can be observed in blue, as well as Nile Red staining the lipid droplets (red channel) (Figure 55).

Figure 55. Confocal laser microscopy images of microgel-loaded Nile Red in cellular media (A549 cell line), blue channel + red channel. $\lambda_{\text{ex}} = 405 \text{ nm}$. White circles point out the areas where fluorescence spectra is recorded.

Spectra of the sheaf-like structures (microgels) and the spherical subcellular structures in the intracellular space were collected (Figure 56) and showed different fluorescence behaviour. Fluorescence emission a maximum of *ca.* 620 nm was detected inside microgels pointing out that slight hydrophilic environment can be found in the microgel. However, the emission maximum inside intracellular lipid droplets is *ca.* 595 nm, this emission maximum points to a very hydrophobic environment (similar as CH_3CN).

It has to be noted that in the microgels, two emission maxima can be observed, one at the blue region (fluorescence of **1**, $\lambda_{\text{em}} = 420 \text{ nm}$) and other in the red region of spectra (fluorescence of NR, $\lambda_{\text{em}} = 620 \text{ nm}$), suggesting that the microgel has not released all the Nile Red cargo. However, inside cellular lipid droplets, more intense fluorescence emission and marked hypsochromic shift emission maxima ($\lambda_{\text{em}} = 590 \text{ nm}$) was detected. This evidence points out that the Nile Red is effectively delivered from microgels into more hydrophobic cavities present inside cells. Moreover, control experiments by the addition of the same

amount of free Nile Red ($7.2 \mu\text{M}$) did not show detectable fluorescence in the same irradiation/detection conditions.

Figure 56. Fluorescence emission microspectroscopy of lipid droplets (red line, white dashed circle in figure 55) and microgel-loaded (black line, white continuous circle in figure 55); $[1] = 20 \mu\text{M}$, $[\text{NR}] = 7.2 \mu\text{M}$, $\lambda_{\text{ex}} = 405 \text{ nm}$.

Acridine Orange

Acridine Orange (AO) is a cationic dye, very well-known as DNA and RNA intercalating agent.¹⁰² It is widely used as bioimaging dye and it is known to stain effectively lysosomes, endosomes and nucleus.¹⁰³ AO has been investigated to act as a antitumoral drug and assayed in photodynamic therapy.^{113–117} Loading of acridine orange into microgels does not alter notably their spectroscopic features, only peak broadening and slight shifts on absorption and emission peaks are detected. Confocal laser microscopy revealed highly loaded microgels inside A549 tumoral cells (Figure 57).

Figure 57. Confocal laser microscopy images of microgel-loaded Acridine Orange in cellular media (A549 cell line). Left: blue channel; Center: Overlapped green+blue channel; Right: Green channel; $\lambda_{\text{ex}} = 405 \text{ nm}$.

Slight release of AO from the microgels was detected. However, the control experiment, in which same amount of AO in free form ($0.45\ \mu\text{M}$) was added, showed highest internalization of free AO. From the microscopy experiments, we can conclude that the affinity of the AO against the microgels is higher than for the cellular environment, resulting in a poor releasing ability. The fact that the AO is a cationic dye and the microgel surface has a negative Z-potential may be one of the reasons for this behaviour. Microspectroscopy of the AO present in the microgels and the AO inside the cells revealed different emission behaviour, probably due to the DNA binding (λ_{em} in microgels $\approx 515\ \text{nm}$; λ_{em} in cells $\approx 535\ \text{nm}$) (Figure 58).¹⁰²

Figure 58. Fluorescence emission microspectroscopy of free (red line) and microgel-loaded (black line); $[1] = 20\ \mu\text{M}$, $[\text{AO}] = 0.45\ \mu\text{M}$, $\lambda_{\text{ex}} = 405\ \text{nm}$.

Hypericin

Hypericin is an anthraquinone derivative that has arisen lots of biological applications, such as antiviral and antibiotic agent, since its isolation from *Hypericum perforatum* and has been classically used in depression treatment. It has been recently demonstrated that hypericin shows preferential accumulation on cancer tissues and, because of its large chromophore conjugated fused structure, is able to absorb in a big range of wavelengths in the visible spectra and to effectively produce reactive oxygen species (ROS) when dissolved in hydrophobic solvents or surfaces. For this reason, hypericin has been demonstrated to be a promising photosensitizer for photodynamic therapy.^{105,118–120}

Loading of hypericin into microgels has been successfully achieved, with a notable variation of absorption spectra as the hypericin was loaded into the microgels (Figure 59 top). Moreover, release experiments with temperature were also carried out in hypericin-loaded microgels and revealed notable shifts and changes in peak ratio (550 nm-600 nm), attributed to the release of hypericin to the medium (Figure 59 bottom).

Figure 59. Top: UV-Vis absorption spectra of free (red dotted line) and microgel-loaded (black continuous line) hypericin. $[1] = 100 \mu\text{M}$ $[\text{Hyp}] = 4.9 \mu\text{M}$ in H_2O . **Inset:** Free (left) and resuspended microgels loaded with hypericin (right). **Bottom:** hypericin release from 1 microgels ($[1] = 100 \mu\text{M}$) with temperature, from 10 to 85 °C.

Confocal microscopy of loaded microgels showed almost completely release of hypericin to the medium, unfortunately no cells could be found in this experiment. Control experiments, in which free hypericin at the same loaded concentration (1 μM) was added revealed that hypericin is preferably located on cellular membrane and is qualitatively similar to the amount released by the microgels (Figure 60).

Figure 60. Confocal laser microscopy images of microgel loaded with hypericin (1 μM) in cellular media (A549 cell line). Channel overlap of red, blue and bright field images, showing microgels and hypericin in the intercellular region. λ_{ex} = 405 nm. Red fluorescence is hypericin and blue fluorescence is the microgel. White circles point out the areas where fluorescence spectra is recorded.

Spectra of cell culture media zone and microgels showed no substantial differences on fluorescence (Figure 61). The analysis of these emission spectra leads us to think that hypericin is in an environment similar to the one inside microgels.

Figure 61. Fluorescence emission microspectroscopy of free (red line) and microgel-loaded (black line) hypericin; [1] = 20 μM , [Hyp] = 1 μM , λ_{ex} = 405 nm.

Derived from this experiment, further biological assays were carried out by attempting photodynamic therapy and will be discussed below (section 5.3.4.6).

Rhodamine 123

Rhodamine 123 (R123) is a cationic dye extensively used as mitochondrial marker for unravelling mechanistic issues within mitochondria, and also used as anticancer drug affecting selectively mitochondria of carcinogenic cells.^{121–125} R123 was loaded into microgels and fluorescence emission spectra revealed a hypsochromic shift of emission maxima from 534 nm in free form to 529 nm when loaded into microgels (Figure 62).

Figure 62. Fluorescence emission spectra of free (red dotted line) and microgel-loaded (black continuous line) R123. ([1] = 20 μ M [Rhod123] = 0.1 μ M) in H_2O .

Although R123 was not very effectively loaded into the microgels (2.3 μ mol/mol), it showed very interesting staining features of mitochondria.¹²⁶ The system was qualitatively compared by CLSM with a control, in which the same amount of free R123 (0.1 μ M) as those calculated (UV-Vis measurements) to be into the microgel sample. Dramatic differences between both samples can be seen. R123-microgel sample resulted in noticeable transport into the cells (fluorescence detection at 520–660 nm) while the control experiment showed no incorporation (Figure 63). The detailed role played by the microgel needs further evaluation in future work but it seems that the high local concentration of R123 in the gel particles, which could adhere to cell surface, could favour the incorporation of the dye into the cells. Alternatively, it should be considered the possibility that the nanogel particles, which coexist with the microgel ones, act as active carriers into the cell.

Figure 63. CLSM images of cell culture of human lung carcinoma cells (A549) in the presence of R123-loaded microgel of **1**. λ_{Ex} 405 nm. Circles indicate the areas where fluorescence spectra were recorded.

Fluorescence spectra at different locations were collected, showing the effective release of R123. Microspectroscopy showed that microgels still have some cargo left and a preferential accumulation of R123 was detected in intracellular organelles.

Figure 64. Confocal microspectroscopy of R123-loaded microgels in cellular media. **Blue triangles:** Control experiment with R123 (0.1 μM); **Green square:** empty space; **Red circles:** intracellular spectrum in presence of microgels, **Black diamonds:** spectrum of R123-loaded micro-nanogels. **[1]** $\approx 40 \mu\text{M}$, **[Rhod123]** $\approx 0.1 \mu\text{M}$.

Rose Bengal

Rose Bengal is an anionic dye extensively used in the biological and biomedical fields. It is used for preventing bacterial growth,¹²⁷ as effective photo repair agent¹²⁸ and has also showed *in vitro* anticancer activity against ovarian cancer,¹²⁹ being a formulation containing this dye in phase III clinical trials for treating metastatic melanoma.¹³⁰ It is an excellent singlet oxygen producer and has been widely used in synthetic chemistry for catalysing alkene photooxidations.^{131,132} More recently, RB has been used as photosensitizer for anticancer photodynamic therapy^{133–135} and antimicrobial photodynamic therapy.^{136,137}

Microgel loading and release abilities of RB have been previously described by means of UV-Vis spectroscopy on section 5.3.4.1. RB loading also can be detected by means of fluorescence emission spectroscopy undergoing large bathochromic shift from free (λ_{em} = 571 nm) to loaded form (λ_{em} = 590 nm) (Figure 65).

Figure 65. Fluorescence emission spectra of free (red dotted line) and microgel-loaded (black continuous line) RB. ([1] = 20 μ M [RB] = 2.2 μ M) in H₂O.

RB loaded microgels were suspended in culture medium and added to A549 cells at a final concentration of 2.2 μ M. Release of RB into intracellular region was not complete and control, performed by addition of 2 μ M of free RB, qualitatively showed more uptake (Figure 66).

Figure 66. CLSM images of cell culture of human lung carcinoma cells (A549) in the presence of RB-loaded microgels. White circles indicating the area where fluorescence spectra was recorded.

Fluorescence emission spectra inside cells and inside the microgels revealed the presence of free RB inside the cells (red line on Figure 67, $\lambda_{em} = 545$ nm) and the presence of RB still loaded on the microgel (black line on Figure 67, $\lambda_{em} = 580$ nm).

Figure 67. Confocal microspectroscopy of RB loaded microgels in cellular media (A549 cells). **Red line:** Fluorescence spectrum of intracellular area. **Blue line:** Fluorescence spectrum of microgel area. $[1] \approx 40 \mu\text{M}$, $[\text{RB}] \approx 2.2 \mu\text{M}$.

Overall, the previous experiments have demonstrated in a very preliminary way the ability of these microgels as carriers into tumour cells and revealed potential applications in the biomedical field.

5.3.4.6 1,8-Naphthalimide microgels as molecular carriers: *in vitro* photodynamic therapy assays

In vitro photodynamic assays were performed with microgels loaded with three different photosensitizers: hypericin, Rose Bengal and R123. The experiments were carried out with HeLa epithelial cervical cancer cells. Microgels of **1** (100 μM) were synthesized and the amount of dye encapsulated was calculated by UV-vis spectroscopy ([RB] = 11 μM , [Rhod 123] = 0.5 μM and [hypericin] = 5 μM). After centrifugation step, resuspension was carried out with culture medium, and this 100 μM stock was used to assay different concentrations of loaded microgels for *in vitro* photodynamic therapy.

In a 96-well plate, 10 different concentrations of the different microgels were added in presence of HeLa cells (30.000 each) and let at rest for 24 hours, in order to internalize the microgels. After this time, removal of non internalized microgels was carried out and a new culture medium was added. Finally, in a first approach, irradiation of the plates was carried out for 2 and 5 minutes (n=6) by two white LED module 2x6 W located at 5 cm of the plates.

Table 8. Concentration of assayed microgels, all containing same amount of **1** but different dye content.

Hypericin-loaded microgels		RB-loaded microgels		Rhod 123-loaded microgels	
[1] / μM	[Hyp] / μM	[1] / μM	[RB] / μM	[1] / μM	[Rhod 123] / μM
20	1,0	20	2,2	20	$1.0 \cdot 10^{-1}$
6.7	$3.5 \cdot 10^{-1}$	6.7	$7.3 \cdot 10^{-1}$	6.7	$3.3 \cdot 10^{-2}$
2.2	$1.2 \cdot 10^{-1}$	2.2	$2.4 \cdot 10^{-1}$	2.2	$1.1 \cdot 10^{-2}$
$7.4 \cdot 10^{-1}$	$3.9 \cdot 10^{-2}$	$7.4 \cdot 10^{-1}$	$8.1 \cdot 10^{-2}$	$7.4 \cdot 10^{-1}$	$3.7 \cdot 10^{-3}$
$2.5 \cdot 10^{-1}$	$1.3 \cdot 10^{-2}$	$2.5 \cdot 10^{-1}$	$2.7 \cdot 10^{-2}$	$2.5 \cdot 10^{-1}$	$1.2 \cdot 10^{-3}$
$8.0 \cdot 10^{-2}$	$4.3 \cdot 10^{-3}$	$8.0 \cdot 10^{-2}$	$9 \cdot 10^{-3}$	$8.0 \cdot 10^{-2}$	$4.1 \cdot 10^{-4}$
$3.0 \cdot 10^{-2}$	$1.4 \cdot 10^{-3}$	$3.0 \cdot 10^{-2}$	$3 \cdot 10^{-3}$	$3.0 \cdot 10^{-2}$	$1.3 \cdot 10^{-4}$
$1.0 \cdot 10^{-2}$	$4.7 \cdot 10^{-4}$	$1.0 \cdot 10^{-2}$	$1 \cdot 10^{-3}$	$1.0 \cdot 10^{-2}$	$4.6 \cdot 10^{-5}$
$3.0 \cdot 10^{-3}$	$1.58 \cdot 10^{-4}$	$3.0 \cdot 10^{-3}$	$3.4 \cdot 10^{-4}$	$3.0 \cdot 10^{-3}$	$1.5 \cdot 10^{-6}$
$1.0 \cdot 10^{-3}$	$5.2 \cdot 10^{-5}$	$1.0 \cdot 10^{-3}$	$1.1 \cdot 10^{-4}$	$1.0 \cdot 10^{-3}$	$5.1 \cdot 10^{-6}$

After the irradiation process, cells were left at 37 °C for 24 hours prior to analyze the cell mortality by MTT assay. This assay is based on the colorimetric detection of formazan (violet color). Formazan is the product of the mitochondrial metabolic reduction of the colorless MTT reactive (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). This reduction can exclusively be performed by living cells. On the one hand, representation of formazan absorption (high values represent alive cells and low values mean dead cells) against [1] showed that RB and R123 loaded microgels do not present mortality increase at the concentrations assayed upon irradiation, compared to the non-irradiated control experiments (in the highest case, 20 μM of microgel, 2.2 μM for RB and 0.1 μM for R123). On the other hand, hypericin loaded microgels showed increased mortality when compared with the control. IC_{50} was determined from the sigmoidal adjust of collected data, to be $0.8 \pm 0.2 \mu\text{M}$ for the microgel and $0.042 \pm 0.005 \mu\text{M}$ for to the loaded hypericin, when irradiating for 5 minutes (Figure 68).

Figure 68. Absorbance values represent the average of six experiments of formazan vs [1], when irradiating samples for 5 min at 5 cm distance of 2x6W LED white light modules.

Further experiments summarized in Table 9 and Figure 69 were carried out, maintaining the irradiation distance but changing irradiation times and comparing hypericin loaded microgels, hypericin loaded nanogels and free hypericin at the same concentration ([hypericin] = 2 μM).

Surprisingly, when increasing irradiation times were assayed (2, 5, 7, 10 and 15 minutes), no tendencies were observed and longer irradiation times did not show significant increases in cell mortality. This behaviour could be attributed to fast photobleaching of the hypericin dye. Moreover, no significant differences were observed when three administration modes (microgels, nanogels and free hypericin) were compared.

Figure 69. MTT assays in HeLa cells. Absorbance of formazan vs [1] for different irradiation times and with different *in vitro* administration formats when irradiating samples at 5 cm distance of 2x6W LED white light modules.

Table 9. IC_{50} of *in vitro* different administration formats and different irradiation times in HeLa cells.

Time	Free hypericin $IC_{50} / \mu M$	Nanogel + hypericin $IC_{50} / \mu M$	Microgel + hypericin $IC_{50} / \mu M$
0	-	-	-
2	0.6 ± 0.2	0.29 ± 0.13	0.13 ± 0.08
5	0.9 ± 0.2	1.2 ± 0.4	0.7 ± 0.2
7	2.7 ± 0.7	2.5 ± 1.0	1.7 ± 0.5
10	1.0 ± 0.2	0.55 ± 0.14	0.73 ± 0.34
15	0.72 ± 0.3	0.70 ± 0.1	1.4 ± 0.4

Additional experiments with healthy HUVEC cells were carried out and showed similar results as the previous described (Table 10): no differences between irradiation times and between administration approach are observed.

Table 10. IC_{50} of *in vitro* different administration formats and different irradiation times in HUVEC cell line.

Time	Free hypericin $IC_{50} / \mu M$	Nanogel + hypericin $IC_{50} / \mu M$	Microgel + hypericin $IC_{50} / \mu M$
0	-	-	-
5	0.4 ± 0.2	0.5 ± 0.2	0.54 ± 0.14
10	0.8 ± 0.2	1.0 ± 0.5	1.06 ± 0.34

As summary, in this section, microgels showed to be capable of loading actives and be used as drug or dye-delivery vectors for tumoral cells. *In vitro* assays did not show real advantages in terms of efficiency in cell mortality. However, *in vivo* experiments might show some hypothetical advantages related to delayed body clearance of nanosized objects and the EPR effect. The loading of the dye into nano/micro structures could give rise to long-lived species allowing sequential irradiations with less frequent administrations. For this reason, further experiments are required *in vivo* to truly assess the possible potential use of these systems in stimuli-responsive drug delivery for photodynamic therapy. Further synthetic efforts should be done in order to design a nano/micro carrier which is able to release efficiently the hypericin photosensitizer.

5.4 4-Amino-1,8-naphthalimide nano(micro)carriers

5.4.1 4-Amino-1,8-naphthalimide derived molecular nanogels

Analogously to 1,8-naphthalimide systems described in section 5.3, gelators **3** and **4** were used to form nano- and microgels (Figure 70).

Figure 70. 4-amino-1,8-naphthalimide derived gelators.

5.4.1.1 Nanogels by precipitation method

Nanogel size study with concentration

Nanogels of **1** could be prepared reliably in the range of concentrations 0.5 to 10 μ M by addition of a DMSO solution into water, as reported above. No nano-objects were detected by DLS until [**3**] achieved 0.5 μ M and microparticles were predominant above 10 μ M. Monitoring the aggregation of **3** by UV-Vis spectroscopy revealed a change in the absorption maxima from

428 nm to 438 nm, which was used to estimate a critical aggregation concentration of around 0.1-0.5 μM (Figure 71).

Figure 71. Normalized UV-Vis Abs spectra of **3** at different concentrations (from 0.5 to 100 μM).

Nanogels could be obtained in a reproducible way and the results are summarized in Figure 72 and Table 11. Nanoparticles of **3** were synthesized and analyzed by means of DLS. Nanoparticles presented hydrodynamic sizes around 100 nm in the range from 0.5 μM to 7.3 μM , and absorption maximum increased from 428 to 433 nm ($\Delta\lambda = 5$ nm) as concentration raised, ascribed to the aggregation process. Above 10 μM bigger particles (around 500 nm) were detected. At higher concentrations, in the range of 24 to 100 μM , voluminous aggregates were observed with the naked eye. Absorption maximum keeps its slight bathochromic shift from 435 until it reaches stabilization at 438 nm ($\Delta\lambda = 3$ nm) with increasing the concentration.

Table 11. Particle size (diameter) variation with the concentration of gelator [**3**] in 10 % DMSO without stabilizer.

[3] / M	Dn / nm	λ_{abs}
$4.88 \cdot 10^{-7}$	107 ± 9	428
$9.76 \cdot 10^{-7}$	89 ± 11	429
$2.44 \cdot 10^{-6}$	75 ± 1	432
$4.88 \cdot 10^{-6}$	109 ± 5	433
$7.32 \cdot 10^{-6}$	95 ± 5	433
$9.76 \cdot 10^{-6}$	491 ± 57	435
$2.44 \cdot 10^{-5}$	Aggregates	436
$4.88 \cdot 10^{-5}$	Aggregates	437
$7.32 \cdot 10^{-5}$	Aggregates	438
$9.76 \cdot 10^{-5}$	Aggregates	438

Figure 72. Top: Representative DLS size distribution histogram by number of nanoparticles sample ($[3] = 4.9 \mu\text{M}$). **Bottom:** Particle size (diameter) variation with the concentration of gelator **1** in 10% DMSO without stabilizer.

Nanogel stability with time

Nanogel colloidal stability was studied for four different concentrations and showed excellent temporal stability towards further aggregation/precipitation, being DLS analysis reproducible for several days. Even after one month, nanoparticles were still present, although with moderately higher average diameter (D_n).

Figure 73. Particle size (diameter) variation of **3** nanogels with time in 10 % DMSO without stabilizer.

Nanogel stability with temperature

Nanogel stability with temperature was studied. The intermolecular forces that maintain the nanogels assembled lead us to think that they will be relatively responsive to temperature. However, DLS measurements were carried out at 4 different temperatures for **[3]** = 1 μ M and again showed that temperature does not affect significantly particle size (Figure 74).

Figure 74. Particle size (diameter) variation of nanogels of **3** with temperature (25°C, 45°C, 65°C, 85°C) in 10% DMSO without stabilizer (**[3]** = 1 μ M).

Nanogel size variation with salt content

Similarly as previously mentioned, the addition of NaCl affects the aggregation process and the nanogel size. Z-potential of nanogels of **3** was calculated by electrophoretic mobility to be -9.8 ± 0.5 mV, which indicates moderate to low stability against aggregation, compared to the nanogels of **1**.^{88,89} When NaCl is added to the system, there is a range of concentrations in which nanogels are insensible to NaCl presence and the size is stable around 200 nm. However, when salt concentration is between 3 and 6 mM, nanogels begin to undergo size increase to achieve around 600-700 nm (Figure 75). It has to be recalled that Z-potential of nanogels formed by **1** is higher than that measured for compound **3** and, accordingly, critical salt concentration for aggregation is found to be 26 mM for **1** and 6.5 mM for **3**.

Figure 75. Particle size (diameter) variation of nanogels of **3** with NaCl concentration.

Nanogel stability with dilution

Dilution tests were performed in order to simulate body injection. Several dilutions were prepared using a at 5 μM preformed nanogels solution (Figure 76). DLS measurements were carried out after 10 minutes of dilution and after 24 hours. After 10 minutes, slight size decrease was observed. Measurements after 24 hours showed no significant size variation until a 0.01 μM concentration was achieved. This concentration represents a limit for the reliable measure of DLS data, due to the extreme dilution conditions. This concentration value is way below the thermodynamic stability of nanogels of **3**, since its minimum aggregation concentration is estimated to be 0.5 μM (from UV-vis measurements) and, therefore, results indicate the presence of kinetically trapped nanoobjects.

Figure 76. Size variation of nanogels of **3** with the dilution after 24 hours.

Electron microscopy of 1,8-naphthalimide derived nanogels

Transmission electron microscopy showed clusters of spherical nanoobjects of approximately 25 ± 5 nm. Same sizes were observed by scanning electron microscopy, but without cluster formation. The size decrease from DLS analysis to microscopy analysis could be ascribed to the fact that the samples were dried prior to perform the analysis in the TEM and SEM and water is removed from the nanogel matrix, indicating intrinsic porosity (Figure 77).

Figure 77. Nanogel particles formed by **3** (5 μ M). **Top row:** TEM **Bottom row:** SEM.

Further analysis were carried out by using cryo-electron microscopy techniques, in which the sample is fast frozen at $\sim 10^6$ °C/s rate to -188 °C to achieve vitreous ice and avoid crystalline ice formation. The most widely used cryogens are liquid ethane or propane, because liquid nitrogen has very low heat capacity. Ethane is also liquid at temperatures just slightly above of those of liquid nitrogen. Therefore, liquid ethane is cold enough (its melting point is -188 °C) to freeze water quickly and correctly, while not boiling off in the process.^{138,139} Nanogels of **3** were analyzed by Cryo-EM images showed spherical nanoobjects with a size distribution of 67

± 15 nm ($n = 20$). In several cases, objects seemed to present nanoparticles embedded into larger structures (Figure 78).

Figure 78. Cryo-EM images of nanogels of **3** (5 μ M). **Right:** selection of vesicular nanogels in which external membrane can be observed.

5.4.2 pH Responsive nanogels by inverse emulsion method

Nanoemulsion method was also used for the nanostructuration of self-assembling molecule **4** as previously mentioned on section 5.3.3. Basic inverse micelles in hexane were acidified to promote compartmentalized self-assembly. Similar variations were observed on UV-Vis and fluorescence spectra depending on protonation, aggregation and environment due to AOT presence (Figure 79).

Figure 79. UV-Vis spectra (left) and fluorescence (right) spectra of nanogel synthesis of **4** by inverse emulsion method. (Black dashed line step 1, red dotted line step 2, and green continuous line step 3); $[2] = 0.25$ mM.

DLS measurements showed the same behaviour as previously reported for compound **2**. On the first stage, a transparent basic nanoemulsion was attained and monodisperse micelles of AOT + **4** showed 6.5 nm diameter. Then, protonation and aggregation of **4** took place and a little contraction of micelles was detected. Gradual removal of AOT by extractions with hexane in the isolation and purification process resulted in a size increase to 69 nm, finally reaching a diameter up to of 250 nm (Figure 80).

Figure 80. Nanogel synthesis of **4** by inverse emulsion method followed by DLS. Acidic emulsion in red, basic emulsion in green, AOT removal in blue and final dispersion in black.

5.4.3 4-Amino-1,8-naphthalimide derived microgels

Microgels of **3** have been synthesized and purified by the DMSO/water precipitation method, at concentrations between 10 to 100 μM , and characterized by transmission electron and confocal laser microscopy. Precipitation procedure was the same as previously described, with 0.1% w/w gelatine acting as stabilizer. TEM analysis showed polydisperse spherical fibrillar microgel structures of $3 \pm 2 \mu\text{m}$ ($n = 15$). These objects were probably formed due to a confined fibre formation because the assemblies appear to be covered of gelatine, which would avoid further aggregation among microgels (Figure 81). Confocal laser spectroscopy provides a more realistic appearance of the microgels, as the methodology examines the wet samples and no drying effects have to be taken into account. CLSM showed spherical particles with fibrillar structures (Figure 82). The microgels of **3** present a thinner fibrillar structure and a seemingly higher porosity than those of **1**. These properties enable to envisage improved active loading performance.

Figure 81. Transmission electron microscopy of xero-microgels of **3** (100 μM) stabilized with gelatine 0.1%.

Figure 82. Confocal laser microscopy of microgels of **3**; [**3**] = 100 μM; [gelatine] = 0.1 % w/w.

5.4.3.1 4-Amino-1,8-naphthalimide microgels as molecular carriers: Loading abilities

Loading of microgels of **3** was investigated by the coprecipitation method. Loading of the same 26 different molecules as in section 5.3.4.1 was assayed and several of them showed good loading abilities (expressed as mmol of loaded dye/mol gelator) inside microgels, followed by UV-Vis and fluorescence spectroscopy (Table 12). In most cases, absorption and fluorescence maximum peak shifts associated to dye entrapment were observed (Figure 83). Analogously to microgels of **1**, microgels of **3** also were good loading agents for hypericin and Protoporphyrin IX, presenting a similar loading ability as **1**. However, microgels of **3** presented lower performance on loading RB (22 vs 60 mmol/mol for **3** and **1**, respectively) and improved the loading of Oxazyme 170 (82.5 vs 18 mmol/mol for **3** and **1**, respectively).

Table 12. Photophysical behaviour of free and loaded dyes on microgels of **3**.

DYE	Abs free 20 μ M / nm	Abs loaded / nm	Loading mmol/mol
Doxorubicin	497	Overlapped	Overlapped
Rose bengal	552	569	22
Fluorescein	487	Overlapped	Overlapped
Hypericin	560	553	52
Oxazyme 170	621.5	568	82.5
Protoporphyrin IX	538.5	541	135
Rhodamine 6G	528	528	13.9
Rhodamine B	553	554	2.5
Toluidine blue	633	639	23.4

Figure 83. Comparison of loaded dyes in microgels of **3** and free dyes by means of UV-Vis spectroscopy. ($[3] = 100 \mu\text{M}$).

5.4.3.2 4-Amino-1,8-naphthalimide microgels as molecular carriers: *In vitro* imaging

Behaviour of molecular microgels was studied in cellular media (A549 lung carcinoma tumour cell line) by means of confocal laser microscopy. Microgels of **3**, which showed sizes between 6-7 μm (Figure 84 right), were added to A5499 tumour cells and incubated for 12 hours, after this time cells were observed under the microscope. Cell internalization of fluorescent gelator was detected upon microgel addition and incubation. It can be observed how the spherical shapes of microgels has been lost in several cases and the yellow fluorescent gelator has stained the cells. However, some intact microgels maintaining the spherical shape could be observed in the images (Figure 84 left, white arrows). A possible explanation for this behaviour could lie on the ability of microgel fibers to intercalate into the cell membrane because of hydrophobic affinity. Further assays must be carried out on health cell lines and with loaded microgels to see if the disassembly of the microgels results on cargo release.

Figure 84. Left: CLSM of microgels of **3** in A549 cell line, white arrows indicating microgel presence; Right: free microgels of **3**; [**3**] = 20 μM .

5.5 Acridine derived nanogels

The use of nanosized materials containing embedded dyes for biomedical application, such as photosensitizers for photodynamic therapy against cancer, was studied in previous sections. In the present section, a different approach was investigated: the photosensitizer is the gelator itself, constituting a system in which the active acts as a self-nanocarrier.

Acridine based compounds are an already well-known tool in Medicine. They have been used in solution as DNA intercalators for bioimaging and therapeutic purposes^{140,141}, for example acridines conjugated with gold or magnetic nanoparticles have been developed for bioimaging, DNA labelling and antimicrobial purposes.^{142,143} Other approach is their use as PDT agents,^{144–148} although much less research has been carried out in this aspect.^{115,117,149–153} For example, recent studies by the group of Premkumar showed the effect of UV-C radiation of acridine units on human breast cancer.¹¹³ Overall, acridine is a very interesting chromophore for PDT applications due to the generation of singlet oxygen with outstanding quantum yields.¹⁵⁴

Here, three acridine-derived compounds (**5**, **6** and **7**), with different polarity and different self-assembly abilities, and a soluble analogue (**8**) are described (Figure 85).

Figure 85. Synthesized acridine-derived compounds.

5.5.1 Nanogels preparation

Compounds **5-7** are low molecular weight molecules that undergo self-assembly and gel formation in different solvents. Compound **8** is a soluble analogue, synthesized for comparison purposes. As previously discussed on Chapter 3, acridine moieties have basic nature and its pK_a is 3.5, as calculated by fluorescence spectroscopic titration (Figure 86). Protonation of acridine moieties at low pH induce a disassembly process and a pronounced emission maximum shift ($\Delta\lambda = 65$ nm), making nanoparticles responsive to pH stimulus. Nanoparticles of the bisacridine compounds were satisfactorily synthesized by the precipitation method, using gelatine as stabilizer.^{155,156}

Figure 86. Left: Fluorescence titration curve for pK_a determination of acridine derivative **7**. Fluorescence emission at 463 nm is monitored. Right: Fluorescence emission spectra of compound **7** at pH = 6 (black line) and at pH = 2 (red line).

A first screening of the synthesized nanoparticles of compounds **5-8** (100 μ M) in water/DMSO mixtures (99/1) was performed by DLS. Compound **7** showed an optimal size range and low polydispersity, with $D_n = 122 \pm 11$ nm. For this reason, compound **7** was selected for further

morphological studies by SEM and TEM (Figure 87 and Table 13). Compound **5** and **6** showed also acceptable sizes for further possible biological applications, with $D_n = 41 \pm 4$ nm and 209 ± 74 nm, respectively. TEM and SEM micrographs of **7** confirmed the results obtained by DLS and showed spherical nanoparticles with size ranges of 135 ± 40 nm and 93 ± 24 by TEM and SEM, respectively. On the other hand, compound **8** did not show nanoobjects by DLS, indicating that complete solubilisation is achieved in the solvent mixture studied.

Figure 87. Physical characterization of Acridine derived nanoparticles. **a)** Representative size distribution by number obtained by DLS analysis of nanoparticles of **7**; **b)** Size distribution of nanoparticles of **7** obtained by SEM analysis; **c)** Size distribution of nanoparticles of **7** obtained by TEM analysis; **d)** SEM micrographs of **7**; **e)** TEM micrographs of **7**.

Table 13. Physical characterization of synthesized acridine based nanoparticles at 100 μ M in H₂O/DMSO (99/1).

Compound	DLS (D_n)	TEM diameter (nm)	SEM
5	41 ± 4	-	23 ± 7
6	209 ± 75	-	-
7	122 ± 11	135 ± 40	93 ± 24
8	soluble	soluble	soluble

5.5.2 Photophysical features

In order to study the aggregation effect in the fluorescence features of the studied compounds, several experiments were carried out using water, acetonitrile and water/acetonitrile mixtures. In acetonitrile, compounds **7** and **8** show the typical absorption of the acridine chromophore at 360 nm, displaying optical features appropriate for UV-A excitation, and weak fluorescence at 419 nm (Figure 88). In a polar solvent such as water, absorption maximum undergoes opposed shifts (365 nm for **7** and 357 nm for **8**) and a baseline increase is observed due to light dispersion for **5**, **6** and **7** (spectra not shown). Fluorescence emission bathochromic shifts were also observed $\Delta\lambda = 20.5$ nm ($\lambda_{em} = 435$ nm) for **7** and $\Delta\lambda = 17$ nm ($\lambda_{em} = 439$ nm) for compound **8**.

Figure 88. Normalized absorption and emission spectra of synthesized compounds in CH₃CN (Top) and H₂O (Bottom). **7** continuous line, **8** dashed line.

It was found that, at low concentrations without aggregation (10 μM) in aerated CH_3CN compounds **5-8**, show a marked fluorescence quenching due to the intersystem crossing (ISC) and to triplet population pathway leading to $^1\text{O}_2$ production. Water addition provokes aggregation (confirmed by absorption baseline increase due to light dispersion)¹⁵⁷ of compounds **5-7** and result in a fluorescence increase for all the compounds. For example, quantum yield attain a 270-fold increase for compound **8** and a 21-fold increase for **7** (Table 14), while compounds **5** and **6** present increases of 8 and 22-fold, respectively.

Table 14. Photophysical properties of synthesized compounds.

Compound	Solvent	Abs λ_{max} (nm)	Em λ_{max} (nm)	Φ_f / %	ϵ ($\text{M}^{-1}\text{cm}^{-1}$)	State
5	CH_3CN	359	439.5	0.4	17939	Solution
	H_2O	361	437.5	3.3	-*	Nanoparticles
6	CH_3CN	359	419.5	0.3	17300	Solution
	H_2O	361	434.5	6.7	-*	Nanoparticles
7	CH_3CN	360	418.5	0.3	16483	Solution
	90% CH_3CN	360	424	1.5		Solution
	70% CH_3CN	360	425	3.8		Solution
	50% CH_3CN	360	426.5	5.2		Solution
	30% CH_3CN	360	428	9.2	-*	Nanoparticles
	10% CH_3CN	368	435	6.3	-*	Nanoparticles
	H_2O	365	439	4.2	-*	Nanoparticles
8	CH_3CN	359	419	0.12	7786	Solution
	90% CH_3CN	359	423	1.3		Solution
	70% CH_3CN	359	424	3.6		Solution
	50% CH_3CN	359	428	6.6		Solution
	30% CH_3CN	359	430	13.5		Solution
	10% CH_3CN	359	432	24.6		Solution
	H_2O	357	435	32.3		Solution

9,10 Diphenylanthracene in cyclohexane as reference for quantum yield $\Phi_f = 0.98$ ¹⁵⁸ calculation; 10 μM of all compounds. *Turbid solutions/dispersions.

5.5.3 Singlet oxygen photogeneration

In this section, the effect of aggregation on the photoproduction efficiency of $^1\text{O}_2$ is assessed. Acridine compounds possess a strong absorption band at 360 nm, which has been employed in the past for PDT.^{115,117,149–153} Compounds **7** and **8** were studied for comparison between an aggregating compound and a soluble one. Acridine derivatives were dissolved (**8**) or suspended (**7**) on 1 cm optical path fluorescence cuvettes and irradiated with an UV-lamp (365 nm, 6W) (Figure 89).

Figure 89. Photooxidation kinetics of 1,5-DHN produced due to the irradiation of **7** and **8** by $^1\text{O}_2$ in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ mixtures, followed by UV-Vis spectroscopy. $[1,5\text{-DHN}] = 80 \mu\text{M}$; $[\mathbf{7}, \mathbf{8}] = 33 \mu\text{M}$.

Singlet oxygen quantum yield was determined by relative comparison with a known photosensitizer (phenalenone in CH₃CN with O₂(¹Δ_g) ≈ 1),¹⁵⁹ by means of 1,5-DHN photooxidation method (Figure 89).¹⁶⁰ Summarized results can be seen in Table 15. In pure acetonitrile, all compounds are at their solubilised state and present ¹O₂ quantum yields very close to 1. As water content rises, quantum yields start to decrease, until reaching Φ_Δ = 0.01 for compound **7** and 0.26 for compound **8**.

Table 15. Photophysical characterization of synthesized acridine based nanoparticles in different water/CH₃CN mixtures.

Compound	Solvent	State	Φ _Δ / %
7	CH ₃ CN	Solution	0.96 ± 0.04
8	CH ₃ CN	Solution	0.97 ± 0.01
Phenalenone*	CH ₃ CN	Solution	1.00 ± 0.02
7	CH ₃ CN/ H ₂ O (9:1)	Solution	0.65 ± 0.02
8	CH ₃ CN/ H ₂ O (9:1)	Solution	0.84 ± 0.04
7	CH ₃ CN/ H ₂ O (1:1)	Solution	0.77 ± 0.01
8	CH ₃ CN/ H ₂ O (1:1)	Solution	0.99 ± 0.03
7	CH ₃ CN/ H ₂ O (1:9)	Nanoparticles	0.032 ± 0.004
8	CH ₃ CN/ H ₂ O (1:9)	Solution	0.41 ± 0.01
7	H ₂ O	Nanoparticles	0.010 ± 0.007
8	H ₂ O	Solution	0.263 ± 0.004

Light source 365nm 6W; air atmosphere; corrected absorption; 80 μM DHN as ¹O₂ probe.

As can be observed in Figure 90, as water enters the system, singlet oxygen quantum yield of **7** drops dramatically when the water content is above 50 % water content. On the other hand, in pure water or culture medium, in which the lifetime of ¹O₂ it is substantially shorter than in CH₃CN (3.45 μs vs 80.9 μs),¹⁶¹ the soluble compound **8** still maintains Φ_Δ = 26 %.

Figure 90. $^1\text{O}_2$ quantum yield evolution of **7** and **8** when increasing H₂O content.

Scheme 2 summarizes the observed photophysical properties described above. The amount of water in the system (the solvent polarity) and aggregation regulate the relaxation pathways for the excited states. In pure acetonitrile, almost all relaxation pathways are through collisional quenching, giving rise to sensitization of triplet molecular oxygen to singlet oxygen. When water enters into the system, two scenarios are possible: i) In compounds that undergo aggregation (**5-7**), fluorescence relaxation pathways are slightly activated and singlet oxygen production is completely halted. ii) For the case in which a non-aggregated compound is studied (**8**), relaxation through intersystem crossing and singlet oxygen formation is pretty diminished, whereas, fluorescence emission relaxation pathway is activated. Summarizing, upon aggregation, a competing pathway is favoured at the expense of energy transfer to triplet oxygen to yield $^1\text{O}_2$. This situation does not occur in the non-aggregated photosensitizer **8**, which retains certain capacity to generate singlet oxygen in aqueous medium.

Scheme 2. Possible relaxation pathways upon irradiation of acridine derivatives in different environments. **Left:** Diagram showing the energy route upon excitation in CH₃CN. **Top right:** Diagram showing the energy route upon excitation in H₂O for non-aggregated compound. **Bottom right:** Diagram showing the energy route upon excitation in H₂O for aggregated compounds. Bold continuous arrows represent the preferential energy relaxation pathways and dashed arrows represent other relaxation through other mechanisms, such as internal conversion, electron transfer among others).

5.5.4 Biological evaluation

Hepatocellular carcinoma cells (Hep3B) were selected to perform the biological studies of acridine derivatives. Cytotoxicity studies revealed that **8** is not toxic at the maximum concentration studied (20 μM), while nanostructured systems (**5-7**) present IC₅₀ values in the low micromolar range (Table 16).

Table 16. IC₅₀ results of compounds **5-8** by MTT method.

Compound	IC ₅₀	State
5	10 μM	Nanoparticles
6	13 μM	Nanoparticles
7	6 μM	Nanoparticles
8	*	Solution

*Above the maximum concentration assayed

Compounds **7** and **8** also have very different properties from the photobiological viewpoint. When Hep3B cells incubated with the compounds were irradiated with light of 365 nm, only **8** was capable of inducing some PDT effect. As it can be seen in Figure 91, Hep3B cells were

incubated for 1 h in the presence of **8** (10 μM) and then irradiated for 3 min. Propidium iodide (PI) was used to test the photodamage, monitored by means of confocal fluorescence laser scanning microscopy (CLSM). Cells stained with PI after irradiations would indicate dead or damaged cells. In the control cells (no photosensitizer added), the number of damaged or dead cells was very low (only several per field), meaning that the irradiation *per se* was not deleterious. However, the number of PI-positive cells was dramatically increased in the cell population irradiated after the uptake of **8**, pointing out the existence of a particularly harmful effect on cell viability when the two stimuli (**8** + irradiation) are combined. For compound **7**, no induced photodamage was detected under the same experimental conditions. This observation confirms that aggregation must be avoided, at the designing stage, for photosensitizers based on acridine since this process hampers the generation of $^1\text{O}_2$. The negative effect of aggregation on the PDT activity is already well known for porphyrins and phthalocyanines, and efforts are devoted to the design of photosensitizers circumventing this process.^{162–164} According to the observations here reported, the same kind of considerations should be taken into account when dealing with acridine-based photosensitizers. Despite the lack of PDT activity of **7**, this compound retains cell internalization ability.

Figure 91. CLSM images of Hep3B cells (30.000 cells/well) stained with propidium iodide (1 μM). **Top row:** Irradiated set of wells in presence of **8** (10 μM); **bottom row:** Irradiated control set of wells in absence of **8**. Irradiation performed with UV lamp at 365nm 6W for 3 minutes.

MTT assays showed that exposure to **7** diminished the cell viability in a concentration-dependent manner, while exposure of **8** had no effect (Table 16). This fact could be related to the best binding ability of bichromophoric acridines as compared to the monochromophoric ones.^{165,166}

The cellular uptake of compound **7** was examined by means of CLSM. As it can be seen in Figure 92, the uptake and subcellular localization of compound **7** was studied by live cell confocal fluorescence microscopy using Hep3B cells. These experiments revealed that after 1 h of incubation with 10 μM of **7**, its fluorescence can be visualized inside cells and tend to localize in the perinuclear area, often in association with the nuclear membrane (Figure 92). Specific co-localization experiments using organelle-specific fluorochromes failed to show correlation of **7** with any of the subcellular compartments studied: ER, mitochondria and lysosomes (data not shown).

Figure 92. CLSM images of Hep3B cells (30.000 cells/well) of **7** (10 μM) incubated for 1 h, stained with Draq5 (1 μM). **(a)** Bright field; **(b)** Merged images of Draq5 fluorescence (nuclei stained in red) and **7** fluorescence (blue), sequentially acquired (**7** fluorescence; λ_{ex} = 405 nm, $\Delta\lambda_{\text{em}}$ =410–550nm; Draq5 fluorescence, λ_{ex} = 488 nm, $\Delta\lambda_{\text{em}}$ = 600–700 nm).

Summarizing, self-assembled nanoparticles formed by an acridine photosensitizer were synthesized by precipitation method. Dual behavior of the chromophore was detected depending on hydrophobic or hydrophilic environment. Apolar environment promotes intersystem crossing and singlet oxygen production. Polar solvent promotes emissive relaxation by fluorescence.

On the one hand, soluble analogue **8** showed notable $^1\text{O}_2$ production in polar solvent and also fluorescence emission behavior ($\Phi_{\Delta} = 26\%$, $\Phi_f = 32.3\%$). On the other hand, aggregation process reduced both mechanisms, showing that in aggregated state, fluorescence emission was reduced due to aggregation-caused quenching mechanism and also $^1\text{O}_2$ production was almost completely shut down ($\Phi_{\Delta} = 1\%$, $\Phi_f = 6.3\%$). Hence, it can be hypothesized that aggregation deactivates the excited states of the hydrophobic compound, discarding its use as a PDT agent. Nevertheless, despite of this lack of photoactivity, suspensions of this molecule, characterized as nano-sized aggregates, have a notable antiproliferative activity, which paves the way for future studies on this class of compounds.

5.6 Conclusions

Miniaturization of macroscopic molecular gels into molecular nano- and microgels and the ability of these molecular particles to load and release actives, as well as act as intracellular carriers was demonstrated as proof of concept. Two different strategies (precipitation method and nanoemulsion method) were explored to synthesize nanogels of five different compounds with different molecular nature: two derived from 4-amino-1,8-naphthalimides, two derived from 1,8-naphthalimide, and three from acridine. In both cases, nanogels were successfully synthesized, with sizes ranging from 60 to 300 nm. These sizes are optimal for systemic circulation and passive exploitation of EPR effect for cancer treatment. Moreover, the stimuli-responsiveness of the nanogels was investigated, showing relatively size insensitivity to temperature, dilution and salt content. Also the stability with time of the molecular nanogels was investigated showing a size increase of around 200 % in one month and aggregation with high concentration of salts (0.3 M).

Particles size was controlled by the concentrations of gelator and stabilizer. Molecular microgels were also synthesized in this way by precipitation method and its loading and release abilities were investigated.

Microgels of **1** (1,8-naphthalimide derivatives) showed high loading capacity of different dyes and photosensitizers, such as Nile Red or hypericin, among others. Microgels of **1** also showed release of these different actives inside cellular media. Hypericin-loaded microgels showed good results when their photodynamic activity was assayed. In a good proof of concept example, irradiation of HeLa epithelial cervical cancer cells in presence of hypericin-loaded microgels showed IC_{50} values in the nanomolar range respect to the hypericin ($0.0042 \pm 0.005 \mu M$) and in the micromolar range respect to the microgel concentration ($0.8 \pm 0.2 \mu M$), calculated by MTT assays. These preliminary results should be further confirmed and extensively investigated to assess its possible use as PDT agent. Additionally, further synthetic efforts could be done in order to design a new nano/micro carrier that would be able to release efficiently the hypericin photosensitizer.

Microgels of **3** (4-amino-1,8-naphthalimide derivatives) were also synthesized and showed good loading capacity of several dyes and photosensitizers. For example, Oxazine 170 and Rose Bengal presented good loading on microgels of **3**.

Finally, a different strategy of nanogel synthesis for potential photodynamic therapy is assayed. In this case, the photosensitizer (acridine) is the gelator itself, constituting a system in which the active acts as a self-nanocarrier. Good results in the preparation of the nanosized system of **7** were achieved getting nanoparticles of around 100 nm. However, their efficiency as PDT agents was discarded. Although aggregation process was detected to reduce PDT efficiency, these nanoparticles showed to have a notable antiproliferative activity, which paves the way for future studies on this class of compounds.

In conclusion, molecular nanogels may represent an interesting alternative to polymeric analogues in Nanomedicine, taking into account their intrinsic reversibility and stimuli-responsive nature. Further work is still in progress to assess the feasibility of different nanogel structures and their capability to act as carriers of actives. Fibrillar nature of microgels was

demonstrated. However, further research should be addressed in order to determine the possible porous nature of molecular nanogels, as no strong evidence was provided to tilt the balance in favour of nanocrystal-like structures or polymeric nanogel-like structure. It seems that depending on the preparation method, fibrillary structures could be achieved in nanoscopic molecular gels.

5.7 Experimental section

5.7.1 Synthesis

5.7.2 Naphthalimide derived nanoparticle experiments

5.7.2.1 Nanogel synthesis

5.7.2.2 Microgel synthesis

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5.7.2.4 Scanning electron microscopy

5.7.2.5 Transmission electron microscopy

5.7.2.6 UV-Vis and Fluorescence spectroscopy

5.7.2.7 Dynamic light scattering measurements

5.7.2.8 Singlet oxygen photogeneration of entrapped dyes

5.7.2.9 Confocal Laser Scanning microscopy (CLSM)

5.7.2.10 Cell culture and *in vitro* dye release studies in A549 cells

5.7.2.11 *In vitro* photodynamic therapy studies.

5.7.3 Acridine derived compounds experiments

5.7.3.1 Nanoparticle synthesis

5.7.3.2 Singlet oxygen photogeneration of acridine derivatives

5.7.3.3 Cell culture of Hep3B cells for tests with acridine derivatives

5.7.3.4 Cell viability of Hep3B cells for tests with acridine derivatives

5.7.3.5 Fluorescence microscopy coupled with static cytometry

5.7.3.6 Confocal microscopy

5.7.3.7 Quantum yield (Φ_F) calculation

5.7.1 Synthesis

Scheme 2. Synthetic route for compound **5.4**. . *ii)* DMAP, THF, reflux 60 °C, 12 h. *iii_b)* Et₃N, C₃H₅ClO₂, THF, 12h. *iv)* H₂, Pd/C, MeOH, 25 °C, 4 h. *v)* 4-nitro-1,8-naphthalic anhydride, CH₃OH, 75 °C, 8 h. *vi)* H₂, Pd/C, MeOH, 12 h. *vii)* LiOH, H₂O/THF 25 °C, 8 h.

Experimental details of synthetic procedures were described in the experimental section of Chapter 3 (Section 3.5.1).

Compound 5.9

^1H NMR (500 MHz, CDCl_3) δ (ppm): 7.41 – 7.29 (m, 5H), 5.32 (d, J = 8.6 Hz, 1H), 5.11 (s, 2H), 4.40 – 4.21 (m, 4H), 3.58 (d, J = 4.5 Hz, 2H), 3.36 (s, 3H), 2.26 – 2.12 (m, 1H), 0.97 (d, J = 6.9 Hz, 3H), 0.90 (d, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ (ppm): 172.1, 156.3, 136.4, 128.6(x2), 128.3, 128.2(x2), 70.4, 67.1, 64.1, 59.1, 59.0, 31.5, 19.0, 17.5.

Compound 5.10

^1H NMR (500 MHz, CDCl_3) δ (ppm): 4.35 – 4.20 (m, 2H), 3.60 (t, J = 4.7 Hz, 2H), 3.38 (s, 3H), 3.33 (d, J = 4.9 Hz, 1H), 2.12 – 1.94 (m, 1H), 0.94 (dd, J = 36.0, 6.9 Hz, 6H).

Compound 5.11

^1H NMR (300 MHz, CDCl_3) δ (ppm): 7.38 – 7.24 (m, 5H), 6.74 (d, J = 7.1 Hz, 1H), 5.69 (t, J = 5.1 Hz, 1H), 5.11 (s, 2H), 4.56 (dd, J = 8.8, 4.9 Hz, 1H), 4.26 (qt, J = 12.0, 4.6 Hz, 2H), 3.90 (d, J = 5.1 Hz, 2H), 3.55 (t, J = 4.7 Hz, 2H), 3.33 (d, J = 0.4 Hz, 3H), 2.22 – 2.09 (m, 1H), 0.91 (d, J = 6.8 Hz, 3H), 0.87 (d, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 171.7, 169.0, 136.2, 128.5, 128.1, 128.0, 70.2, 67.1, 64.0, 58.8, 57.1, 44.5, 31.2, 18.8, 17.6.

Compound 5.12

^1H NMR (500 MHz, CDCl_3) δ (ppm): 8.48 (s, $J = 43.6$ Hz, 1H), 4.33 (s, $J = 23.0$ Hz, 1H), 4.23 (d, $J = 19.7$ Hz, 2H), 3.97 (s, 2H), 3.56 (s, 2H), 3.30 (s, 3H), 2.12 (s, 1H), 0.89 (s, 6H). ^{13}C NMR (126 MHz, CDCl_3) δ (ppm): 172.0, 167.1, 70.1, 64.1, 58.6, 58.4, 41.1, 30.4, 18.7, 18.0.

Compound 5.13

^1H NMR (500 MHz, CDCl_3) δ (ppm): 8.77 (d, $J = 8.6$ Hz, 1H), 8.69 (d, $J = 7.2$ Hz, 1H), 8.65 (d, $J = 8.0$ Hz, 1H), 8.35 (d, $J = 8.0$ Hz, 1H), 7.98 – 7.88 (m, 1H), 6.61 (d, $J = 8.5$ Hz, 1H), 4.93 (dd, $J = 67.9, 15.4$ Hz, 2H), 4.65 (dd, $J = 8.7, 4.7$ Hz, 1H), 4.39 – 4.13 (m, 2H), 3.58 (t, $J = 4.7$ Hz, 1H), 3.35 (s, 3H), 2.21 (ddd, $J = 19.6, 13.2, 6.7$ Hz, 1H), 0.96 (dd, $J = 15.4, 6.9$ Hz, 6H). ^{13}C NMR (126 MHz, CDCl_3) δ (ppm): 171.9, 166.2, 163.0, 162.2, 149.7, 132.7, 130.1, 129.8, 129.5, 129.2, 126.5, 123.8, 123.6, 122.6, 70.2, 64.2, 58.9, 57.4, 42.9, 31.6, 18.9, 17.6. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{22}\text{H}_{24}\text{N}_3\text{O}_8^+$ 458.1563; found 458.1554 $[\text{M}+\text{H}]^+$ ($\Delta = 2.0$ ppm).

Compound 5.16

HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{22}\text{H}_{25}\text{N}_3\text{O}_6\text{Na}^+$ 450.1641; found 450.1639 $[\text{M}+\text{Na}]^+$ ($\Delta = 0.4$ ppm).

Compound 5.4

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.62 (d, J = 8.3 Hz, 1H), 8.17 (d, J = 8.4 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.44 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.72 (s AB system, J = 15.9 Hz, 2H), 4.18 (dd, J = 8.5, 6.0 Hz, 1H), 2.06 (td, J = 13.0, 6.5 Hz, 1H), 0.91 (dd, J = 6.4, 4.0 Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 173.4, 167.7, 164.1, 163.2, 153.3, 134.5, 131.5, 130.3, 129.9, 124.4, 122.1, 119.9, 108.7, 107.9, 57.7, 42.1, 30.7, 19.6, 18.5. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{19}\text{H}_{20}\text{N}_3\text{O}_5^+$ 370.1403; found 370.1407 [$\text{M}+\text{Na}$] $^+$ (Δ = 1.1 ppm).

Compounds 5.1, 5.2, and 5.3

Next compounds were described in previous chapters:

The compounds have been renumbered for coherence with the chapter numeration.

Scheme 3. Synthetic route for compound **5.6** and **5.7**. *iii_a)* THF, 4 h, 25 °C. *iv)* H₂, Pd/C, MeOH, 25 °C, 4 h. *viii)* CH₂Cl₂, 0 °C, Oxalyl chloride, DMF (cat.), 18h, then, THF, 16 h.

Experimental details of synthetic procedures were described in the experimental section of Chapter 3. (Section 3.5.1).

Compound 5.16

NMR spectra were consistent with those described in the literature.¹⁶⁷

¹H NMR (300 MHz, *d*₆-DMSO) δ (ppm): 0.82 (d, 12H, *J* = 6.6 Hz), 1.22 (br s, 4H), 1.35 (br s, 4H), 1.90 (m, 2H), 2.94-3.09 (m, 4H), 3.77 (t, 2H, *J* = 9.0 Hz), 5.01 (s, 4H), 7.15 (d, 2H, *J* = 8.4 Hz), 7.28-7.33 (m, 10H), 7.83 (br s, 2H); ¹³C NMR (75 MHz, *d*₆-DMSO) δ (ppm): 18.4, 19.3, 26.2, 29.1, 30.4, 38.5, 60.5, 65.4, 127.6, 127.7, 128.3, 137.1, 156.0, 170.8.

Compound 5.17

NMR spectra were consistent with those described in the literature.¹⁶⁷

¹H NMR (300 MHz, *d*₆-DMSO) δ (ppm): 0.81 (d, 12H, *J* = 6.3 Hz), 1.20 (br s, 8H), 1.25 (br s, 4H), 1.89 (m, 2H), 2.95-3.08 (m, 4H), 3.76 (t, 2H, *J* = 8.0 Hz), 5.00 (s, 4H), 7.13 (d, 2H, *J* = 8.4 Hz), 7.33 (br s, 10H), 7.81 (br s, 2H); ¹³C NMR (75 MHz, D₆-DMSO) δ (ppm): 18.4, 19.4, 26.4, 28.8, 29.1, 30.4, 38.5, 60.4, 65.4, 127.6, 127.7, 128.3, 137.1, 156.0, 170.8.

Compound 5.18

NMR spectra were consistent with those described in the literature.¹⁶⁷

¹H NMR (300 MHz, CDCl₃) δ (ppm): 0.68 (d, 6H, *J* = 7.1 Hz), 0.84 (d, 6H, *J* = 6.8 Hz), 1.20 (br s, 8H), 1.37 (m, 4H), 2.11 (m, 2H), 3.03-3.12 (m, 6H), 7.31 (br s, 2H); ¹³C NMR (75 MHz, CDCl₃) δ (ppm): 15.9, 19.5, 26.2, 29.3, 30.7, 38.4, 59.9, 173.9; ESI-MS *m/z* 315.3 (*M* + H⁺).

Compound 5.19

NMR spectra were consistent with those described in the literature.¹⁶⁷

^1H NMR (300 MHz, CDCl_3) δ (ppm): 0.72 (d, 6H, J = 6.8 Hz), 0.89 (d, 6H, J = 7.1 Hz), 1.21 (s, 8H), 1.38-1.43 (m, 8H), 2.17 (m, 2H), 3.09-3.18 (m, 6H), 7.30 (br s, 2H); ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 15.9, 19.6, 26.7, 29.0, 29.5, 30.7, 38.8, 60.0, 174.0; ESI-MS m/z = 172.2 ($\text{M} + 2\text{H}^+$).

Compound 5.6

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 9.14 (d, J = 8.2 Hz, 2H), 8.25 – 8.15 (m, 6H), 8.13 (d, J = 7.9 Hz, 2H), 7.95 (d, J = 7.9 Hz, 2H), 7.87 (t, J = 7.2 Hz, 4H), 7.72 – 7.58 (m, 4H), 4.51 (t, J = 7.8 Hz, 2H), 3.29 – 3.10 (m, 4H), 2.15 – 2.00 (m, 2H), 1.61 – 1.45 (m, 4H), 1.46 – 1.33 (m, 2H), 0.98 (dd, J = 11.0, 6.8 Hz, 11H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 171.0, 166.8, 148.7, 142.9, 131.0, 131.0, 129.8, 129.6, 127.1, 126.8, 126.6, 126.0, 122.7, 122.4, 59.8, 39.0, 30.2, 29.7, 26.7, 19.9, 19.0. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{44}\text{H}_{49}\text{N}_6\text{O}_4^+$ 725.3815; found 725.3820 [$\text{M} + \text{H}$] $^+$ (Δ = 0.7 ppm).

Compound 5.7

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 9.12 (d, J = 8.3 Hz, 1H), 8.29 – 8.08 (m, 4H), 7.97 (d, J = 7.2 Hz, 1H), 7.92 – 7.83 (m, 2H), 7.72 – 7.55 (m, 2H), 4.53 (t, J = 7.9 Hz, 1H), 3.28 – 3.04 (m, 3H), 2.17 – 2.02 (m, J = 13.7, 6.7 Hz, 1H), 1.59 – 1.44 (m, 2H), 1.44 – 1.28 (m, 4H), 0.99 (dd, J = 11.0, 6.8 Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 170.98(x2), 166.74(x2), 148.66(x4), 142.89(x2), 130.97(x4), 129.71(x4), 127.05(x4), 126.01(x4), 122.61(x4), 59.76(x2), 39.02(x2), 30.23(x2), 29.62(x2), 29.23(x2), 26.82(x2), 19.86(x2), 19.00(x2). HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{46}\text{H}_{52}\text{N}_6\text{O}_4^+$ 753.4050; found 753.4136 [$\text{M} + \text{H}$] $^+$ (Δ = 1.1 ppm).

Scheme 4. Synthetic route for compound **5.8**. . *iii_a)* THF, 4 h, 25 °C. *iv)* H_2 , Pd/C, MeOH, 25 °C, 4 h. *viii)* CH_2Cl_2 , 0 °C, Oxalyl chloride, DMF (cat.), 18h, then, THF, 16 h.

Compound 5.8

^1H NMR (400 MHz, CDCl_3) δ (ppm): 8.22 (d, $J = 8.8$ Hz, 2H), 8.01 (d, $J = 8.6$ Hz, 2H), 7.77 (ddd, $J = 8.8, 6.6, 1.3$ Hz, 2H), 7.63 – 7.49 (m, 2H), 6.87 (d, $J = 8.7$ Hz, 1H), 6.16 (t, 1H), 4.64 (dd, $J = 8.7, 7.3$ Hz, 1H), 3.45 – 3.17 (m, 2H), 2.38 – 2.15 (m, 1H), 1.72 – 1.47 (m, 2H), 1.12 (dd, $J = 12.4, 6.8$ Hz, 6H), 0.99 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ (ppm): 170.3, 167.4, 148.7(x2), 140.2, 130.4(x2), 129.9(x2), 127.0(x2), 125.0(x2), 122.3(x2), 77.2, 59.7, 41.5, 31.0, 22.9, 19.5, 18.7, 11.4. HRMS (ESI-TOF, positive mode): m/z calcd. for $\text{C}_{22}\text{H}_{26}\text{N}_3\text{O}_2^+$ 364,1947; found 364.2027 $[\text{M}+\text{H}]^+$ ($\Delta = 0.5$ ppm).

5.7.2 Naphthalimide derived nanoparticle experiments

5.7.2.1 Nanogel synthesis

On a representative example, 1 mL of **1** 0.1 mM (0.04 mg/ml) in DMSO is added dropwise, using a syringe pump at 0.6 mL/min rate over a glass vial containing 9 mL of mili-Q water filtered over polyvinylidene difluoride filter (PVDF, 0.22 μ m mesh). The addition is performed under vigorous stirring to achieve a 10 μ M dispersion of **1** in a 9/1 H₂O/DMSO solvent ratio.

5.7.2.2 Microgel synthesis

On a representative example, 1 mL of stock solution of **1** or **2** (1 mM, 0.4 mg/mL) in DMSO is added dropwise, using a syringe pump at 0.6 mL/min rate, over a glass vial with 9 mL of filtered mili-Q water containing 0.02 % w/w gelatine (acting as stabilizer). The addition is carried out under vigorous stirring to achieve a 100 μ M solution of **1** or **2** at 9/1 H₂O/DMSO solvent ratio. The dispersion becomes turbid and is left stirring for 5min. The purification step is performed by centrifugation at 6000 rpm for 60 min (x3), adding 10 mL of fresh water each centrifugation. A white pellet is formed of microgel particles, which can be dispersed in pure water, PBS or cell culture medium prior to use.

5.7.2.3 Loaded microgel synthesis

The loading process is performed by coprecipitation-entrapment method. The synthesis and purification step is similar as previously described, only changing the first step. 200 μ l of dye solution (RB or R123) 1 mM in DMSO is mixed with 1mL of the stock solution of **1**, 1 mM (0.4 mg/ml) and then precipitated and purified as mentioned above.

5.7.2.4 Scanning electron microscopy

Field-emission scanning electron micrographs were taken on a JEOL 7001F microscope equipped with a digital camera. The corresponding gel dispersions were placed on top of an aluminium specimen mount stub, fast frozen with liquid N₂ and lyophilized overnight. The sample was then sputtered with Pt (Baltec SCD 500) for 30 seconds.

5.7.2.5 Transmission electron microscopy

Transmission electron micrographs were taken on JEOL 2100 microscope with thermionic gun LaB6 200KV equipped with Gatan Orius high resolution CCD camera. TEM samples were prepared over carbon formvar copper grids by putting a drop of nano/microgel suspension and incubate for 5 minutes. The suspension excess was removed with filter paper.

For Cryo-TEM technique, JEM-2200FS/CR transmission electron microscope (JEOL, Japan), equipped with an UltraScan 4000 SP (4008×4008 pixels) cooled slow-scan CCD camera (GATAN, UK) was used. A drop of the nanogel suspension was placed on the TEM grid and a automated vitrification robot Vitrobot™ was used to freeze the sample in liquid ethane.

5.7.2.6 UV-Vis and Fluorescence spectroscopy

Solutions and suspensions were measured in a JASCO FP-8300 spectrofluorimeter and JASCO V-630 spectrophotometer for UV-Vis measurements using 3 mL quartz cuvettes with 10 mm path length.

5.7.2.7 Dynamic light scattering measurements

DLS measurements were performed with Malvern Zetasizer nano ZS instrument. 3mL of nanogel/microgel suspensions in pure water were measured in disposable 1 cm plastic length path cuvettes.

5.7.2.8 Singlet oxygen photogeneration of entrapped dyes

Photo-oxidation reactions were performed under air inside 3 mL fluorescence quartz cuvettes (1 cm light path) containing aerated aqueous solutions of the singlet oxygen trap ABDA (3 mL, 50 μ M) and RB loaded microgels ($\approx$ 5.6 μ M). Irradiations were carried out using a LED lamp cylindrical reactor (100 W, *ca.* 400-700 nm emission output) placed 12 cm away from the cuvette equipped with a heating-stir plate. The evolution of the photoreactions was monitored over time by means of UV-Vis spectroscopy (decrease of absorption at $\lambda_{\text{abs}} = 400$ nm). The initial points of the kinetic traces were fitted to a pseudo-first order model ($\ln A/A_0 = -k_{\text{obs}} \cdot t$, where A is the absorption of ABDA at a certain time t and A_0 is the initial absorption of

ABDA). Control experiments were performed by using ABDA in the absence of the photosensitizer.

5.7.2.9 Confocal Laser Scanning microscopy (CLSM)

Experiments were performed on inverted confocal microscope Leica TCS SP8. Images were obtained with HC PL APO CS2 63x/1.40 oil immersion objective. Excitation of samples was performed with sequential diode laser excitation 405 nm and 514nm and fluorescence was acquired with PMT detector. Microspectroscopy was performed with $\Delta\lambda = 5$ nm. Samples were prepared on sterilized Ibidi μ -Slide 8 Well Glass Bottom: # 1.5H (170 μ m +/- 5 μ m) Schott glass.

5.7.2.10 Cell culture and in vitro dye release studies in A549 cells

Human lung carcinoma cells (A549) were obtained from American Type Culture Collection and were grown at 37 °C in a humidified 5% CO₂, 95% air incubator. A549 cells were grown in Dulbecco's modified Eagle's medium, DMEM/High glucose modified (+4500 mg/L Glucose, +110 mg/l Sodium pyruvate w/o L-Glutamine) (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cell culture media was purchased from Hyclone (South Logan, Utah, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, UK). Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St Louis, MO, USA). Plastics for cell culture were supplied by Thermo Scientific™ BioLite. A549 cells at 70–80% confluence were collected and 2.5×10^4 cells were placed in a Ibidi μ -Slide 8 Well Glass Bottom well plate in 300 μ L of medium.

500 μ L of dye-loaded nano/micro gel suspensions were mixed with 800 μ L cell culture media (DMEM 1x no glucose, no glutamine, no red phenol (Gibco, Grand Island, NY, USA)) [1] $\approx$ 40 μ M. After 48 h, cells were washed and treated with 300 μ L of the previous solution and incubate for 12 hours at 37 °C.

5.7.2.11 In vitro photodynamic therapy studies.

In vitro photodynamic assays were performed with three different microgel-loaded photosensitizers that demonstrated to have good loading and release capabilities: hypericin,

RB and Rhodamine 123 loaded microgels. The experiment was carried out in HeLa epithelial cervical cancer cells. Microgels of **1** (100 μ M) were synthesized and the loading of the three different dyes was calculated by UV-vis spectroscopy. After centrifugation step, resuspension was carried out with cell culture media and this 100 μ M stock was used to assay different concentrations of loaded microgels ability to undergo *in vitro* photodynamic therapy.

In a 96-well plate, 10 different concentrations of the different microgels were added in presence of HeLa cells (30.000 cells/well) and let rest for 24 hours in order to internalize the possible microgels. After this time, removal of non internalized microgels was carried out and new cell culture media was added. Finally, in a first approach, irradiation of the plates was carried out for the desired time ($n = 6$) by 2 white LED module 2x6 W located at 5 cm of the plates. After the irradiation process, cells were left at 37 $^{\circ}$ C for 24 hours prior to analyze the cell mortality by MTT assay. Additional experiments with healthy HUVEC cells were carried out.

5.7.3 Acridine derived compounds experiments

5.7.3.1 Nanoparticle synthesis

For acridine derivatives: On a representative example, 100 μ L of **1** (10 mM) in DMSO is added dropwise, using a syringe pump at 0.6 mL/min rate, over a glass vial containing 9.9 ml of mili-Q water filtered over polyvinylidene difluoride filter (PVDF, 0.22 μ m mesh). The addition is performed under vigorous stirring to achieve a 100 μ M dispersion of **1** in a 99/1 H₂O/DMSO solvent ratio.

5.7.3.2 Singlet oxygen photogeneration of acridine derivatives

Photo-oxygenation reactions were performed inside open 3 ml fluorescence quartz cuvettes containing singlet oxygen trap 1,5-DHN (3 mL, 80 μ M) solutions in different ratios of aerated H₂O/CH₃CN. **1** and **2** concentrations were adjusted to have the same absorption at 365 nm (around 0.58 absorbance units) and irradiations were performed with continuous stirring under UV-lamp irradiation 6 W placed in contact with the cuvette side. The evolution of the photoreactions was monitored over time by means of UV-vis absorption spectrophotometry

(decrease of absorbance at 298 nm). The initial points of the kinetic traces were fitted to a pseudo-first order model ($\ln C/C_0 = -k_{\text{obs}} \cdot t$, where C is the concentration of 1,5-DHN at a certain time t and C_0 is the initial concentration of 1,5-DHN).

The calculated rate constants were compared with a well-known photosensitizer, perinaphthenone (also known as phenalene), which possess a singlet oxygen quantum yield in CH_3CN ($\Phi_{\Delta} = 1$ in acetonitrile). Again the absorption was adjusted to be the same as the acridine samples (0.58 units at 365 nm) and the photooxygenation reaction performed in the exact same manner.

5.7.3.3 Cell culture of Hep3B cells for tests with acridine derivatives

Experiments using cells were performed with the human hepatoblastoma cell line Hep3B (ATCC HB-8064) cultured in minimal essential medium (MEM), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 1 mM non-essential amino acids, 1mM sodium pyruvate. Although Hep3B are a reliable cellular model for this type of research, for comparison we also employed the primary cell line HUVEC (Human Umbilical Vein Endothelial Cells) to assess the general cellular toxicity of the particles; in order to rule out the possibility of the effect being related to the cancerous nature of Hep3B. HUVEC were isolated from fresh human umbilical cords by extraction with collagenase and cultured in EMG-2 medium supplemented with 2.5 $\mu\text{g}/\text{mL}$ fungizone-amphotericin B and BulletKit components (Clonetics, Lonza, Walkersville, MD, USA) according to the manufacturer's instructions. The protocol employed complied with European Community guidelines for the use of human experimental models and was approved by the Ethics Committee of the University of Valencia. All cell cultures were maintained in the presence of penicillin (50 units/mL) and streptomycin (50 $\mu\text{g}/\text{mL}$). Cells were used for experiments at passage number lower than 25 (for Hep3B) or lower than 3 (for HUVEC) and treatments were performed in complete cell culture medium. Unless stated otherwise, all the reagents employed in cell culture were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All cell cultures were maintained in an incubator (IGO 150, Jouan, Saint-Herblain Cedex, France) at 37 °C, in a humidified atmosphere of 5% CO_2 /95% air (AirLiquide Medicinal, Valencia, Spain).

5.7.3.4 Cell viability of Hep3B cells for tests with acridine derivatives

The colorimetric assay MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), frequently employed to assess viability of cultured cells, is based on cells ability to reduce a soluble yellow tetrazolium salt to blue formazan crystals. This reduction takes place only when mitochondrial reductase enzymes are active, and is thus also marker of cell viability related to mitochondrial function. To assess the general toxicity of the compounds, cells were seeded in 96-well plates (Falcon, BD, Durham, USA) at 15000 cells/well of two cellular models, Hep3B and HUVEC, using 5 technical replicates per treatment. In 100 μ L of complete culture medium/well. MTT reagent (Roche Diagnostics, Mannheim, Germany) was added (10 μ L/well) for the last 3h of the treatment. Cells were solubilized with DMSO (100 μ L/well, 5 min, 37 °C) and absorbance was measured using a “Multiscan” plate-reader spectrophotometer (Thermo Labsystems, ThermoScientific, Rockford, IL, USA). Results were obtained subtracting the absorbance at 690 nm (background absorbance) from the 570 nm absorbance.

5.7.3.5 Fluorescence microscopy coupled with static cytometry

Hep3B cells (30000 cells/well) were seeded in 48-well plates (Falcon, BD, Durham, USA) the day before the experiment. Then, cells were incubated in presence of **2** (1, 10 or 20 μ M) for 1h at 37 °C, medium was refreshed and irradiation performed for 3 min (a UV lamp was used, 230V-50/60 Hz, 365 nm). Cells were washed with sterile HBSS and fluorochromes added: 2 μ M Hoechst 33342 (Sigma-Aldrich, Steinheim, Germany) to stain nuclei and propidium iodide (PI, 0.3 μ L/well) to label dead or damaged cells (purchased in the form of Annexin-V-FLUOS Staining kit, Roche Diagnostics, Mannheim, Germany). After 20 min-incubation, cells were washed with HBSS and immediately visualized. All treatments were performed in 2 technical replicates and 20 images per well were recorded with a fluorescence microscope (IX81, Olympus, Hamburg, Germany) coupled with a static cytometry software “ScanR” version 2.03.2 (Olympus).

5.7.3.6 Confocal microscopy

Hep3B cells (30000/well) were seeded in 8-well chambered coverglass w/cvr (Thermo Fisher Scientific, Rochester, New York) the day before the experiment. Then, cells were incubated

with 10 μM of **7** or **8** for 1 h at 37 $^{\circ}\text{C}$, cell culture medium was refreshed and cells were immediately visualized using a confocal fluorescence microscope -Leica TCS-SP2 confocal laser scanning unit with argon and helium-neon laser beams and attached to a Leica DMIRBE inverted microscope. Excitation was performed at 360 nm and emission at 420 nm. For the experiments with irradiation, a UV lamp was used (230V-50/60 Hz, 365nm) and exposure was performed during 3min. PI was then added in HBSS (0.3 μL in 300 μL of HBSS) and cells were immediately visualized.

Confocal fluorescence microscopy was also employed to assess the subcellular localization of compound **7** after its cellular up-take. To this end, cells were incubated with 10 μM of **7** for 1h at 37 $^{\circ}\text{C}$ and fluorochromes to mark different organelle were added in the last 30 min of the treatment (1 μM of ERtracker Red, Mitotracker Red, LysoTracker Red (Invitrogen, Oregon, USA) or Draq5 (Thermo Fisher Scientific, Rockford, USA). Excitation and emission wavelengths were 543 nm and 568-700 nm respectively, for ERtracker Red, Mitotracker Red and LysoTracker Red, and 633 nm and 651-830 nm in the case of Draq5.

Images were captured at 63x or 40x magnification with HCxPL APO 40.0 x 1.32 oil UV objective or HCxPL APO 40.0x 1.25-0.75 OIL CS objective, respectively.

5.7.3.7 Quantum yield (Φ_F) Calculation

Fluorescence quantum yields were calculated using 9,10-Diphenylanthracene (DPA) in cyclohexane as standard and using the following equation.

$$\Phi_x = \Phi_{st} \cdot \frac{A_{st}}{A_x} \cdot \frac{F_x}{F_{st}} \cdot \frac{n_x^2}{n_{st}^2} \cdot \frac{D_x}{D_{st}}$$

Quantum yield	Standard Unknown	Φ_{st} Φ_x
Absorbance at Ex wavelength	Standard Unknown	A_{st} A_x
Area of emission spectrum	Standard Unknown	F_{st} F_x
Refractive index value of solvent	Standard Unknown	n_{st} n_x
Dilution ratio	Standard Unknown	D_{st} D_x

5.8 References

- (1) Oeppen, J.; Vaupel, J. W. *Science* (80-.). **2002**, 296 (5570), 1029.
- (2) Fleming, A. *Br. J. Exp. Pathol.* **1929**, 226.
- (3) WHO | Cancer <http://www.who.int/mediacentre/factsheets/fs297/en/> (accessed May 19, 2017).
- (4) Persidis, A. *Nat. Biotechnol.* **1999**, 17 (Supp), 94.
- (5) Kahn, J. *Natl. Geogr. Mag.* **2006**.
- (6) van der Waals, J. D.; Rowlinson, J. S. *On the continuity of the gaseous and liquid states*; North-Holland: Amsterdam ; Oxford, 1988.
- (7) Bear, R.; Rintoul, D. *Viruses* <https://cnx.org/contents/tK8Muj08@6/Viruses> (accessed Jul 11, 2017).
- (8) Binnig, G.; Rohrer H. *IBM J. Res. Dev.* **1986**, 30 (4), 355.
- (9) Binnig, G.; Gerber, C.; Stoll, E.; Albrecht, T. R.; Quate, C. F. *Europhys. Lett.* **1987**, 3 (12), 1281.
- (10) Lin, W. *Chem. Rev.* **2015**, 115 (19), 10407.
- (11) Juliano, R. *Nat. Rev. Drug Discov.* **2013**, 12 (3), 171.
- (12) Brayden, D. J. *Drug Discov. Today* **2003**, 8 (21), 976.
- (13) Hare, J. I.; Lammers, T.; Ashford, M. B.; Puri, S.; Storm, G.; Barry, S. T. *Adv. Drug Deliv. Rev.* **2017**, 108, 25.
- (14) Chen, G.; Roy, I.; Yang, C.; Prasad, P. N. *Chem. Rev.* **2016**, 116 (5), 2826.
- (15) Aguilar, Z. P. *Nanomaterials for medical applications*; Elsevier Ltd: Oxford, 2013.
- (16) Au, J. L.; Jang, S. H.; Zheng, J.; Chen, C. T.; Song, S.; Hu, L.; Wientjes, M. G. *J. Control. Release* **2001**, 74 (1–3), 31.
- (17) Wang, S.; Huang, P.; Chen, X. *ACS Nano* **2016**, 10 (3), 2991.
- (18) Mao, Z.; Zhou, X.; Gao, C. *Biomater. Sci.* **2013**, 1 (9), 896.
- (19) Sun, T.; Zhang, Y. S.; Pang, B.; Hyun, D. C.; Yang, M.; Xia, Y. *Angew. Chem. Int. Ed.* **2014**, 53 (46), 12320.
- (20) Matsumura, Y.; Maeda, H. *Cancer Res.* **1986**, 46 (12), 6387.

- (21) Bergers, G.; Benjamin, L. E. *Nat. Rev. Cancer* **2003**, 3 (6), 401.
- (22) Decuzzi, P.; Pasqualini, R.; Arap, W.; Ferrari, M. *Pharm. Res.* **2009**, 26 (1), 235.
- (23) Qiu, Y.; Liu, Y.; Wang, L.; Xu, L.; Bai, R.; Ji, Y.; Wu, X.; Zhao, Y.; Li, Y.; Chen, C. *Biomaterials* **2010**, 31 (30), 7606.
- (24) Bertrand, N.; Leroux, J.-C. *J. Control. Release* **2012**, 161 (2), 152.
- (25) Harush-Frenkel, O.; Debotton, N.; Benita, S.; Altschuler, Y. *Biochem. Biophys. Res. Commun.* **2007**, 353 (1), 26.
- (26) Yu, D.; Zhang, Y.; Zou, G.; Cui, X.; Mao, Z.; Gao, C. *Biointerphases* **2014**, 9 (3), 31015.
- (27) Chonn, A.; Cullis, P. R.; Devine, D. V. *J. Immunol.* **1991**, 146 (12), 4234.
- (28) Torchilin, V. P. Springer, Berlin, Heidelberg, 2010; pp 3–53.
- (29) Danhier, F.; Feron, O.; Préat, V. *J. Control. Release* **2010**, 148 (2), 135.
- (30) Fox, M. E.; Szoka, F. C.; Fréchet, J. M. J. *Acc. Chem. Res.* **2009**, 42 (8), 1141.
- (31) Sun, H.; Wong, E. H. H.; Yan, Y.; Cui, J.; Dai, Q.; Guo, J.; Qiao, G. G.; Caruso, F. *Chem. Sci.* **2015**, 6 (6), 3505.
- (32) Yi, X.; Shi, X.; Gao, H. *Phys. Rev. Lett.* **2011**, 107 (9), 98101.
- (33) Gunaseelan, S.; Gunaseelan, K.; Deshmukh, M.; Zhang, X.; Sinko, P. J. *Adv. Drug Deliv. Rev.* **2010**, 62 (4–5), 518.
- (34) Giri, S.; Trewyn, B. G.; Lin, V. S. *Nanomedicine* **2007**, 2 (1), 99.
- (35) Kresge, C. T.; Leonowicz, M. E.; Roth, W. J.; Vartuli, J. C.; Beck, J. S. *Nature* **1992**, 359 (6397), 710.
- (36) Friedman, R. *JNCI J. Natl. Cancer Inst.* **2011**, 103 (19), 1428.
- (37) Wu, P.; Yan, X.-P. *Chem. Soc. Rev.* **2013**, 42 (12), 5489.
- (38) Liu, Z.; Chen, K.; Davis, C.; Sherlock, S.; Cao, Q.; Chen, X.; Dai, H. *Cancer Res.* **2008**, 68 (16).
- (39) Jain, S.; Hirst, D. G.; O’Sullivan, J. M. *Br. J. Radiol.* **2012**, 85 (1010), 101.
- (40) Tang, L.; Cheng, J. *Nano Today* **2013**, 8 (3), 290.
- (41) Lalwani, G.; Henslee, A. M.; Farshid, B.; Lin, L.; Kasper, F. K.; Qin, Y.-X.; Mikos, A. G.; Sitharaman, B. *Biomacromolecules* **2013**, 14 (3), 900.

- (42) Torchilin, V. P. *Nat. Rev. Drug Discov.* **2005**, 4 (2), 145.
- (43) Alley, S. C.; Okeley, N. M.; Senter, P. D. *Curr. Opin. Chem. Biol.* **2010**, 14 (4), 529.
- (44) Lu, Z.-R.; Shiah, J.-G.; Sakuma, S.; Kopecková, P.; Kopecek, J. *J. Control. Release* **2002**, 78 (1–3), 165.
- (45) Henderson, I. C.; Bhatia, V. *Expert Rev. Anticancer Ther.* **2007**, 7 (7), 919.
- (46) Gradishar, W. J. *Expert Opin. Pharmacother.* **2006**, 7 (8), 1041.
- (47) Sun, T.; Zhang, Y. S.; Pang, B.; Hyun, D. C.; Yang, M.; Xia, Y. *Angew. Chem. Int. Ed.* **2014**, 53 (46), n/a.
- (48) Allen, T. M.; Cullis, P. R. *Adv. Drug Deliv. Rev.* **2013**, 65 (1), 36.
- (49) Sercombe, L.; Veerati, T.; Moheimani, F.; Wu, S. Y.; Sood, A. K.; Hua, S. *Front. Pharmacol.* **2015**, 6, 286.
- (50) Barenholz, Y. *J. Control. Release* **2012**, 160 (2), 117.
- (51) Petre, C. E.; Dittmer, D. P. *Int. J. Nanomedicine* **2007**, 2 (3), 277.
- (52) O'Brien, M. E. R.; Wigler, N.; Inbar, M.; Rosso, R.; Grischke, E.; Santoro, A.; Catane, R.; Kieback, D. G.; Tomczak, P.; Ackland, S. P.; Orlandi, F.; Mellars, L.; Alland, L.; Tendler, C. *Ann. Oncol.* **2004**, 15 (3), 440.
- (53) Silverman, J. A.; Deitcher, S. R. *Cancer Chemother. Pharmacol.* **2013**, 71 (3), 555.
- (54) Lao, J.; Madani, J.; Puértolas, T.; Álvarez, M.; Hernández, A.; Pazo-Cid, R.; Artal, Á.; Torres, A. A. *J. Drug Deliv.* **2013**, 2013, 1.
- (55) Zhang, X.; Rehm, S.; Safont-Sempere, M. M.; Wuerthner, F. *Nat. Chem.* **2009**, 1 (8), 623.
- (56) Patri, A. K.; Majoros, I. J.; Baker, J. R. *Curr. Opin. Chem. Biol.* **2002**, 6 (4), 466.
- (57) Gillies, E.; Frechet, J. *Drug Discov. Today* **2005**, 10 (1), 35.
- (58) Nanjwade, B. K.; Bechra, H. M.; Derkar, G. K.; Manvi, F. V.; Nanjwade, V. K. *Eur. J. Pharm. Sci.* **2009**, 38 (3), 185.
- (59) Zhao, P.; Astruc, D. *ChemMedChem* **2012**, 7 (6), 952.
- (60) Soleyman, R.; Adeli, M. *Polym. Chem.* **2015**, 6 (1), 10.
- (61) Kedar, U.; Phutane, P.; Shidhaye, S.; Kadam, V. *Nanomedicine Nanotechnology, Biol. Med.* **2010**, 6, 714.

- (62) Mahmud, A.; Xiong, X.-B.; Aliabadi, H. M.; Lavasanifar, A. *J. Drug Target.* **2007**, *15* (9), 553.
- (63) Oerlemans, C.; Bult, W.; Bos, M.; Storm, G.; Nijsen, J. F. W.; Hennink, W. E. *Pharm. Res.* **2010**, *27* (12), 2569.
- (64) Song, C. X.; Labhasetwar, V.; Murphy, H.; Qu, X.; Humphrey, W. R.; Shebuski, R. J.; Levy, R. J. *J. Control. Release* **1997**, *43* (2–3), 197.
- (65) Ishii, H.; Furuse, J.; Nagase, M.; Maru, Y.; Yoshino, M.; Hayashi, T. *Jpn. J. Clin. Oncol.* **2003**, *33* (11), 570.
- (66) Tahara, Y.; Akiyoshi, K. *Adv. Drug Deliv. Rev.* **2015**, *95*, 65.
- (67) Chacko, R. T.; Ventura, J.; Zhuang, J.; Thayumanavan, S. *Adv. Drug Deliv. Rev.* **2012**, *64* (9), 863.
- (68) Chan, M.; Almutairi, A. *Mater. Horiz.* **2016**, *3* (1), 21.
- (69) Hamidi, M.; Azadi, A.; Rafiei, P. *Adv. Drug Deliv. Rev.* **2008**, *60* (15), 1638.
- (70) Kabanov, A. V.; Vinogradov, S. V. *Angew. Chem. Int. Ed.* **2009**, *48* (30), 5418.
- (71) Ryu, J.-H.; Jiwanich, S.; Chacko, R.; Bickerton, S.; Thayumanavan, S. *J. Am. Chem. Soc.* **2010**, *132* (24), 8246.
- (72) Jiwanich, S.; Ryu, J.-H.; Bickerton, S.; Thayumanavan, S. *J. Am. Chem. Soc.* **2010**, *132*, 10683.
- (73) Akiyoshi, K.; Deguchi, S.; Moriguchi, N.; Yamaguchi, S.; Sunamoto, J. *Macromolecules* **1993**, *26* (12), 3062.
- (74) Takehiro Nishikawa; Kazunari Akiyoshi, A.; Sunamoto*, J. *J. Am. Chem. Soc.* **1996**, *118*(26), 6110.
- (75) Tsuji, K.; Hamada, T.; Uenaka, A.; Wada, H.; Sato, E.; Isobe, M.; Asagoe, K.; Yamasaki, O.; Shiku, H.; Ritter, G.; Murphy, R.; Hoffman, E. W.; Old, L. J.; Nakayama, E.; Iwatsuki, K. *Cancer Immunol. Immunother.* **2008**, *57* (10), 1429.
- (76) Kawabata, R.; Wada, H.; Isobe, M.; Saika, T.; Sato, S.; Uenaka, A.; Miyata, H.; Yasuda, T.; Doki, Y.; Noguchi, Y.; Kumon, H.; Tsuji, K.; Iwatsuki, K.; Shiku, H.; Ritter, G.; Murphy, R.; Hoffman, E.; Old, L. J.; Monden, M.; Nakayama, E. *Int. J. Cancer* **2007**, *120* (10), 2178.
- (77) Raghavan, S. R. *Langmuir* **2009**, *25* (15), 8382.
- (78) Ding, D.; Li, K.; Liu, B.; Tang, B. Z. *Acc. Chem. Res.* **2013**, *46* (11), 2441.
- (79) Hong, Y.; Meng, L.; Chen, S.; Leung, C. W. T.; Da, L. T.; Faisal, M.; Silva, D. A.; Liu, J.;

- Lam, J. W. Y.; Huang, X.; Tang, B. Z. *J. Am. Chem. Soc.* **2012**, *134* (3), 1680.
- (80) Wu, Y.; Huang, S.; Zeng, F.; Wang, J.; Yu, C.; Huang, J.; Xie, H.; Wu, S.; Kim, S. A.; Song, S. Y.; Kim, K. S.; Cho, S. W.; Kim, H.; Paik, Y. K. *Chem. Commun.* **2015**, *51* (64), 12791.
- (81) Mandal, A. K.; Sreejith, S.; He, T.; Maji, S. K.; Wang, X.-J.; Ong, S. L.; Joseph, J.; Sun, H.; Zhao, Y. *ACS Nano* **2015**, *9* (5), 4796.
- (82) Baba, K.; Pudavar, H. E.; Roy, I.; Ohulchanskyy, T. Y.; Chen, Y.; Pandey, R. K.; Prasad, P. N. *Mol. Pharm* **2006**, *4* (2), 289.
- (83) Baba, K.; Kasai, H.; Nishida, K.; Nakanishi, H. In *Nanocrystals*; Sciyo, 2010.
- (84) Zhang, J.; Li, S.; An, F.-F.; Liu, J.; Jin, S.; Zhang, J.-C.; Wang, P. C.; Zhang, X.; Lee, C.-S.; Liang, X.-J. *Nanoscale* **2015**, *7* (32), 13503.
- (85) Cao, Y.; Li, Y.; Hu, X.-Y.; Zou, X.; Xiong, S.; Lin, C.; Wang, L. *Chem. Mater.* **2015**, *27* (3), 1110.
- (86) Jana, A.; Devi, K. S. P.; Maiti, T. K.; Singh, N. D. P. *J. Am. Chem. Soc.* **2012**, *134* (18), 7656.
- (87) Malvern. *Zetasizer Nano Series User Manual*; Malvern, 2009; Vol. 2.
- (88) Salopek, B.; Krasić, D.; Filipović, S. *Rudarsko-geolosko-naftni zbornik*; Sveuciliste U Zagrebu, 1992; Vol. 4.
- (89) Riddick, T. *Control of colloid stability through zeta potential*; Livingston: Wynnewood Pa., 1968.
- (90) Hozumi, A.; Sugimura, H.; Yokogawa, Y.; Kameyama, T.; Takai, O. *Colloids Surfaces A Physicochem. Eng. Asp.* **2001**, *182*, 257.
- (91) Beattie, J. K.; Djerdjev, A. M.; Warr, G. G.; Eastoe, J.; Jungwirth, P.; Ivanov, I. B.; Borwankar, R. P. *Faraday Discuss.* **2009**, *141* (0), 31.
- (92) Nebot, V. J.; Ojeda-Flores, J. J.; Smets, J.; Fernández-Prieto, S.; Escuder, B.; Miravet, J. F. *Chem. - A Eur. J.* **2014**, *20* (44), 14465.
- (93) Kunz, W.; Henle, J.; Ninham, B. W. *Curr. Opin. Colloid Interface Sci.* **2004**, *9* (1), 19.
- (94) Xia, L.-W.; Xie, R.; Ju, X.-J.; Wang, W.; Chen, Q.; Chu, L.-Y. *Nat. Commun.* **2013**, *4* (2226), 1.
- (95) Blachechen, L. S.; Silva, J. O.; Barbosa, L. R. S.; Itri, R.; Petri, D. F. S. *Colloid Polym. Sci.* **2012**, *290* (15), 1537.
- (96) Bueno, V. B.; Bentini, R.; Catalani, L. H.; Petri, D. F. S. *Carbohydr. Polym.* **2013**, *92* (2), 1091.

- (97) Horn, D.; Rieger, J. *Angew. Chemie* **2001**, *40* (23), 4330.
- (98) Ryu, J.-H.; Chacko, R. T.; Jiwanich, S.; Bickerton, S.; Prakash Babu, R.; Thayumanavan, S. *J. Am. Chem. Soc* **2010**, *132*, 17227.
- (99) Torchilin, V. P. *J. Control. Release* **2001**, *73* (2–3), 137.
- (100) Sackett, D. L.; Wolff, J. *Anal. Biochem.* **1987**, *167* (2), 228.
- (101) Greenspan, P.; Mayer, E. P.; Fowler, S. D. *J. Cell Biol.* **1985**, *100* (3), 965.
- (102) Fotia, C.; Avnet, S.; Kusuzaki, K.; Roncuzzi, L.; Baldini, N. *Curr. Pharm. Des.* **2015**, *21* (28), 4088.
- (103) Moriyama, Y.; Takano, T.; Ohkuma, S. *J. Biochem.* **1982**, *92* (4), 1333.
- (104) Sayed, M.; Krishnamurthy, B.; Pal, H. *Phys. Chem. Chem. Phys.* **2016**, *18* (35), 24642.
- (105) Lima, A. M.; Pizzol, C. D.; Monteiro, F. B. F.; Creczynski-Pasa, T. B.; Andrade, G. P.; Ribeiro, A. O.; Perussi, J. R. *J. Photochem. Photobiol. B Biol.* **2013**, *125*, 146.
- (106) Paczkowski, J.; Neckers, D. C. *Macromolecules* **1985**, *18* (12), 2412.
- (107) Derosa, M. C.; Crutchley, R. J. *Coord. Chem. Rev.* **2002**, *233–234*, 351.
- (108) Linden, S. M.; Necken, D. C. *J. Am. Chem. Soc* **1988**, *110*, 1257.
- (109) Hélène, C.; Toulmé, J. J. *Biochim. Biophys. Acta* **1990**, *1049* (2), 99.
- (110) Hélène, C. *Anticancer. Drug Des.* **1991**, *6* (6), 569.
- (111) Song, G.; Xing, F.; Qu, X.; Chaires, J. B.; Ren, J. *J. Med. Chem.* **2005**, *48* (10), 3471.
- (112) Greenspan, P.; Mayer, E. P.; Fowler, S. D. *J. Cell Biol.* **1985**, *100*, 965.
- (113) Pitchaimani, A.; Renganathan, A.; Cinthaikini, S.; Premkumar, K. *RSC Adv.* **2014**, *4* (42), 22123.
- (114) Zdolsek, J. M. *APMIS* **1993**, *101* (1–6), 127.
- (115) Hiruma, H.; Katakura, T.; Takenami, T.; Igawa, S.; Kanoh, M.; Fujimura, T.; Kawakami, T. *J. Photochem. Photobiol. B Biol.* **2007**, *86*, 1.
- (116) Molero, M. L.; Hazen, M. J. *J. Plant Physiol.* **1988**, *132* (5), 636.
- (117) Satonaka, H.; Kusuzaki, K.; Matsubara, T.; Shintani, K.; Nakamura, T.; Matsumine, A.; Iino, T.; Uchida, A. *Oncol. Lett.* **2010**, *1* (1), 69.
- (118) Zeisser-Labouèbe, M.; Lange, N.; Gurny, R.; Delie, F. *Int. J. Pharm.* **2006**, *326* (1–2),

- 174.
- (119) Barras, A.; Boussekey, L.; Courtade, E.; Boukherroub, R. *Nanoscale* **2013**, 5 (21), 10562.
- (120) Feinweber, D.; Verwanger, T.; Brüggemann, O.; Teasdale, I.; Krammer, B. *Photochem. Photobiol. Sci.* **2014**, 13 (11), 1607.
- (121) Chen, L. B. *Annu. Rev. Cell Biol.* **1988**, 4 (1), 155.
- (122) Huang, M.; Camara, A. K. S.; Stowe, D. F.; Qi, F.; Beard, D. A. *Ann. Biomed. Eng.* **2007**, 35 (7), 1276.
- (123) Schneckeburger, H.; Stock, K.; Lyttek, M.; Strauss, W. S. L.; Sailer, R. *Photochem. Photobiol. Sci.* **2004**, 3 (1), 127.
- (124) Lampidis, T. J.; Bernal, S. D.; Summerhayes, I. C.; Chen, L. B. *Cancer Res.* **1983**, 43 (2).
- (125) Ronot, X.; Benel, L.; Adolphe, M.; Mounolou, J. C. *Biol. Cell* **1986**, 57 (1), 1.
- (126) Millot, J.-M.; Sharonov, S.; Manfait, M. *Cytometry* **1994**, 17, 50.
- (127) Smith, N. R.; Dawson, V. T. *Soil Sci.* **1944**, 58 (6), 467.
- (128) Chan, B. P.; Chan, O. C. M.; So, K.-F. *Acta Biomater.* **2008**, 4 (6), 1627.
- (129) Koevary, S. B. *Int. J. Physiol. Pathophysiol. Pharmacol.* **2012**, 4 (2), 99.
- (130) Testori, A.; Faries, M. B.; Thompson, J. F.; Pennacchioli, E.; Deroose, J. P.; van Geel, A. N.; Verhoef, C.; Verrecchia, F.; Soteldo, J. J. *Surg. Oncol.* **2011**, 104 (4), 391.
- (131) Pan, Y.; Kee, C. W.; Chen, L.; Tan, C.-H. *Green Chem.* **2011**, 13 (10), 2682.
- (132) Ravelli, D.; Fagnoni, M. *ChemCatChem* **2012**, 4 (2), 169.
- (133) Panzarini, E.; Inguscio, V.; Dini, L. *Cell Death Dis.* **2011**, 2 (6), 169.
- (134) Hamblin, M. R.; Hasan, T. *Photochem. Photobiol. Sci.* **2004**, 3 (5), 436.
- (135) Panzarini, E.; Inguscio, V.; Fimia, G. M.; Dini, L. *PLoS One* **2014**, 9 (8), e105778.
- (136) Costa, A. C. B. P.; Rasteiro, V. M. C.; Pereira, C. A.; Rossoni, R. D.; Junqueira, J. C.; Jorge, A. O. C. *Mycoses* **2012**, 55 (1), 56.
- (137) Rossoni, R. D.; Junqueira, J. C.; Santos, E. L. S.; Costa, A. C. B.; Jorge, A. O. C. *Lasers Med. Sci.* **2010**, 25 (4), 581.
- (138) Thompson, R. F.; Walker, M.; Siebert, C. A.; Ranson, N. A. *Methods* **2016**, 100, 3.
- (139) Tivol, W. F.; Briegel, A.; Jensen, G. J. *Microsc. Microanal.* **2008**, 14 (5), 375.

- (140) Cholewiński, G.; Dzierzbicka, K.; Kolodziejczyk, A. M. *Pharmacol. Reports* **2011**, 63, 305.
- (141) Rescifina, A.; Zagni, C.; Varrica, M. G.; Pistarà, V.; Corsaro, A. *Eur. J. Med. Chem.* **2014**, 74, 95.
- (142) Mitra, P.; Chakraborty, P. K.; Saha, P.; Ray, P.; Basu, S. *Int. J. Pharm.* **2014**, 473 (1), 636.
- (143) Liu, C.-H.; Sahoo, S. L.; Tsao, M.-H. *Colloids Surfaces B Biointerfaces* **2014**, 115, 150.
- (144) Delcros, J. G.; Tomasi, S.; Carrington, S.; Martin, B.; Renault, J.; Blagbrough, I. S.; Uriac, P. *J. Med. Chem.* **2002**, 45 (23), 5098.
- (145) Shinozuka, K.; Seto, Y.; Sawai, H. *J. Chem. Soc. Chem. Commun.* **1994**, 23 (11), 1377.
- (146) Blagbrough, I. S.; Taylor, S.; Taylor, S.; Carpenter, M. L.; Novoselskiy, V.; Shamma, T.; Haworth, I. S. *Chem. Commun.* **1998**, 18 (8), 929.
- (147) Ferreira, R.; Artali, R.; Farrera-Sinfreu, J.; Albericio, F.; Royo, M.; Eritja, R.; Mazzini, S. *Biochim. Biophys. Acta* **2011**, 1810 (8), 769.
- (148) Farrera-Sinfreu, J.; Aviñó, A.; Navarro, I.; Aymamí, J.; Beteta, N. G.; Varón, S.; Pérez-Tomás, R.; Castillo-Avila, W.; Eritja, R.; Albericio, F.; Royo, M. *Bioorganic Med. Chem. Lett.* **2008**, 18 (7), 2440.
- (149) Guetzoyan, L.; Ramiandrasoa, F.; Dorizon, H.; Desprez, C.; Bridoux, A.; Rogier, C.; Pradines, B.; Perrée-Fauvet, M. *Bioorganic Med. Chem.* **2007**, 15 (9), 3278.
- (150) Cisáriková, A.; Barbieriková, Z.; Janovec, L.; Imrich, J.; Hunáková, L.; Bačová, Z.; Paulíková, H. *Bioorg. Med. Chem.* **2016**, 24, 2011.
- (151) Čížeková, L.; Grolmusová, A.; Ipóthová, Z.; Barbieriková, Z.; Brezová, V.; Hunáková, L.; Imrich, J.; Janovec, L.; Dvinová, I.; Paulíková, H. *Bioorg. Med. Chem.* **2014**, 22 (17), 4684.
- (152) Iwamoto, Y.; Yoshioka, H.; Yanagihara, Y. *Chem. Pharm. Bull. (Tokyo)*. **1987**, 35 (6), 2478.
- (153) Eberhard, J.; Peuntinger, K.; Rath, S.; Neumann, B.; Stammler, H.-G.; Guldi, D. M.; Mattay, J. *Photochem. Photobiol. Sci.* **2014**, 13, 380.
- (154) Scurlock, R. D.; Ogilby, P. R. *J. Photochem. Photobiol. A Chem.* **1993**, 72 (1), 1.
- (155) Auweter, H.; Haberkorn, H.; Heckmann, W.; Horn, D.; Lüddecke, E.; Rieger, J.; Weiss, H. *Angew. Chem. Int. Ed.* **1999**, 38 (15), 2188.
- (156) Auweter, H.; André, V.; Horn, D.; Lüddecke, E. *J. Dispers. Sci. Technol.* **1998**, 19 (2–3), 163.
- (157) Bohren, C. F.; Huffman, D. R. *Absorption and scattering of light by small particles*;

- Wiley, 2004.
- (158) Morris, J. V.; Mahaney, M. A.; Huberr, J. R. *J. Phys. Chem.* **1976**, *80* (9), 969.
- (159) Schmidt, R.; Tanielian, C.; Dunsbach, R.; Wolff, C. *J. Photochem. Photobiol. A Chem.* **1994**, *79* (1–2), 11.
- (160) Takizawa, S.; Aboshi, R.; Murata, S. *Photochem. Photobiol. Sci.* **2011**, *10* (6), 895.
- (161) Bregnhøj, M.; Westberg, M.; Jensen, F.; Ogilby, P. R. *Phys. Chem. Chem. Phys.* **2016**, *18* (33), 22946.
- (162) Bennett, L. E.; Ghiggino, K. P.; Henderson, R. W. *J. Photochem. Photobiol. B.* **1989**, *3* (1), 81.
- (163) Rossetti, F. C.; Lopes, L. B.; Carollo, A. R. H.; Thomazini, J. A.; Tedesco, A. C.; Bentley, M. V. L. *J. Control. Release* **2011**, *155* (3), 400.
- (164) Siraj, N.; Kolic, P. E.; Regmi, B. P.; Warner, I. M. *Chem. - A Eur. J.* **2015**, *21* (41), 14440.
- (165) Far, S.; Kossanyi, A.; Verchère-Béaur, C.; Gresh, N.; Taillandier, E.; Perrée-Fauvet, M. *European J. Org. Chem.* **2004**, *2004* (8), 1781.
- (166) Borgions, F.; Ghysels, D.; Van Aerschot, A.; Rozenski, J.; Herdewijn, P. *Helv. Chim. Acta* **2006**, *89* (6), 1194.
- (167) Becerril, J.; Bolte, M.; Burguete, M. I.; Galindo, F.; García-España, E.; Luis, S. V.; Miravet, J. F. *J. Am. Chem. Soc.* **2003**, *125* (22), 6677.

Chapter 6

**Upconverting
nanoparticles
embedded in
molecular gels**

Chapter 6: Upconverting nanoparticles embedded in molecular gels

6.1 Motivation

The synergistic combination of organic and inorganic components affords composite or hybrid materials, which present several physicochemical properties of interest. Initial examples of hybrid materials can be dated back to the interest of humankind in art and decoration. For example, Maya blue is a hybrid of the blue dye indigo and clay and some ancient paints consisted in a protein base (albumin or casein) or an oil base (linseed oil) mixed with a powdered pigment. As usual, nature provides the most beautiful examples and has also a clear lead in developing structural and functional hybrid materials. For instance, bones are a three-phase composite of organic collagen fibres, inorganic crystalline hydroxyapatite crystals and bone matrix. Other examples include dentin (apatite and fibrils of type I collagen), nacre (calcium carbonate in the form of aragonite and chitin), and wood (a fibrous composite composed of parallel columns of cells which are supported by cellulose wound spirally in one direction and embedded in a matrix of a complex poly-phenolic resin called lignin).¹

We are currently not able to imitate the complex hierarchy of natural hybrid materials and nanocomposites. However, we are only at the beginning of understanding how natural materials are assembled and how we can use these biological concepts. During the last few decades, extensive fundamental research on these versatile materials has provoked an exponential rise in publications and patents and pushed forward new applications and features not known up to date. In this chapter, we report a fundamental study of a new hybrid material based on the structural and energetic relationships between two areas of interest: fluorescent molecular gels and upconverting nanoparticles (UCNPs).

6.2 Introduction to upconversion phenomenon

6.2.1 Upconversion mechanisms in inorganic/ionic materials

Upconversion (UC) expression is used to describe the phenomenon in which radiation with a given wavelength is converted to a shorter wavelength, namely, the radiation frequency is increased. The seminal idea of upconversion mechanisms that give birth to the whole upconversion field was proposed by Bloembergen in 1959, when he envisaged that infrared photons could be detected through sequential absorption within the levels of a given ion (from rare earth elements) in a solid. He named the detector as infrared quantum counter (IRQC) and based the detection in the superexcitation phenomena. He postulated that there was little chance that, with incoherent pumping, the same single doping ion would receive two photons in sequence during the first excited-state lifetime. The experimental demonstration of this effect had to wait for laser excitations of fibers with confined doping in the center. Later in 1966, Auzel suggested that energy transfer between rare earth ions, both of them being in an excited state, played an important role in the upconversion initial step.²

Among different upconversion mechanisms in inorganic rare earth crystals,² three of them should be highlighted due to their relatively notable efficiency: Energy transfer upconversion (ETU), excited-state absorption (ESA), and photon avalanche (PA) (Figure 1).

Figure 1. UC processes for lanthanide-doped crystals: **a)** excited-state absorption, **b)** energy-transfer upconversion, **c)** photon avalanche.

These three mechanisms are based on the consecutive absorption of two or more photons by metastable long-lived excited levels populating high-energy states and their relaxation through short wavelength radiation emission. In the case of ESA, the emitting ions sequentially absorb at least two photons of suitable energy to reach the high energy emitting level. In ETU, one photon is absorbed by the ion and subsequent energy transfer from neighbouring ions results in the population of a highly excited state of the emitting ion. The photon avalanche effect, was first discovered by Chivian in 1979.³ Also based on sequential energy transfers, but of the downconversion type (usually called cross-relaxation), whereas the upconversion step itself is due to ESA.

6.2.2 Upconversion mechanisms in organic/organometallic materials

Aside from the upconversion mechanisms seen before for rare earth materials, other interesting upconversion mechanism should be highlighted, principally occurring in organic materials: the sensitized triplet-triplet annihilation (TTA) mechanism. This phenomenon was firstly introduced by Parker and Hatchard in early 60s.⁴ They observed upconverted fluorescence from a donor(sensitizer)/acceptor(annihilator) mixture composed of phenantrene/naphthalene or proflavin hydrochloride/anthracene. TTA overall mechanism is based firstly on the Dexter-based triplet-triplet energy transfer (TTET), requiring close contact between two components (donor/acceptor) and long-lived triplet states, followed by a triplet-triplet annihilation (TTA), which is a bimolecular process involving two annihilator triplet states (Figure 2).

Figure 2. State energy level diagram of TTA upconversion processes leading to singlet delayed fluorescence. TTET is triplet-triplet energy transfer; TTA is triplet-triplet annihilation. ISC is intersystem crossing.⁵

This field was recently expanded by the introduction of various organometallic triplet sensitizers, spanning the absorption from the visible to NIR region of the spectrum. The interplay of triplet excited states makes the overall mechanism sensible to oxygen (as is well known that collision with oxygen provokes triplet state deactivation), so several strategies were followed to construct optical materials insensible to aerated ambient. For instance, the donor/acceptor entrapment on polymeric matrixes.⁶ However, this strategy blocks the diffusion of the excited triplet molecules and requires high-power excitation. To solve this problem, Duan and co-workers proposed to entrap donor and acceptor pairs in self-assembled fibrillar networks of N,N'-bis(octadecyl)-L-boc-glutamic diamide (LBG), a supramolecular gelator. Nanofibres are used as adaptative nanohosts in organic media, which accommodate donor and acceptor molecules to provide air-stable-photon upconversion gels via hydrophobic interactions (Figure 3).⁷ Overall, TTA mechanism on self-assembled matrixes is an interesting approach for developing upconversion materials with applications ranging from sunlight powered photovoltaics and photocatalysis to bioimaging and phototherapy.⁸⁻¹² TTA upconversion is not studied in this thesis but is undoubtedly an interesting topic to focus in future research.

Figure 3. A schematic representation of the unit structure of the upconversion gel system. Donor (red) and acceptor (blue) molecules are incorporated in the gelator nanofibers as extended domains. The donor molecules are excited by long-wavelength light, followed by a sequence of triplet-triplet energy transfer (TTET) from the donor to the surrounding acceptor, triplet energy migration among the acceptor molecules, triplet-triplet annihilation (TTA) and delayed fluorescence from the upconverted singlet state of acceptor.⁷

6.2.3 Upconverting nanoparticles

The most efficient UC mechanisms are present in solid-state materials doped with rare-earth ions. In these materials, UC process goes through metastable states with a lifetime of microseconds, whereas the two-photon absorption mechanism in organic fluorophores involves virtual short lived excited states in the femtosecond scale. This feature enables the use of continuous wave lasers for excitation and lower power densities for UC with rare-earth doped materials ($1\text{--}103\text{ W}\cdot\text{cm}^{-2}$, while for organic two-photon systems power in the range of $106\text{--}109\text{ W}\cdot\text{cm}^{-2}$).¹³ Although, bulk upconverting lanthanide-based crystals have been known for decades, there has been a resurgence of their study associated to the preparation of upconverting nanoparticles (UCNPs) about one decade ago.^{14,15} The rise of nanoscience increased interest in the design of colloidal, non-toxic, nanocrystalline UCNPs. Ideally, upconverters should present tunable emission, large anti-Stokes shift, sharp emission bandwidth and high photostability. Typically, the upconversion nanoparticles are formed by a host lattice, a dopant, a co-dopant or activator and surface covered with organic moieties to prevent aggregation. In Figure 4, different TEM images of oleic acid coated $\text{NaYF}_4\text{:Yb,Tm}$ UCNP with different doping ion concentration (0.5–8 % mol) are shown, presenting different shapes and sizes around 40 nm.

Figure 4. TEM images of $\text{NaYF}_4\text{:Yb,Tm}$ nanoparticles (Yb doping concentration 20 mol %). The Tm doping concentrations are from a–j, respectively: 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6 and 8 mol %.¹⁶

6.2.3.1 Selection of host materials

The choice of the host lattice is very important because it determines the distance between the dopant ions, their relative spatial position, their coordination numbers and the type of

anions surrounding the dopant. Host materials with a hexagonal (β -phase) are densely packed and allow for a more efficient energy transfer between the dopant ions than the loosely packed cubic phase (α -phase).¹⁷ The purity of the phase is an important variable too, pure hexagonal phases exert uniform crystal-field around their dopant ions and maximize the upconversion efficiency. Impurities may increase multiphonon relaxation rates, resulting in the reduction of energy transfer ability.¹⁸ Despite the vast knowledge available on various host materials, NaYF₄ is the most common matrix described for UCNPs. In fluoride materials, long lifetimes of the excited states are usually observed because of the low phonon energies (*ca.* 350 cm⁻¹) of the crystal lattice.¹⁹ However, additional effects should be considered in nanoparticles, as additional non-radiative decay pathways exist for ions at, or near, the surface.

6.2.3.2 Selection of suitable dopants

The dopant ion is the most important counterpart in the upconversion process. The emitted upconverted luminescence (UCL) largely depends on the type and the amount of ions doped. As discussed above, rare earth metals, such as lanthanides, are suitable candidates as dopants because they possess long-lived excited states with energy gaps quite similar, which enables sequential excitation with a single monochromatic light source. The most common lanthanide emitter ions with an energy-level structure suitable for this type of excitation include Er³⁺, Tm³⁺ and Ho³⁺. Er³⁺ possess the optimum energy gaps for multiple photon absorption and shows green fluorescence emissions from the ²H_{11/2} level and red fluorescence from the ⁴F_{9/2} level. Blue emission is observed in systems doped with Tm³⁺, after the absorption of four photons (Figure 5).

Figure 5. Energy-level diagram, upconversion excitation and visible emission schemes for the systems containing Yb^{3+} and Er^{3+} or Tm^{3+} . Arrows indicate radiative and nonradiative energy transfer and multiphonon relaxation processes.²⁰

A high concentration of dopants can induce emission quenching. The upper limit of concentration for dopants depends on the exact distance among the lattice sites occupied by lanthanide ions. For most upconversion materials, the concentration of Er^{3+} does not exceed 3% and in the case of Tm^{3+} 0.5 %. Because of the low proportion of doping agents and its low absorption cross-section, strongly absorbing ions (sensitizers) are added into the material lattice to ensure an efficient energy transfer to the emitting actives.¹⁸

6.2.3.3 Codoping systems activators and sensitizers

The most used codoping agent for UCNPs is Yb^{3+} , which is introduced into the host lattice in high concentrations (18–20%). The large absorption cross-section and the possession of suitable metastable single state of Yb^{3+} makes this ion the standard sensitizer for Tm^{3+} and Er^{3+} doped upconversion materials. Both ion couples ($\text{Er}^{3+}/\text{Yb}^{3+}$ and $\text{Tm}^{3+}/\text{Yb}^{3+}$), show the highest upconversion efficiency when doped into hexagonal-phase ($\beta\text{-NaYF}_4$). Apart from high upconversion efficiencies, the emission of Yb^{3+} , Er^{3+} codoped phosphors saturates only at high excitation densities.²¹

6.2.3.4 Photochemical feature modification and surface chemistry

Strategies to expand the range of emission colours have been investigated by doping the NaYF_4 host with all three ions Yb^{3+} , Tm^{3+} , and Er^{3+} and adjusting their concentrations. For example, Wang and Liu obtained colloids with bluish to whitish fluorescence emission upon IR irradiation (Figure 6).²⁰

The surface chemistry of UCNP ultimately determines its solubility, stability, biocompatibility and pharmacokinetic features. Furthermore, the capability of anchoring different functional groups, targeting ligands, photosensitizers and drugs is extremely surface-dependent. The UCNP surface chemistry has been improved by anchoring polyethylenimine (PEI), PEGylation, amphiphilic PEG block copolymers, PEG-phospholipids, chitosan, BSA, oleic acid or silica coating.²⁰

Figure 6. Typical strategies and surface molecules (monomers) used for making hydrophilic UC nanoparticles with pendant functional groups **e)** $\text{NaYF}_4:\text{Yb}^{3+}, \text{Tm}^{3+}$ (20 and 0.2 mol%), **f–j)** $\text{NaYF}_4:\text{Yb}^{3+}, \text{Tm}^{3+}, \text{Er}^{3+}$ (20, 0.2, and 0.2–1.5 mol %), and **k–n)** $\text{NaYF}_4:\text{Yb}^{3+}, \text{Er}^{3+}$ (18–60 and 2 mol%). The samples were excited at 980 nm with a 600 mW diode laser. Image adapted from refs. 20 and 22.

6.2.4 Biomedical applications

UCNPs linked to biological macromolecules have been investigated as fluorescent markers for imaging biological processes. They benefit from a narrow particle size distribution, high photostability, narrow emission bandwidths, good biocompatibility, high penetration depth and a high signal-to-noise ratio, owing to the weak autofluorescence background generated by NIR excitation. Biological applications of UCNPs include specific detection of biomolecules, contrast for optical imaging and their use as light nano-transducers for cancer therapy.²²

6.2.4.1 *In vitro* detection

The first demonstration of the potential of upconversion materials in diagnosis was provided by Tanke and co-workers in 2001. They used a rare-earth-doped yttrium oxysulfide for selective detection of specific nucleic acid sequences (Human Papillomavirus Type 16), taking advantage of the strong interaction between biotin and avidin (or streptavidin).^{23–25} Other alternative strategy for biosensing is the FRET-based detection approach. For instance, Li and coworkers developed for the first time a system that uses FRET between bioconjugated UCNPs and gold NPs. In this case, FRET system is formed by biotinylated UCNPs ($\text{NaYF}_4:\text{Yb}^{3+}, \text{Er}^{3+}$) and biotinylated Au NPs. The absorption plasmon of Au nanoparticles at 520 nm fits the emission band of the UCNPs. The close proximity of both biotinylated particles turns on the FRET mechanism and produces emission quenching when avidin is added, owing to the sensitive and selective interaction between avidin and biotin. The luminescence of the system after excitation with NIR light was gradually quenched with increasing amounts of avidin added to the system, this system could be used in the detection of trace amounts of avidin (Figure 7).²⁶

Figure 7. Scheme of the FRET system, with phosphor-biotin nanoparticles as energy donors and Au-biotin nanoparticles as energy acceptors, in the analysis of avidin. ET=energy transfer.²⁶

6.2.4.2 *In vivo* detection

A seminal paper about the *in vivo* application of UCNPs was published by Zhang and co-workers. They introduced upconverting polyethyleneimine (PEI) coated $\text{NaYF}_4:\text{Yb}$, Er UCNPs into living mice for cellular and tissue imaging and studied their biodistribution and biocompatibility. It was found that UCNPs could be detected even in tissue depths of around 10 mm.²⁷ In further works, Zhang and Jiang used FRET taking place between rare-earth codoped NaYF_4 UCNPs and intercalating dye BOBO-3 for the intracellular investigation of siRNA in living cells (Figure 8).²⁸ This work paved the way for the use of this systems for the detection of some other biomolecules, such as nucleic acids and proteins, in both PBS buffer and living cells.

Figure 8. (A) TEM image of silica/ $\text{NaYF}_4:\text{Yb}$, Er upconversion nanoparticles. (B) Schematic drawing of FRET-based UCNP/siRNA-BOBO3 complex system.²⁸

6.2.4.3 *Upconverting nanoparticles as photodynamic agents*

Among all the possible applications, upconverting nanoparticles are successfully used as photodynamic agents for cancer therapy.^{29–33} For instance, Zhang and co-workers prepared $\text{NaYF}_4:\text{Yb}^{3+}, \text{Er}^{3+}$ UCNPs coated with a thin layer of silica as shell, which contained the photosensitizer MC-540. The upconverting system acted as a nanotransducer from IR excitation to visible emission and transferring the energy to a photosensitizer, in order to produce reactive oxygen species that kill cancer cells *in vitro*. Furthermore, selectivity through tumoral tissue was achieved by functionalizing the nanoparticle surface with a tumor targeting anti-MUC1/episialin antibody. *In vitro* PDT was performed by irradiating MCF-7/AZ cells with a NIR light, causing cell shrinkage and membrane damage.³⁴ Further *in vivo* advances regarding this approach were described by Zhang et. al., by using UCNP targeted to tumor-associated antigens on cancer cells, which could be imaged with a high signal-to-background ratio.

Photodynamic therapy was carried out by the modification of the nanoparticles with the photosensitizer zinc phthalocyanine (ZnPc) and merocyanine 540 (M540), inducing tumor cell death upon low energy irradiation (Figure 9).³⁵

Figure 9. **A)** Schematic representation of coated upconverting nanoparticle and its action mechanism when irradiated at 960 nm. **B)** TEM image of silica-coated upconverting nanoparticles, scale bar 50 nm. **C)** Schematic diagram showing targeted UCNP-based PDT in a mouse model of melanoma intravenously injected. UCNPs surface was modified with folic acid (FA) and PEG moieties. Scale bar, 10 mm. Adapted from ref. 35.

Prud'homme and Ju et al. prepared PEGylated composite systems containing UCNPs and meso-tetraphenyl porphyrin. The overlap between emission of UCNPs and photosensitizer absorption ensures the energy transfer from UCNPs to the photosensitizer, which generates singlet oxygen upon IR excitation at 978 nm. In a PDT experiment, a 75 % of HeLa cancer cells death was achieved after incubation for 45 min and subsequent irradiation.³⁶ To improve the tumor-targeting capacity, Gu et al. coated the surface of oleic acid-capped UCNPs (OA-UCNPs) with folate-modified amphiphilic chitosan. The photosensitizer used was Zn(II) phthalocyanine, anchored through hydrophobic interactions. These nanoparticles demonstrated an enhanced targeting of folate-overexpressed tumors.³⁷ Cui and co-workers reported chitosan wrapped UCNPs attaining high loading of ZnPc photosensitizer via hydrophobic interactions (10.8%).³⁸

Figure 10. Scheme of the synthesis of FASOC-UCNP-ZnPc nanoconstruct and folate-mediated binding to tumor cells with folate receptor overexpressed.³⁸

Further and advanced assays for cancer therapy using upconverting nanoparticles were performed by combination of techniques and therapies. The first attempt to combine bimodal imaging (MR/UCL) and trimodal therapy (Chemo-/ radio- /photodynamic) in a single UCNP-based nanotheranostic system was reported by Fan and co-workers. They synthesized an 80 nm nanocomposite containing Gd³⁺-doped UCNPs. Particles were covered with mesoporous silica which contained hematoporphyrin (as photosensitizer and radiosensitizer) and the chemotherapeutic drug docetaxel (Dtxl). Tumours in mouse models were quickly eradicated due to the synergistic effect of combined therapies (Figure 11).³⁹

Figure 11. Schematic illustration of the synthetic procedure of UCMSNs. Gd-UCNPs were prepared by epitaxial growth of a NaGdF₄ layer on NaYF₄:Yb/Er/Tm through a typical thermal decomposition process. Then, Gd-UCNPs were coated with a dense silica layer by a reverse microemulsion method, designed as Gd-UCNPs@SiO₂. Subsequently, a mesoporous silica shell was deposited on Gd-UCNPs@SiO₂, via the template of CTAC, designed as Gd-UCNPs@SiO₂@mSiO₂. Finally, UCMSNs were successfully obtained based on a “surface-protected hot water etching” strategy.³⁹

6.3 Introduction to hybrid materials

The IUPAC define hybrid materials as follows: “A hybrid material is composed of an intimate mixture of inorganic components, organic components, or both types of component”.⁴⁰ This definition has been nuanced by several experts in the field and has been reformulated: “A hybrid material consists of at least two components – usually an inorganic and an organic component – that are molecularly dispersed in the material.” The intimate mixture of the components on the molecular scale can change many properties compared to those of nanocomposites. For example optical and mechanical properties can be very different although similar components are mixed.⁴¹

6.3.1 Hybrid materials: Expanding features

The preparation of hybrid materials aims to obtain improved properties. Ashby and Bréchet proposed a material-property chart which schematizes the possible outcomes when preparing hybrid materials (Figure 12).⁴² Several scenarios are possible when designing hybrid materials: the new material may show the best properties of both components (point A), follow the rule of mixtures (point B), show a domination of the weaker link (point C) or present the worst properties of both materials (point D).

Figure 12. I) The possibilities of hybridization. The properties of the hybrid material reflect those of their component materials, combined in one of several possible ways; II) Metals and polymers are mixed to form elastomer-metal hybrids. A hole in material-property space is filled.⁴²

6.3.2 Nanoparticles@molecular gels hybrid materials

Several studies have been devoted to hybrid NPs-supramolecular gel systems. For example, the incorporation of noble (Ag and Au) metal NPs into molecular gels has been extensively studied, yielding new optoelectronic materials,^{43,44} antibacterial soft materials^{45–47} and even as catalysts.⁴⁸ Supramolecular gels have shown their utility as scaffold to support NPs,^{49–51} which, in several cases, were prepared *in situ*.^{52,53}

Interesting studies have evaluated the reciprocal influence in the photoluminescence when mixing molecular gel networks and nanoparticles. For example, Del Guerzo and coworkers described hybrid material by combining the self-assembled 2,3-didecyloxyanthracene (DDOA)

gelator and thiol-capped gold nanoparticles. Homogeneous NP distribution and variable absorbance in the visible range were observed. In this system, efficient energy transfer from fluorescent gelator to AuNPs is observed, quenching the luminescence of the DDOA nanofibers (Figure 13).⁵⁴ Analogous relationship was shown between DDOA gels and organic capped ZnO-NPs, which showed light-harvesting behavior leading to photo-induced processes at extremely low loading of ZnO-NPs.⁵⁵ An opposite behavior was reported by Yamaguchi and coworkers. They used two-component gels, using pseudoenantiomeric ethynylhelicene oligomers containing a disulfide group as attachment to gold nanoparticles. The hybrid materials exhibited a novel emission at 600–800 nm from the gold nanoparticles. The emission reversibly disappeared upon sol formation with heating, which was accompanied by an enhancement of the emission at 450 nm.⁵⁶

Figure 13. Left: Fluorescence spectra of hybrid gels in *n*-butanol with the addition of hexanethiol capped gold nanoparticles (DDOA : 6.5 mM, C6NP : 0–0.62 μ M, λ_{ex} = 384 nm). Right: TEM images of a xerogel from a DDOA/*n*-butanol gel. Scale bars: A: 1000 nm; B: 200 nm; C: 50 nm; D: 20 nm.⁵⁴

Further investigations have been reported combining molecular gels and core/shell semiconductor nanocrystals of CdSe/ZnS, namely quantum dots (QDs). For example, Galindo et. al. incorporated CdSe/ZnS QDs in an organogel formed by pseudopeptidic macrocycle. The core-shell QDs preserved their photophysical properties in the organogel medium. When the core of QDs (CdSe) are dispossessed of the external shell and embedded within the fibrillar network, enhancement of fluorescence intensity (up to 528 %) and fluorescence lifetime (up to 1.7-fold) are reported (Figure 14).⁵⁷

Figure 14. Left: Fluorescence emission spectra and lifetimes of QDs (with ZnS shell (red) and without ZnS shell (blue)) in presence and absence of gel matrix. **Top right:** AFM of xerogel containing TC533 QDs. Scan area: $5.5 \mu\text{m} \times 5.5 \mu\text{m}$. **Bottom right:** TEM micrographs of the fibrillar structure of cyclophane xerogel containing ACS531 QDs. Scale bar 20 nm.⁵⁷

QDs embedded in gel matrixes also showed further applications in sensing. For example, Galindo et. al. reported a nitric oxide sensor based on nanoparticles doped hybrid organogels, which were prepared using a pseudopeptidic macrocycle and CdSe/ZnS quantum dots (QDs).⁵⁸

The combination of UCNPs with molecular gels has been scarcely studied. Only two reports directly related to this kind of hybrid materials are available in the literature. UCNP could be dispersed in a supramolecular gel matrix formed by a peptide derivative, reinforcing the gel structure.⁵⁹ In this case, the gel matrix was acting merely as a passive scaffold. In another report, a photoactive supramolecular gel, formed by a derivative of trans-stilbene, experienced energy transfer from core-shell $\text{NaYF}_4:\text{Yb/Tm}$ UCNP upon IR excitation. In this case, the UV absorption of the gelator overlaps with the UV-emission of the UCNP upon 980 nm laser excitation, and the fluorescence of the aggregates in the visible region adds up to the direct visible light upconversion of the UCNP (Figure 15).⁶⁰ Unlike the results reported in this thesis, the reversibility of the hybrid gel was not studied and the emission of gelator is overlapped with that of the UCNP, making the enhancement difficult to analyse and quantify.

Figure 15. Left: SEM image and emission spectra of **a)** neat UCNP in chloroform solution at various NIR excitation powers ($\lambda_{\text{ex}} = 980 \text{ nm}$), and **b)** the corresponding spectra of the hybrid gel (1.4 wt % UCNP and 2.6 wt % gelator).⁶⁰

6.4 Preparation and study of UCNPs@Molecular gel hybrid materials

Here, the incorporation of IR light upconverting $\text{NaYF}_4\text{:Yb/Tm}$ nanoparticles (UCNPs) in a naphthalimide-derived fluorescent supramolecular gel network is described. The high loading capacity of molecular gels regarding hydrophobic dyes and their fluorescent features are combined with the low energy and tissue penetrating IR excitation showed by UCNPs. Optical communication between the UCNPs and the organic chromophore in gel phase is studied, as well as the light manipulation process associated to the reversible assembly/disassembly of the fibrillar network. Additionally, the sizing down of the hybrid material up to nanometric level and the incorporation of dyes and photosensitizers for application in photodynamic therapy (PDT) was successfully achieved during a short stay in Prof. Yong Zhang's group at the National University of Singapore (NUS).

Details regarding UCNPs preparation are not discussed here. They were obtained either from Dr. Beatriz Julian's group at University Jaume I, which prepared them following a simple two-phase solvothermal procedure reported in literature,^{61,62} or from Zhang's group.

6.4.1 UCNPs@gel physical characterization

To prepare the hybrid gels, two different gelators from our laboratories were used: a 1,8-naphthalimide derivative (**1**) and a 4-amino-1,8-naphthalimide derivative (**2**). Two different UCNPs were assayed for the preparation of the hybrids: $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$ and $\text{NaYF}_4:\text{Yb}^{3+}/\text{Er}^{3+}$. In both cases, UCNPs surface is covered with oleic acid. All the possible four combinations showed efficient interaction between molecular gels and UCNPs, but detailed extensive experiments were only performed for the systems **NaYF₄:Yb³⁺/Tm³⁺@1** and **NaYF₄:Yb³⁺/Tm³⁺@2**. *n*-Butanol was used as solvent, as it is amenable of gelation and good dispersions of the UCNPs are obtained.

Gels of **1**, which present AIE (as described in chapter 4), present light absorbance at $\lambda_{\text{max}} = 335$ nm and emission at $\lambda_{\text{max}} = 410$ nm (Figure 16 top left). The minimum concentration required to gelate in *n*-butanol (mgc) was 5 mM, with a transition from gel to solution (T_{gel}) taking place at 65 °C, using vial inversion test. UCNPs@gel could be prepared by direct suspension of UCNPs in 2 mL of *n*-butanol and sonication for 5 min. Then, the desired amount of **1** was added and the closed system heated to 100 °C until its complete solubilisation. After resting at room temperature for 10 minutes, the gel was formed (see experimental section for further details).

To compare the influence of the presence of UCNPs in gel stability, a variable temperature study of fluorescence emission spectra was carried out (Figure 16). The inflection point of the representations $\lambda_{\text{em max}}$ vs *T* was taken as T_{gel} and it showed similar values when UCNPs are present, 68 vs 66 °C. However, UCNPs induce a broader disassembly temperature range (58-78 °C for **NaYF₄:Yb³⁺/Tm³⁺@1** vs 62-69 °C for **1** alone). This behaviour could be ascribed to the extra stability inferred by the UCNPs in gel matrix, by enhancing the fibre entanglement in junctions. Such effect is in agreement with studies for other hybrid organogels doped with different nanoparticles.^{51,55,57,59,63,64}

Intimate interaction between gel fibers and UCNPs was visualized by electron microscopy analysis (Figure 17 and Figure 18). TEM revealed fibre entanglement, commonly observed in supramolecular gels with UCNPs attached to the fibers. It could be argued that oleic acid units

covering UCNPs provide anchorage points to the surface of the fibers, as described, for example, for the interaction with polymers.^{65,66}

Figure 16. Top left: Absorption (dotted line) and emission spectra (continuous line) of **1** (25 μM) in *n*-butanol. Top right: Pictures of the hybrid **NaYF₄:Yb³⁺/Tm³⁺@1** gel under natural light, UV lamp excitation and 980 nm laser irradiation. Bottom left: Emission spectra (λ_{ex} = 335 nm) variation with temperature of **1** in *n*-BuOH ΔT = 5 °C. Bottom right: Emission spectra (λ_{ex} = 335 nm) variation with temperature of **NaYF₄:Yb³⁺/Tm³⁺@1** gel in *n*-BuOH ΔT = 5 °C.

Figure 17. Transmission electron microscopy image of the hybrid **NaYF₄:Yb³⁺/Tm³⁺@1**.

Figure 18. Transmission electron microscopy image of the hybrid $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}@2$.

6.4.2 Energy transfer of upconverted emission in UCNPs@gel

The hybrid systems were designed in such a way that the absorption of the gelators overlaps with the emission bands of the UCNPs, located at *ca.* 350 nm in the case of $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}@1$ (Figure 19A and 20C). In the case of compound **2**, the overlap encompasses broader regions of the spectrum because the absorption of the gelator is extended to the visible region, also including the signals around 490 nm (Figure 20D).

Figure 19. (A) Overlaid absorption spectrum of compound **1** in *n*-butanol (dotted line) and emission spectrum (continuous line) of Tm-UCNPs ($\lambda_{\text{exc}} = 980$ nm). (B) Jablonsky diagram of the electronic transitions reported. Energy levels from left to right correspond to Yb^{3+} , Tm^{3+} and gelator.

When constructing the hybrid materials, the spectral overlap and the close proximity between both components enables an efficient energy transfer. This process can be noticed by the vanishing of the signals present in the UV region around 350 nm for **NaYF₄:Yb³⁺/Tm³⁺@1** and those signals located at 350 nm and 490 nm for **NaYF₄:Yb³⁺/Tm³⁺@2** (Figure 20A and B, respectively).

Figure 20. A) Normalized emission spectrum ($\lambda_{\text{ex}} = 980 \text{ nm}$) of **NaYF₄:Yb³⁺/Tm³⁺@1** gel in *n*-BuOH; B) Normalized emission spectrum ($\lambda_{\text{ex}}=980\text{nm}$) of **NaYF₄:Yb³⁺/Tm³⁺@2** gel in *n*-BuOH; C) and D) Normalized emission spectrum ($\lambda_{\text{ex}} = 980 \text{ nm}$) of Tm UCNP suspension in *n*-BuOH (black line) and excitation spectrum of gel of **1** ($\lambda_{\text{em}} = 407 \text{ nm}$) and **2** ($\lambda_{\text{em}} = 533 \text{ nm}$) at 2.4 mg/ml, respectively. The black arrow indicates those signals vanishing when comparing UCNP in absence and in presence of gel (top images). Detection of emission spectrum were recorded with an optic fibre-based detector.

Next, the emission of UV-Vis light from the gelator fibres upon irradiation of the UCNP-loaded gels with 980 nm IR light was deeply studied by changing the setup and using a PMT detector (see experimental details for further information). For **NaYF₄:Yb³⁺/Tm³⁺@1** it was found again that the emission of the UCNPs at *ca.* 350 nm disappears and as expected, a weak emission band at around 410 nm emerges resulting of ET from UCNPs to gelator (Figure 21 left). The same behaviour was observed when investigating the emission spectra of **NaYF₄:Yb³⁺/Tm³⁺@2**

(Figure 21 right). Upon comparison of the emission of free nanoparticles with that of the hybrid material, it can be observed how the signals around 350 nm and 490 nm disappear and an emission at 530 nm appears, resulting from energy transfer from UCNPs to 4-amino-1,8-naphthalimide gelator **2**.

Figure 21. Left: Overlay of the emission spectra of UCNPs (dotted line) and hybrid $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$ @1; Right: Overlay of the emission spectra of UCNPs (dotted line) and hybrid $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$ @2. $\lambda_{\text{exc}} = 980 \text{ nm}$.

6.4.3 Fluorescence output modulation with temperature in UCNPs@gel

Intrinsic reversibility of molecular gels could be used for modulating the energy transfer and output emission of the hybrid materials. Interestingly, $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$ @1 only originates IR-promoted emission at 410 nm in the gel state (Figure 22), being this process cancelled when the gel is disassembled at 80°C . This behaviour is directly related to the AIE properties of compound **1**. The system showed good reversibility, and several heating-cooling cycles could be performed, restoring the upconversion to 410 nm at low temperatures (Figure 23). Consequently, the system formed by $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$ @1 constitutes a thermally regulated light upconverting soft material.

Figure 22. Right: Emission spectra ($\lambda_{\text{exc}} = 980 \text{ nm}$) of $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$ @1 gel in *n*-BuOH, centred on the reversible gel emission with the temperature. Left: Reversible emission of the fibres of $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$ @1 gel in *n*-BuOH at $\lambda = 407 \text{ nm}$ ($\lambda_{\text{exc}} = 980 \text{ nm}$).

Figure 23. Variation of the emission intensity at 410 nm for the hybrid system $\text{NaVF}_4\text{:Yb}^{3+}/\text{Tm}^{3+}\text{@1}$ upon heating-cooling cycles. Dotted line is used as guide to the eye ($\lambda_{\text{ex}} = 980 \text{ nm}$).

It is important to remark that the light emitted by UCNPs at *ca.* 350 nm is absorbed by compound **1**, either when the hybrid system is in the gel state at 30°C or in the sol state at 80°C (see Figure 22 right). This fact has implications for the mechanism of energy transfer occurring in the system. At 80 °C, the fibers are disassembled and spatial proximity between UCNPs and naphthalimide units is precluded, discarding a dipole-dipole energy transfer mechanism like Förster resonance energy transfer (FRET).⁶⁷ Therefore, a photon reabsorption process, also known as inner filter effect, emerges as the most plausible mechanism for energy transfer between UCNPs and the gel. The inner filter effect has been used in sensing applications based on UCNPs.⁶⁸

Aside of the previous results, it is noteworthy that the emission efficiency of the UCNPs in the presence of the supramolecular gel is clearly improved. Variable temperature studies were carried out for suspensions of the UCNPs in *n*-butanol, in the presence and absence of compound **1**. As can be seen in Figure 24, the emission of the UCNPs at 475 nm, measured in the range 20-90 °C, varies weakly, being moderately higher at low temperatures. However, in the presence of gelator **1**, a very notable dependence with temperature was detected. It is

important to recall that, in the range 20-90 °C, a progressive thermal disassembly of the gel network takes place and the system changes from a gel to a solution. For the sake of comparison, it was decided to analyze the behavior of both samples, with and without gelator, normalizing the results to the emission intensity measured at 90 °C for pure UCNPs. This seems reasonable because in both cases free and disperse UCNPs and no aggregates are present. Notably, the normalized fluorescence intensity at 20 °C in the presence of the gelator is much higher than the one measured only in the presence of UCNPs, with a 6-fold increase measured (Figure 24). The process was found to be reversible and after a heating-cooling cycle, the emission was restored to its initial values. These results indicate an improvement of the emission efficiency of UCNPs, ascribable to their interaction with the fibrillar network of the organogelator **1**. A plausible explanation for this behaviour is based on the partial isolation from the solvent experienced by the UCNPs upon adsorption on the gel fibers, avoiding in this way strong quenching effects from the high vibrational states of hydroxyl groups of *n*-BuOH. Such quenching effects have been demonstrated unequivocally in the case of water molecules and other alcohols^{69,70}

Figure 24. Variable temperature study of emission intensity at 475 nm of UCNPs (suspended in *n*-butanol) and hybrid **NaYF₄:Yb³⁺/Tm³⁺@1** gel. Values are normalized taken as reference the intensities measured at 90 °C for both systems ($\lambda_{\text{exc}} = 980 \text{ nm}$).

6.4.4 UCNPs@gel temporal stability

UCNPs suspensions in the absence of gel aggregates presented a two-fold fluorescence emission decrease after 20 hours (Figure 25 right). However, the aging of $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}@1$ does not provoke precipitation, indicating that fibrillary network precludes the aggregation of the UCNPs. Interestingly, in the hybrid system aging enhances UCNPs emission (Figure 25 left). This fact can be rationalized again by the above mentioned isolation of UCNPs from the solvent, considering that the system evolves to a better incorporation of UCNPs on the gel fibers, avoiding in this way strong solvent-caused quenching effects.^{69,70}

Figure 25. Stability measurements of $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}$ (right) and in hybrid material $\text{NaYF}_4:\text{Yb}^{3+}/\text{Tm}^{3+}@1$ (left) after 20 hours.

6.5 Hybrid nanomaterials: UCNPs@nanogels

In this section, a tricomponent nanomaterial composed of a nanogel particle, UCNP and a dye/photosensitizer is prepared. It has to be highlighted that each component of the nanomaterial has a very concrete function: i) Nanogels are envisaged to act as stimuli-responsive molecular “glue”, which can entrap both UCNPs and photosensitizer in aqueous media and put them in close disposition. ii) UCNPs are introduced in the system to act as phototransducers from IR to UV excitation. iii) Photosensitizers are introduced in the system to produce singlet oxygen ($^1\text{O}_2$) and act as potential photodynamic agents.

6.5.1 UCNPs@Nanogels

Core-shell $\text{NaYF}_4:\text{Yb}^{3+}/\text{Er}^{3+}$ UCNPs were provided by Prof. Zhang’s group at National University of Singapore. These NPs were selected for the present study because they present high fluorescence quantum yields, as the presence of the undoped shell minimizes luminescence

quenching attributed to the core surface defects. Additionally, these NPs have an emission spectra suitable for photosensitizer excitation, having a intense fluorescence band in the green region of the spectrum (≈ 530 nm).

These UCNPs were encapsulated in nanogels formed by **1** in aqueous media. The preparation starts with gel formation in an organic solvent in the presence of UCNPs. Then, lyophilisation affords a xerogel that is sonicated in the presence of aqueous media to afford UCNPs-loaded nanogel particles. This procedure is adapted from that reported by J. Li.^{71,72} As a representative example, 3 mg of **1**, 200 μ L of a suspension of NaYF₄:Yb³⁺/Er³⁺ UCNPs in cyclohexane (27 mg/mL) and 1 mL of 2-propanol were placed in a screw-capped vial. The mixture was heated until total solubilisation. Gel formation was achieved by fast cooling on ultrasonic bath for 10 seconds. Under optical fluorescence microscopy it can be observed how UCNPs are distributed homogeneously within the gel matrix (Figure 26), providing green fluorescence upon IR irradiation.

Figure 26. Optical microscopy 40x of **1** gels + NaYF₄:Yb³⁺/Er³⁺ in 2-propanol, from left to right: under IR irradiation at 980 nm (UCNP green emission), UV irradiation (blue gel emission).

The preparation of UCNPs@nanogel systems involved freeze-drying of the gels and resuspension of the xerogels in 2 mL of PBS (10 min stand + 10 min sonication). Isolation of the hybrid nanoparticles was performed by centrifugation at 6000 rpm, which removed large particles and afforded a colloidal solution of **UCNPs@1** (Figure 27).

Figure 27. Schematic representation of transition from macroscopic xerogel with embedded UCNP to UCNP@NGs nanomaterials.

UCNPs@1 colloids present a whitish appearance, as can be seen on Figure 28. Turbidity of the samples depends on the concentration of UCNPs. Dispersions showed very good DLS fittings, presenting a number averaged diameter (D_n) of 59 nm and an intensity averaged diameter (D_i) of 151 nm (Figure 28). Remarkably, the suspensions are stable after 8 days, in accordance with the negative Z-potential measured, -60 mV, which avoids particle aggregation. CLSM enabled the visualization of the hybrid particles, revealing discrete objects with fluorescence from the gelator (blue) and the UCNPs (green).

Figure 28. Size distribution by DLS analysis (by number and by intensity) of **UCNP@1** NGs in PBS; **Inset:** cuvettes containing nanogels with different amounts of UCNPs (0 mg, 6 mg, 12 mg, 25 mg).

Figure 29. 40x and 100x microscopy images of bulk gels and nanogels containing UCNPs.

IR absorption spectra of freeze-dried colloidal **UCNPs@1** were recorded to gain insight in the interactions between gelator and UCNPs. Figure 30 shows that, in the presence of UCNPs, the relative intensity of IR absorption bands corresponding at C-H stretching (from 3095 to 3098, 2924 to 2926 and from 2854 to 2856 cm^{-1}) and C=O stretching (from 1698 to 1701 cm^{-1}) region of gelator **1** changes. Hydrophobic interactions between oleic acid present on the surface of UCNPs and the hydrophobic aliphatic region of **1** may explain these results.

Figure 30. Selected regions of IR spectra, showing interactions between hydrophobic regions of **1** and UCNPs surface.

It is noteworthy that UCNPs incorporation into nanogels enables their stable dispersion in aqueous media (PBS). As shown in Figure 31, in the absence of nanogels, UCNPs can't be dispersed into water and no emission is found. On the contrary, nice IR-promoted emission is observed when trapped into the nanogels.

Figure 31. Fluorescence emission spectra of **UCNP@1** Ng's in PBS (black line) and control UCNPs in PBS (red line), upon excitation at 980 nm.

An important issue is the quantification of UCNPs and gelator in the obtained colloidal dispersions. For the estimation of the composition of the synthesized hybrid nanomaterials, UV-Vis and fluorescence spectroscopies were used and linear calibration curves were constructed using **1** in PBS (containing 1 % of DMSO) and NaYF₄:Yb³⁺/Er³⁺ UCNPs in cyclohexane, as can be seen in Figure 32. It was found that, in a typical preparation procedure, the concentration of gelator **1** was *ca.* 100 μ M (0.04 mg/ml) and that of UCNPs, *ca.* 0.2 mg/ml.

Figure 32. Calibration curve for concentration determination of **1** (left) and UCNPs (right) in hybrid systems.

6.5.2 UCNPs+Dye/Photosensitizer@Nanogels

Figure 33. Schematic representation of transition from macroscopic gel with embedded UCNPs and Photosensitizer (PS) to PS+UCNP@NGs nanomaterials.

The incorporation of a known hydrophobic dye (Nile red) or a hydrophobic photosensitizer (hypericin) together with UCNPs into the nanogels was assayed (Figure 33). The systems were designed in order that the IR-promoted emission of the UCNPs is absorbed by the dye or photosensitizer, to produce light emission or singlet oxygen, respectively. Such types of systems are interesting in the areas of light manipulation and IR-promoted photodynamic therapy, respectively because IR excitation is a non-ionizing radiation that does not cause tissue damage and has high tissue penetration due to the low absorption in this spectral region.

6.5.2.1 Nile Red

Nanogel particles were prepared as previously described above and the addition of the dye was performed at the beginning of the procedure, before the formation of the gel. The ternary system **Nile Red+UCNP@1** NGs resulted in a moderate reduction of Z-potential (-45 mV)

compared to colloidal **UCNPs@1** NGs (-60 mV), but this value is sufficient to provide temporal stability to the colloid. DLS revealed that the number averaged diameter of the particles increases with the presence of Nile red, from 151 nm to 282 nm. Tentatively, this behaviour can be attributed to particle aggregation, favored by Nile Red presence in the surface of the particles.

Fluorescence optical microscopy showed colocalization of Nile Red, gelator and UCNPs, as seen by the overlap of their emissions. The optical images revealed homogeneously disperse particles with some clustering pointed with arrows at Figure 34. Fluorescence spectra also confirmed the incorporation of Nile Red into the nanogels, presenting a fluorescence maximum at 403 nm for nanogels of **1** (Figure 35 left) and at 630 nm for Nile Red emission (Figure 35 right), indicating a relatively low hydrophobic environment. Besides, control UCNP without gelator showed no fluorescence at all in the same conditions.

Figure 34. 100x microscopy images under IR irradiation (980 nm) and UV irradiation (nanogel and NR emission), showing coexistence of nanogels, Nile Red and UCNPs.

Figure 35. Fluorescence spectra of **Nile Red+UCNP@1** NGs (black lines) and comparison with UCNP in presence of NR (red lines). **Left:** NGs emission $\lambda_{\text{ex}} = 330$ nm; **Right:** NR emission inside NGs $\lambda_{\text{ex}} = 530$ nm.

To confirm the possible energy transfer between UCNPs and entrapped dyes within gel matrix, emission spectra upon IR irradiation was recorded (Figure 36). Results showed energy transfer between UCNPs to gelator (vanishing signal at 370 nm) and from UCNPs to Nile Red (decrease of signal at 410 and 530 nm and appearance of signal at 620 nm).

Figure 36. Left: Fluorescence emission spectra upon IR irradiation of **1** gel in 2-propanol containing entrapped UCNPs and Nile Red (Black line), versus UCNPs entrapped within gelatine gel without Nile Red (red dashed line). Right: Photograph of **1** gel containing UCNPs and Nile Red upon IR excitation (980 nm). [**1**] = 2 mM, [NR] = 50 μ M.

6.5.2.2 Hypericin

Introduction of a photosensitizer for singlet oxygen production was also studied for its possible application in PDT upon IR irradiation (Figure 37). For this purpose, the same procedure as for introducing Nile Red was carried out (dye introduction before gel formation).

Figure 37. Schematic representation of **Photosensitizer+UCNP@1** NGs and its potential mechanism of action. Green spheres: UCNPs; Red spheres: photosensitizer; blue fibres: **1**; Pink arrow: IR irradiation; orange arrow: energy transfer.

Hypericin is poorly soluble in water, but it can be incorporated in aqueous media by encapsulation into the nanogels. UV-Vis spectra revealed a hypericin encapsulation of *ca.* 5.5 % w/w (3 μ M of hypericin in 70 μ M of nanogels of **1**), which is considered a good loading based on literature reports for nanocarriers and microcarriers of all chemical natures.^{73–75} Loading of hypericin was further confirmed by the detection of a notable displacement of absorption peaks, revealing blue shifts and sharper absorption bands, meaning that hypericin is probably entrapped in hydrophobic cavities (Figure 38). Interestingly, energy transfer was also observed in the ternary system **Hypericin+UCNP@1** NGs upon IR irradiation (λ_{ex} = 980 nm) between UCNPs and hypericin, with an emission peak detected at λ = 600 nm and a slight decrease of the green signal at λ = 530 nm (Figure 39).

Figure 38. Absorption spectrum of **Hypericin+UCNP@1** NGs; [**1**] = 70 μ M; [Hypericin] = 3 μ M.

Figure 39. Fluorescence emission spectra upon IR irradiation at 980 nm of **Hypericin+UCNP@1** NGs (left) and upon irradiation at 560 nm (right), showing presence of hypericin and a slight energy transfer.

The energy transfer resulted, additionally to the light emission from hypericin, in a decay through the triplet state, affording singlet oxygen generation. Singlet oxygen production was studied using the benchmark photooxidation of anthracene-9,10-dipropionic acid disodium salt (ADPA). The irradiation was carried out inside the spectrophotometer at 2W and the evolution over time was followed (Figure 40). Clearly, in the presence of **Hypericin+UCNP@1** NGs, upon IR-irradiation, singlet oxygen is provoking ADPA photooxidation, reflected in a decrease in the fluorescence emission of ADPA ($k = 11,6 \pm 1,2 \text{ min}^{-1}$), with a 7-fold higher constant than the control sample. In the control experiments with binary systems, **Hypericin@1** nanogels and **UCNP@1** nanogels, no significant photooxidation was observed ($k = 1,6 \pm 0,5 \text{ min}^{-1}$ and $k = 0.05 \pm 0.4$, respectively).

Figure 40. Bottom Left: Photooxidation reaction of ADPA 2.5 μM in presence of **Hypericin+UCNP@1** NGs in PBS upon IR irradiation (980 nm); Bottom Right: Control sample of **Hypericin@1** NGs. Inset: $\text{Ln}(F/F_0)$ at 406 nm vs time (black dots for **Hypericin+UCNP@1** NGs ($k = 11.6 \pm 1.2 \text{ min}^{-1}$) and red dots for **1 hypericin@NGs** ($1.6 \pm 0.5 \text{ min}^{-1}$). Irradiation times: 0, 2, 4, 6, 8, 10, 13, 16, and 20 min. $[1] = 70 \mu\text{M}$, $[\text{Hypericin}] = 3 \mu\text{M}$.

6.5.2.3 Preliminary cellular studies

Uptake and photodynamic therapy assays of the tricomponent nanomaterial in mouse urothelial carcinoma cell line (MB49) are currently being carried out in the National University of Singapore. Preliminary imaging study for **Hypericin+UCNP@1** visualization showed good uptake of the nanomaterial. Optical microscopy images are shown in Figure 41.

Figure 41. Left: Optical fluorescence microscopy images under IR excitation (2W, 980 nm, 6s exposure time) of MB49 cells incubated for 5 min with **Hypericin+UCNP@1**. Right: Fluorescence microscopy images of same MB49 under UV excitation (DAPI (blue) and Alexa fluor 488 (green) for nucleus and cytoskeleton stain).

At the left row, MB49 cells under exposure to 980 nm excitation can be observed showing yellow fluorescence, clearly pointing out that the nanomaterial has entered by endocytosis inside the cell. In the right row, it can be observed the same cells stained with DAPI (blue) and Alexa fluor 488 (green) (nucleus and cytoskeleton stain respectively) under UV irradiation.

6.6 Conclusions

New hybrid systems, combining supramolecular photonic gels of naphthalimide derivatives and upconverting NaYF₄:Yb/Tm nanoparticles (UCNPs), have been prepared. The hybrid system reversibly, manipulates light as a result of an optical communication between the UCNPs and the photoactive gel network. Radiative excitation energy transfer occurs very efficiently, affording a transformation of the light emitted from the nanoparticles. The emitted radiation, in the range of the absorption of the gel chromophore, is complete removed and a new emission from the 1,8-naphthalimide acceptor is observed at 410 nm, resulting in a light harvesting process. This process is only active in the form of gel, as a result of the aggregation induced emissive properties of the supramolecular gelator. Reversible gel disassembly is promoted by temperature changes, getting a temperature regulated tuneable photonic soft material. Additionally, the interaction of the UCNPs with the self-assembled fibers fosters the emissive relaxation pathways versus non-radiative decays, originating an outstanding improvement of their emission efficiency. This effect can be tentatively ascribed to isolation of nanoparticles from the solvent upon interaction with the gel fibers, together with IR light scattering produced by the self-assembled gel network. Hybrid UCNPs-gel systems as those described here, are envisaged to be used in applications related to IR-based sensing or light manipulation in general, especially taking into account the possibility of their miniaturization in the form of micro/nanogels for biomedical applications.

Taking as starting point the previous approach, the synthesis of a new hybrid nanomaterial, which can be suspended in water (**Dye+UCNP@1** NGs), was developed with the idea of applying it in photodynamic therapy in deep tissues. Good dispersion and stabilization of oleic acid coated core-shell UCNPs was achieved by incorporation into organic nanoparticles (nanogels). Hybrid nanomaterials showed sizes in the range of 60 to 200 nm and a good loading ability of hydrophobic photosensitizer hypericin (5% w/w) and Nile Red dye. The

colocalization of nanoparticles within the nanogel and the photosensitizer (or dye) was detected by fluorescence optical microscopy and allowed an efficient energy transfer upon IR irradiation. This energy transfer has been detected by fluorescence spectroscopy, by means of the decrease of the green signal at 540 nm of the UCNPs and the rising of the signal at 600 nm from hypericin (or Nile Red). The singlet oxygen production upon IR irradiation further confirms the energy transfer process and reveals very interesting potential uses as photodynamic agents in deep tissues. Preliminary studies of cell internalization also revealed the cellular uptake of the nanomaterials and further experiments are currently being carried out in NUS for determination of their PDT performance against MB49 mouse bladder urothelial carcinoma cell line.

6.7 Experimental section

- 6.7.1 Synthesis
- 6.7.2 Upconverting nanoparticle preparation
- 6.7.3 Hybrid UCNPs-1 gel preparation
- 6.7.4 Fluorescence spectroscopy
- 6.7.5 UV-Vis spectroscopy
- 6.7.6 Transmission electron microscopy

6.7.1 Synthesis

Compound **6.1** is the same as **4.1** and compound **6.2** is the same as **3.1** (also mentioned in the text as **1-NH2**) (see section 4.5.1 and section 3.5.1 respectively for description). Compounds have been renumbered for coherence with the chapter numeration. Experimental details of synthetic procedures were described in the experimental section of Chapter 3 (Section 3.5.1).

6.7.2 Upconverting nanoparticle preparation

Details regarding UCNPs preparation are provided either from the Dr. Beatriz Julian's group at University Jaume I, which prepared them following a simple two-phase solvothermal procedure reported in literature,^{61,62} or from Zhang's group.³³

Nanocrystals were synthesized following a two-step solvothermal procedure reported in literature, as follows.⁶¹ In a first step, a mixture of lanthanide stearates as the precursor was prepared. Typically, 4 mmol of $\text{Y}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$, 0.9 mmol of $\text{Yb}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$, 0.025 mmol of $\text{Tm}(\text{NO}_3)_3 \cdot \text{H}_2\text{O}$ and 15 mmol of stearic acid were dissolved under reflux in 100 mL of absolute ethanol. Subsequently, a solution of 15 mmol NaOH in 5 mL of water and 10 mL of ethanol were dropwise injected into the reaction vessel. After 30 minutes of refluxing under fast

stirring, the hot precipitate was filtered under vacuum, washed with abundant water and EtOH, and dried at 75 °C overnight.

1.9156 g of the Y-doped stearate precursor was emulsified in 30 mL EtOH, 20 mL OAc and 10 mL Milli-Q water previously stirred, until clear solution. Then, 0.84 g of NaF was added as source of sodium and fluorine ions. The whole mixture was transferred in a Teflon-lined 125 mL autoclave, sealed and solvothermally treated at 200 °C for 48 h. Once the autoclave was cooled to room temperature, the precipitate was collected by centrifugation and then washed twice with absolute ethanol, once with water to remove any excess of NaF and a last washing with acetone to make the particles dry quicker. The powders were finally dried in air at 75 °C, collected and stored for further treatments.

6.7.3 Hybrid UCNPs-1 gel preparation

20 mg of UCNPs were suspended in 2 mL of *n*-butanol and sonicated for 5 min in a screw-capped vial (diameter = 2 cm). Then 6×10^{-3} mmol of **1** were added and the closed system heated to 100°C until its complete solubilisation. The system was allowed to rest at room temperature for 10 minutes and the gel was formed. For measurements in spectrofluorometer, the hot solution was transferred to a cuvette and allowed to cool down for 10 minutes.

6.7.4 Fluorescence spectroscopy

Irradiation at 980 nm was achieved with a 1 W lasing power with a continuous diode laser. Luminescence studies of pure UCNPs were carried out initially in vials using an optical fiber connected to a spectrofluorometer. Additional experiments on the hybrid system were carried out using the setup shown below using a spectrofluorometer JASCO FP-8300 with peltier temperature control (ETC-815), using 10 mm pathlength and 3 mL quartz cuvettes. Temperature experiments were carried out *in situ* using the peltier temperature control module. Each increment of temperature was followed of 5 minutes of stabilization upon the temperature was reached.

6.7.5 UV-Vis spectroscopy

UV-Vis measurements were carried out in a JASCO V-630 spectrophotometer equipped with a Peltier accessory ETCS-761 at 25 °C, unless otherwise is indicated. The samples were placed inside 1 cm suprasil quartz cuvettes for diluted solutions and 1 mm path length for optically dense solutions and gels. Data interval acquisition was set to $\Delta\lambda = 1$ nm, with UV-vis bandwidth 1.5 nm and scan speed set at 400 nm/min.

6.7.6 Transmission electron microscopy

Transmission electron micrographs were taken on a JEOL 2100 microscope with thermionic gun LaB₆ 200 KV equipped with Gatan Orius high resolution CCD camera. TEM samples were prepared over 200 mesh Formvar carbon copper grids by putting a little portion of gel on top of the grid and then incubated for 5 minutes. The excess of gel was removed with filter paper by capillarity. Grids were observed without staining the samples.

6.8 References

- (1) Currie, H. A.; Patwardhan, S. V.; Perry, C. C.; Roach, P.; Shirtcliffe, N. J. In *Hybrid Materials: Synthesis, Characterization, and Applications*; 2006; pp 255–299.
- (2) Auzel, F. *Chem. Rev.* **2004**, *104* (1), 139.
- (3) Chivian, J. S.; Case, W. E.; Eden, D. D. *Appl. Phys. Lett.* **1979**, *35* (2), 124.
- (4) Parker, C. A. *Trans. Faraday Soc.* **1964**, *60* (0), 1998.
- (5) Singh-Rachford, T. N.; Castellano, F. N. *Coord. Chem. Rev.* **2010**, *254* (21–22), 2560.
- (6) Simon, Y. C.; Weder, C. J. *Mater. Chem.* **2012**, *22* (39), 20817.
- (7) Duan, P.; Yanai, N.; Nagatomi, H.; Kimizuka, N. J. *Am. Chem. Soc.* **2015**, *137* (5), 1887.
- (8) Ajayaghosh, A.; Praveen, V. K.; Vijayakumar, C. *Chem. Soc. Rev.* **2008**, *37* (1), 109.
- (9) Calzaferri, G.; Huber, S.; Maas, H.; Minkowski, C. *Angew. Chem. Int. Ed.* **2003**, *42* (32), 3732.
- (10) Choi, M.-S.; Yamazaki, T.; Yamazaki, I.; Aida, T. *Angew. Chem. Int. Ed.* **2004**, *43* (2), 150.
- (11) Nakashima, T.; Kimizuka, N. *Adv. Mater.* **2002**, *14* (16), 1113.
- (12) Vadrucci, R.; Weder, C.; Simon, Y. C. *Mater. Horiz.* **2015**, *2* (1), 120.
- (13) Haase, M.; Schäfer, H. *Angew. Chemie - Int. Ed.* **2011**, *50* (26), 5808.
- (14) Heer, S.; Lehmann, O.; Haase, M.; Güdel, H.-U. *Angew. Chemie Int. Ed.* **2003**, *42* (27), 3179.
- (15) Yan, C.; Zhao, H.; Perepichka, D. F.; Rosei, F. *Small* **2016**, *12* (29), 3888.
- (16) Liu, Y.; Lu, Y.; Yang, X.; Zheng, X.; Wen, S.; Wang, F.; Vidal, X.; Zhao, J.; Liu, D.; Zhou, Z.; Ma, C.; Zhou, J.; Piper, J. A.; Xi, P.; Jin, D. *Nature* **2017**, *543* (7644), 229.
- (17) Krämer, K. W.; Biner, D.; Frei, G.; Güdel, H. U.; Hehlen, M. P.; Lüthi, S. R. *Chem. mater.* **2004**, *16*, 1244.
- (18) Wang, F.; Liu, X. *Chem. Soc. Rev.* **2009**, *38* (4), 976.
- (19) Naccache, R.; Yu, Q.; Capobianco, J. A. *Adv. Opt. Mater.* **2015**, *3* (4), 482.
- (20) Wang, F.; Liu, X. *J. Am. Chem. Soc.* **2008**, *130* (17), 5642.
- (21) Page, R. H.; Schaffers, K. I.; Waide, P. A.; Tassano, J. B.; Payne, S. A.; Krupke, W. F.; Bischel, W. K. *J. Opt. Soc. Am. B* **1998**, *15* (3), 996.
- (22) Wang, F.; Banerjee, D.; Liu, Y.; Chen, X.; Liu, X. *Analyst* **2010**, *135* (8), 1839.

- (23) Corstjens, P.; Zuiderwijk, M.; Brink, A.; Li, S.; Feindt, H.; Niedbala, R. S.; Tanke, H. *Clin. Chem.* **2001**, *47* (10), 1885.
- (24) Wilchek, M.; Bayer, E. A. *Anal. Biochem.* **1988**, *171* (1), 1.
- (25) Bayer, E. A.; Wilchek, M. *J. Chromatogr. A* **1990**, *510*, 3.
- (26) Wang, L.; Yan, R.; Huo, Z.; Wang, L.; Zeng, J.; Bao, J.; Wang, X.; Peng, Q.; Li, Y. *Angew. Chemie* **2005**, *117* (37), 6208.
- (27) Chatterjee, D.; Rufaihah, A.; Zhang, Y. *Biomaterials* **2008**, *29* (7), 937.
- (28) Jiang, S.; Zhang, Y. *Langmuir* **2010**, *26* (9), 6689.
- (29) Lucky, S. S.; Soo, K. C.; Zhang, Y. *Chem. Rev.* **2015**, *115* (4), 1990.
- (30) Peng, H.-Q.; Niu, L.-Y.; Chen, Y.-Z.; Wu, L.-Z.; Tung, C.-H.; Yang, Q.-Z. *Chem. Rev.* **2015**, *115* (15), 7502.
- (31) Zhu, X.; Feng, W.; Chang, J.; Tan, Y.-W.; Li, J.; Chen, M.; Sun, Y.; Li, F. *Nat. Commun.* **2016**, *7*, 10437.
- (32) Lim, E.; Kim, T.; Paik, S.; Haam, S.; Huh, Y.; Lee, K. *Chem. Rev.* **2015**, *115* (1), 327.
- (33) Idris, N. M.; Jayakumar, M. K. G.; Bansal, A.; Zhang, Y. *Chem. Soc. Rev.* **2015**, *44* (6), 1449.
- (34) Zhang, P.; Steelant, W.; Kumar, M.; Scholfield, M. *J. Am. Chem. Soc.* **2007**, *129* (15), 4526.
- (35) Idris, N. M.; Gnanasammandhan, M. K.; Zhang, J.; Ho, P. C.; Mahendran, R.; Zhang, Y. *Nat. Med.* **2012**, *18* (10), 1580.
- (36) Shan, J.; Budijono, S. J.; Hu, G.; Yao, N.; Kang, Y.; Ju, Y.; Prud'Homme, R. K. *Adv. Funct. Mater.* **2011**, *21* (13), 2488.
- (37) Cui, S.; Yin, D.; Chen, Y.; Di, Y.; Chen, H.; Ma, Y.; Achilefu, S.; Gu, Y. *ACS Nano* **2013**, *7* (1), 676.
- (38) Cui, S.; Chen, H.; Zhu, H.; Tian, J.; Chi, X.; Qian, Z.; Achilefu, S.; Gu, Y. *J. Mater. Chem.* **2012**, *22*, 4861.
- (39) Fan, W.; Shen, B.; Bu, W.; Chen, F.; He, Q.; Zhao, K.; Zhang, S.; Zhou, L.; Peng, W.; Xiao, Q.; Ni, D.; Liu, J.; Shi, J. *Biomaterials* **2014**, *35*, 8992.
- (40) Alemán, J. V.; Chadwick, A. V.; He, J.; Hess, M.; Horie, K.; Jones, R. G.; Kratochvíl, P.; Meisel, I.; Mita, I.; Moad, G.; Penczek, S.; Stepto, R. F. T. *Pure Appl. Chem.* **2007**, *79* (10), 1801.

- (41) Kickelbick, G.; Wiley InterScience (Online service). *Hybrid materials: synthesis, characterization, and applications*; Wiley - VCH, 2007.
- (42) Ashby, M. F.; Bréchet, Y. J. M. *Acta Mater.* **2003**, *51* (19), 5801.
- (43) Cametti, M.; Džolić, Z. *Chem. Commun.* **2014**, *50* (61), 8273.
- (44) van Herrikhuyzen, J.; George, S. J.; Vos, M. R. J.; Sommerdijk, N. A. J. M.; Ajayaghosh, A.; Meskers, S. C. J.; Schenning, A. P. H. J. *J. Am. Chem. Soc.* **2007**, *129*, 1825.
- (45) Mandal, S. K.; Brahmachari, S.; Das, P. K. *Chempluschem* **2014**, *79* (12), 1733.
- (46) Guan, Y.; Chen, J.; Qi, X.; Chen, G.; Peng, F.; Sun, R. *Ind. Eng. Chem. Res.* **2015**, *54* (30), 7393.
- (47) Dutta, S.; Kar, T.; Mandal, D.; Das, P. K. *Langmuir* **2013**, *29* (1), 316.
- (48) Nanda, J.; Biswas, A.; Adhikari, B.; Banerjee, A. *Angew. Chemie Int. Ed.* **2013**, *52* (19), 5041.
- (49) Chakrabarty, A.; Maitra, U.; Das, A. D. *J. Mater. Chem.* **2012**, *22* (35), 18268.
- (50) Nanda, J.; Adhikari, B.; Basak, S.; Banerjee, A. *J. Phys. Chem. B* **2012**, *116* (40), 12235.
- (51) Coates, I. A.; Smith, D. K. *J. Mater. Chem.* **2010**, *20* (32), 6696.
- (52) Das, D.; Maiti, S.; Brahmachari, S.; Das, P. K. *Soft Matter* **2011**, *7* (16), 7291.
- (53) Chakrabarty, A.; Maitra, U. *J. Phys. Chem. B* **2013**, *117* (26), 8039.
- (54) Sangeetha, N. M.; Bhat, S.; Raffy, G.; Belin, C.; Loppinet-Serani, A.; Aymonier, C.; Terech, P.; Maitra, U.; Desvergne, J.-P.; Del Guerzo, A. *Chem. Mater.* **2009**, *21* (14), 3424.
- (55) Das, R. K.; Bhat, S.; Banerjee, S.; Aymonier, C.; Loppinet-Serani, A.; Terech, P.; Maitra, U.; Raffy, G.; Desvergne, J.-P.; Del Guerzo, A. *J. Mater. Chem.* **2011**, *21* (8), 2740.
- (56) Yamamoto, K.; An, Z.; Saito, N.; Yamaguchi, M. *Chem. - A Eur. J.* **2013**, *19* (32), 10580.
- (57) Wadhavane, P. D.; Galian, R. E.; Izquierdo, M. A.; Aguilera-Sigalat, J.; Galindo, F.; Schmidt, L.; Burguete, M. I.; Pérez-Prieto, J.; Luis, S. V. *J. Am. Chem. Soc.* **2012**, *134*, 20554.
- (58) Wadhavane, P. D.; Izquierdo, M. A.; Galindo, F.; Burguete, M. I.; Luis, S. V. *Soft Matter* **2012**, *8*, 4373.
- (59) Wu, J.; Tian, Q.; Hu, H.; Xia, Q.; Zou, Y.; Li, F.; Yi, T.; Huang, C. *Chem. Commun.* **2009**, 0 (27), 4100.
- (60) Tong, X.; Xiang, J.; Shi, F.; Zhao, Y. *Adv. Opt. Mater.* **2016**, *4* (9), 1392.

- (61) Wang, M.; Liu, J.-L.; Zhang, Y.-X.; Hou, W.; Wu, X.-L.; Xu, S.-K. *Mater. Lett.* **2008**, *63* (2), 325.
- (62) Gonell, F.; Haro, M.; Sánchez, R. S.; Negro, P.; Mora-Seró, I.; Bisquert, J.; Julián-López, B.; Gimenez, S. *J. Phys. Chem. C* **2014**, *118*, 11279.
- (63) Srinivasan, S.; Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. *Angew. Chem. Int. Ed.* **2008**, *47* (31), 5746.
- (64) Ray, S.; Das, A. K.; Banerjee, A. *Chem. Commun.* **2006**, *26*, 2816.
- (65) Yi, G.-S.; Chow, G.-M. *Chem. Mater.* **2007**, *19* (3), 341.
- (66) Wu, T.; Boyer, J.-C.; Barker, M.; Wilson, D.; Branda, N. R. *Chem. Mater.* **2013**, *25* (12), 2495.
- (67) Riittamäki, T.; Hyppänen, I.; Kankare, J.; Soukka, T. *J. Phys. Chem. C* **2011**, *115* (36), 17736.
- (68) Sun, L.-N.; Peng, H.; Stich, M. I. J.; Achatz, D.; Wolfbeis, O. S. *Chem. Commun.* **2009**, *108* (33), 5000.
- (69) Boyer, J.-C.; Manseau, M.-P.; Murray, J. I.; van Veggel, F. C. J. M. *Langmuir* **2010**, *26* (2), 1157.
- (70) Liu, C.; Wang, H.; Li, X.; Chen, D. *J. Mater. Chem.* **2009**, *19* (21), 3546.
- (71) Yan, X.; Cui, Y.; He, Q.; Wang, K.; Li, J. *Chem. Mater.* **2008**, *20* (4), 1522.
- (72) Yan, X.; Cui, Y.; Qi, W.; Su, Y.; Yang, Y.; He, Q.; Li, J. *Small* **2008**, *4* (10), 1687.
- (73) Chacko, R. T.; Ventura, J.; Zhuang, J.; Thayumanavan, S. *Adv. Drug Deliv. Rev.* **2012**, *64* (9), 863.
- (74) Ryu, J.-H.; Chacko, R. T.; Jiwanich, S.; Bickerton, S.; Prakash Babu, R.; Thayumanavan, S. *J. Am. Chem. Soc.* **2010**, *132*, 17227.
- (75) Torchilin, V. P. *J. Control. Release* **2001**, *73* (2–3), 137.

Chapter 7
**Reactivity of 4-amino-
1,8-naphthalimide
gelators with NO**

7 Reactivity of ANI gelators with NO

7.1 Motivation

Nitric oxide (NO) is, chemically speaking, an austere molecule, but biologically speaking, it contains a lot of power in its structure. Nitric oxide is a very effective agent in the regulation of complex cellular functions and, most likely, played a crucial role in the early stages of the evolution of life. It is thought that in the first stages of life, NO may have played a role as a defense mechanism for primitive microorganisms in order to fight the oxidative stress provoked by O₃ present in the early atmosphere. Mammalian cells produce NO from L-arginine and the origin of this biosynthesis might be related to the essential process of nitrification/denitrification in prokaryotic cells. In last few decades, the interest in NO has blossomed. Nitric oxide was considered the "Molecule of the Year" in 1992 by the journal Science and the 1998 Nobel Prize in Physiology and Medicine dealt with the discovering of nitric oxide's role a signalling molecule.¹⁻⁴ After more than a century of use of nitrate-derived drugs such as nitroglycerine and amyl nitrite, it was relatively recently found that these species are precursors of nitric oxide.

7.2 Introduction

Nitric oxide (NO) is a molecule playing numerous roles at the biological level.⁵ For example, it has been identified as a messenger molecule,⁶ a vasodilator⁷ and antibacterial agent.^{8,9} NO plays a crucial role in many important systems of mammals such as platelets and white cells stabilization, regulation of the cardiac contractility, ventilation in lungs, endocrine secretion, glomerular perfusion, rennin secretion and cell-to-cell communication.¹⁰ Therefore, possible alterations on the production or regulation NO can cause several disorders in body operation. For instance, NO has been recently associated to disorders such as cancer¹¹ and Alzheimer's disease.¹² Hence, its detection by instrumental techniques is of paramount importance, not only to develop diagnostic tools but also for the understanding of the biochemical mechanisms of action of this elusive molecule. A series of approaches have been followed to analyze NO in biological samples such as the use of electron spin resonance¹³

electrochemistry¹⁴ or fluorescence spectroscopy.¹⁵ The later technique has unique advantages such as high sensitivity and spatiotemporal resolution.¹⁶ A great number of NO fluorescent probes have been described in the literature so far^{17–34} and a series of excellent reviews summarize different chemical strategies followed by researchers in this field.^{15,35–38} Probably, the most frequently used strategy to design and synthesize a fluorescent sensor for NO is the utilization of probes containing the *o*-phenylenediamine moiety. The reactivity of this type of compounds towards nitric oxide in aerated medium is known to yield a triazole aromatic derivative, whose optical properties are well distinct from the original diamine molecule (Figure 1).

Figure 1. Representative example structures of NO turn-on sensors based on *ortho*-phenylenediamine moiety designed by **A)** Sasaki¹⁹ **(C)** Kojima¹⁷ and **(D)** Galindo.²⁰ **B)** Fluorescence turn-on spectra upon NO addition of compound designed by Sasaki.

However, although this approach has been extremely useful in the past for elucidating the mechanisms of action of NO, has some disadvantages. One of such drawbacks is the complexity of the synthetic routes devised to make the sensor. Another one is the selectivity issue, since it has been reported in some cases that molecules such as dehydroascorbic acid

(DHA) and glyoxal lead to emissive products that can be wrongly interpreted as NO positive assays.^{39–43} An alternative consists in the use of aromatic monoamine compounds, which react to produce diazonium salts and subsequent deamination under certain circumstances. This deamination strategy has been already explored in a limited number of cases. For instance, Wang and coworkers developed a fluorescein derivative bearing a single amine group, which upon reaction with NO in aerated aqueous medium led to a deaminated product with strong fluorescence (Figure 2).⁴⁴

Figure 2. Single aromatic amine approach for NO-turn on sensors based on fluorescein.⁴⁴

More recently, Guo and coworkers have reported bodipy and tricarbo-cyanine monoamine dyes that react with NO in aerated medium at a notable velocity, being the p-methoxy aniline group the susceptible moiety for deamination process.⁴⁵ Our group reported a series of dyes following analogous principles adapted to the pyrilium fluorophore.²⁴ The simplicity of introducing single amine groups in the sensing system is really advantageous from the synthetic point of view and it has allowed to produce alternative or non conventional NO sensing structures in an unsophisticated way, like for instance the metal-organic-framework (MOF) developed by the group of Gosh.⁴⁶ The same strategy is also valid for the detection of nitrite anion (NO_2^-) since this species, in acidic medium, is able to perform the conversion of the amine groups into the diazonium moiety. Thus, systems for the analysis of NO_2^- include,

for example, functionalized gold nanoparticle bearing aniline units⁴⁷ or upconversion nanoparticles loaded with the dye Neutral Red, which has a primary amine in its structure. The latter sensing mechanism is based in a very creative strategy that takes profit of the FRET between negatively charged UCNP (green signal from 500 to 550 nm) and positively charged ($-\text{NH}_3^+$) Neutral Red, which absorbs at this wavelength. The electrostatic attraction puts the particle-dye together and turns off the green signal due to efficient FRET. The presence of NO triggers the deamination reaction and subsequent disruption of FRET process (Figure 3).⁴⁸

Figure 3. A) Schematic illustration for the principle of detection of NO_2^- by FRET. B) Spectral mechanism for the detection of NO_2^- : the upconversion spectrum of UCNP (black line) and the UV-vis spectra of NR before (red line) and after (pink line) the reaction with NO_2^- . C) Evolution of upconversion spectra of the NR-UCNPs system ($\lambda_{\text{exc}} = 980 \text{ nm}$) with increasing NO_2^- concentrations. Inset: calibration curve of I_{539}/I_{654} versus NO_2^- concentration.⁴⁸

Considering the promising perspectives of this new approach for the development of nitric oxide and nitrite sensors, it seems clear that in the future a great number of probes based on monoaminated compounds could be developed.

One of the most popular fluorophores used so far in the area of chemical sensing and bioimaging is 4-amino-1,8-naphthalimide. It has been used to develop sensors and a number of materials with several functions.^{49–58} We envisaged that the amino group present in this fluorophore could be deaminated by reaction with NO in aerated medium (and, logically also

by acidic nitrite) and hence this moiety could be used for the development of optical probes for both NO and NO_2^- (Figure 4).

Figure 4. Hypothesis of deamination process induced by NO reaction.

In this chapter, we explore the feasibility of that idea by testing eight derivatives of 4-amino-1,8-naphthalimide (ANI), synthesized using different architectural motifs based on our previous experience on self-assembled systems (Figure 5).⁵⁹ The aim of testing such a structural variety was to determine the influence of the non-fluorogenic part of the molecule on the optical response of the system under study, in order to design future optimized fluorescent sensors based on this fluorophore. We also investigated the effect of aggregation on nitric oxide responsiveness.

Figure 5. Compounds under study

7.3 Aggregation behaviour

Three compounds were chosen for study their aggregation behaviour: **2**, **3** and **6**. Compound **2** showed no aggregation in the experimental conditions used (H₂O:DMSO 9:1, 10 μ M), giving transparent solutions even at concentrations in the milimolar range. On the other hand, compounds **3** and **6** form nanoparticles under those conditions, which could be detected both by dynamic light scattering (DLS) (Figure 6) and transmission electron microscopy (TEM) or scanning electron microscopy (SEM). Averaged size distributions by number obtained by DLS revealed monomodal distributions corresponding to nanoparticles with a diameter of 131 ± 5 nm in the case of compound **6** and 422 ± 14 nm in the case of compound **3**. TEM and SEM images of nanoparticles (Figure 7a and 7d) are in agreement with this observations showing spherical objects whose size is in agreement with that observed by DLS. In the case of compound **3**, the nanoparticles could be visualized by confocal laser scanning microscopy (CLSM), revealing a uniform distribution of rather monodisperse fluorescent particles (Figure 7b). It has to be mentioned that the formation of nanoparticles in aqueous media by the deaminated analogue of compound **3** has been recently described by us.⁶⁰ When moving from micromolar to milimolar concentration range compound **6** forms molecular gels composed of self-assembled fibrillar networks (Figure 7c) while compound **3** affords amorphous solids.

Figure 6. Size distribution by number (Dn) obtained by DLS analysis of compound **3** and **6** showing nanoparticles of 422 ± 14 nm, and 131 ± 5 nm respectively.

Figure 7. (a) TEM images of nanoparticles of **3** showing spherical nanoparticles of around 400 nm; **inset**: detail of one nanoparticle; (b) CLSM showing fluorescent nanoparticles of **3**. (c) TEM Images of gels of **6** showing fibrillar structures (d) SEM images of nanoparticles of **6**.

The analysis of the fluorescence of nanoparticles formed by **6** and **3** suggest that their arrangement of ANI units differ. As can be seen in Figure 8, the fluorescence maximum of **6** is at 546 nm (yellow light) while that of **3** is heavily shifted in a hypsochromic way to 507 nm (green light) showing additionally a shoulder. These differences can be ascribed tentatively to a different relative position of the ANI units in the aggregates.

Figure 8. Fluorescence spectra of **3** (continuous black line) and **6** (dashed red line) at 10 μM in $\text{H}_2\text{O}:\text{DMSO}$ (9/1). λ_{ex} = 433nm.

7.4 Reactivity towards NO and NO_2^-

Compounds **1-8** were dissolved in water with a 10 % of DMSO with a concentration of 10 μM . Their reactivity towards NO or NO_2^- was assayed. A preliminary test with **6** in the presence of NO excess (bubbled) was conducted, revealing a complete quenching of the fluorescence emission of this compound at 546 nm. The same results were obtained with nitrite 0.5 mM (in 2 % CH_3COOH). Comparison of the ^1H NMR spectrum of this reaction with spectrum obtained for the deaminated analogue **9**, prepared as reported elsewhere,⁶⁰ confirm that deamination was the main reaction. Deamination of **6** was also confirmed by mass spectrometry (Figure 9 right). Therefore, the reactivity towards NO of the ANI fluorophore in the compounds here described proceeds via deamination, as early reported for synthetic applications⁶¹⁻⁶³ and not following other reaction pathways that could take place such as hydroxylation.^{64,65}

Figure 9. ^1H NMR (left) and HRMS (right) of compound **6** before and after exposure to NO and its comparison with the model compound **9**.

Figure 10. Model compound.

Next, the reactivity of **1-8** towards NO was compared. To this end, 1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium (NONOate) was used as NO donor. To solutions/dispersions of **1-8** (10 μM), different amounts of NONOate were added and the response was monitored by steady state fluorescence spectroscopy. The intensity of emission at 546 nm was plotted against the concentration of released NO (assuming that 1.5 molecules of NO are generated by each molecule of NONOate).⁶⁶ The formation of the nitrosating specie nitrous anhydride (N_2O_3) is a requisite for the reaction to occur. For clarity, compounds **2**, **3** and **6** have been chosen to explain the results because they present three notably different aggregation behaviour and response upon exposure to NO. Figure 11a-b shows the fluorescence spectra recorded for **6** and **3**, respectively. Figure 11c shows the graphical representation of the variation of fluorescence intensity for increasing amounts of NO for **2**, **3** and **6**. Noticeably, while compounds **2** and **6** react with NO resulting in fluorescence quenching, compound **3** is found to be unreactive. As listed in Table 1, the limit of detection (LOD) of NO calculated from the graphs at Figure 11 for compounds **2** and **6** is respectively 0.5 and 2.0 μM . The 4-fold

difference can be linked to the reduced reactivity of the ANI in the nanoparticles formed by **6**. The other compounds studied showed the same behaviour as **6**, since they are prone to aggregate in the working conditions (Table 1 and Figure 12). However, there is a notable variability in the shape of the response curve depending on the structure of the molecule (since the studied systems differ only in the nature of the pendant organic residue). Hence, it can be inferred that the association between different molecules of a fluorescent compound is controlling the extent of deamination reaction.

Figure 11. Top: Representative variation of fluorescence spectra of compound **6** (a) and **3** (b) with the concentration of NO. **Inset:** vials under UV light (365 nm) upon exposure to different concentrations of NO. **Bottom:** Fluorescence intensity sigmoid fitting in response to NO exposure of studied compounds. $[1-3] = 10 \mu\text{M}$; $[\text{NO}] = 0, 0.37, 0.74, 1.8, 3.7, 7.5, 18.5, 37.5, 73.5, 111 \mu\text{M}$.

Regarding the intriguing reactivity difference between **3** and **6**, it seems that ANI units are hidden in the nanoparticles formed by **3** in such a way that reaction with NO cannot take place. The different fluorescence emission spectra obtained for **3** and **6** above mentioned (Figure 8) supports the different nature of the aggregates formed by these compounds. It has to be noticed that compound **3** is much more hydrophobic (ClogP = 7.7) than **6** (ClogP = 2.5) and, therefore, rather strong aromatic stacking interactions are most likely present in the aggregates.

Table 1. Characterization of studied compounds and LOD towards NO (NO₂)

Compound	Solvent	λ_{em} / nm	LOD* NO (NO ₂) / μ M
1	H ₂ O:DMSO (9:1)	544	2.5 (3.5)
2	H ₂ O:DMSO (9:1)	545	0.5
3	H ₂ O:DMSO (9:1)	507	Not sensitive
4	H ₂ O:DMSO (9:1)	544	5.0
5	H ₂ O:DMSO (9:1)	546	1.6
6	H ₂ O:DMSO (9:1)	546	2.0
7	H ₂ O	544	1.5 (9.7)
8	H ₂ O	543	1.0

*Errors in LOD are estimated to be 5 %

Figure 12. Fluorescence intensity sigmoidal fitting in response to NO exposure of studied compounds **1-8**; [sensors] = 10 μ M; [NO] = 0, 0.37, 0.74, 1.8, 3.7, 7.5, 18.5, 37.5, 73.5, 111 μ M.

7.5 Conclusions

The aggregation in aqueous media of three ANI derivatives, **2**, **3** and **6**, and their reactivity towards dissolved nitric oxide in aerated medium have been studied. DLS, electron microscopy and CLSM studies revealed that compounds **6** and **3** aggregate to form nanoparticles with diameters respectively of *ca.* 130 and 420 nm. It has been found that the ANI fluorophore experiences deamination upon exposure to NO in aerated aqueous systems, with the concomitant fluorescence turn-off process. Nanoparticle formation reduces the reactivity of **3**, in comparison to that of the non-aggregating specie **2**. Remarkably, in the case of **6** aggregation blocks the reactivity towards NO. This difference can be rationalized considering that the nanoparticles formed by **3** and **6** present rather different relative dispositions of the ANI units, as hinted by their notable different fluorescence spectra.

Further studies on the reactivity towards NO of eight structurally different compounds showed that this deamination mechanism is found to be dependent on the architecture of the molecule and its supramolecular organization. Systems with tendency to form self-assembled structures which orientate the reactive amine towards the aqueous solvent, display an attenuated reactivity (i.e. compounds **1**, **4**, **5**, **6**, **7** and **8**). Others are completely insensitive, that is the case of those whose reactive amine is completely buried inside hydrophobic regions (compound **3**). Nevertheless these prone to be solvated and independent from other neighboring molecules are more suitable for the deamination reaction (for instance, compound **2**). The sensitivity of the studied molecules lies within the micromolar range, which could be improved in a future work by activation of the intrinsically electron deficient 1,8-naphthalimide ring. Although the turn-off behavior is not the ideal one for a fluorescent probe, it could be reversed and converted into an off-on one by combination with a complementary fluorophore and using the Förster Resonance Energy Transfer (FRET) mechanism, as other sensors reported in the literature.^{6,59,67,68}

7.6 Experimental section

7.6.1 Synthesis of Glycine-valine ANI derivatives

Scheme 1. Synthetic route for compound **7.4**, **7.5**; *iii_a*) Cyclohexylamine or 1-aminopropane, THF, 4 h, 25 °C. *iii_b*) Et₃N, C₃H₅ClO₂, THF, Cbz-Gly-OH, 12h. *iv*) H₂, Pd/C, MeOH, 25 °C, 10 h. *v*) 4-nitro-1,8-naphthalic anhydride, CH₃OH, 75 °C, 8 h. *vi*) H₂, Pd/C, MeOH, 12 h.

Experimental details of synthetic procedures were described in the experimental section of Chapter 3. (Section 3.5.1)

Compound 7.9 = 4.14

NMR spectra were consistent with those described in the literature (described before, see compound 4.14). The compound has been renumbered for coherence with the chapter numeration.

Compound 7.10 = 4.15

NMR spectra were consistent with those described in the literature (described before, see compound 4.15). The compound has been renumbered for coherence with the chapter numeration.

Compound 7.11

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 7.83 (d, J = 7.6 Hz, 1H), 7.69 (d, J = 8.9 Hz, 1H), 7.45 (t, J = 5.8 Hz, 1H), 7.39 – 7.28 (m, 5H), 5.03 (s, 2H), 4.16 – 4.08 (m, 1H), 3.66 (d, J = 6.0 Hz, 2H), 3.57 – 3.47 (m, 1H), 1.94 – 1.83 (m, 1H), 1.76 – 1.59 (m, 5H), 1.53 (d, J = 12.4 Hz, 1H), 1.31 – 1.04 (m, 5H), 0.81 (dd, J = 11.3, 6.7 Hz, 6H). ^{13}C NMR (126 MHz, d_6 -DMSO) δ (ppm): 170.1, 169.3, 156.9, 137.5, 128.8, 128.2, 128.1, 65.9, 57.9, 47.9, 43.9, 32.9, 32.6, 31.4, 25.7, 25.0, 24.9, 19.5, 18.6.

Compound 7.12

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 7.89 (d, J = 7.8 Hz, 1H), 4.15 (t, J = 7.7 Hz, 1H), 3.60 – 3.43 (m, 1H), 3.10 (s, 2H), 1.87 (td, J = 13.6, 6.9 Hz, 1H), 1.67 (s, 4H), 1.54 (d, J = 12.3 Hz, 1H), 1.33 – 1.02 (m, 5H), 0.82 (t, J = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 172.9, 170.1,

57.2, 47.8, 45.0, 32.9, 32.6, 31.9, 25.7, 25.0, 24.9, 19.5, 18.6. HRMS (ESI-TOF, positive mode): m/z calcd. For $C_{13}H_{25}N_3O_2^+$ 256.2025; found 256.2020 $[M+H]^+$ (Δ = 2.0 ppm).

Compound 7.13

1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 8.72 (d, J = 8.5 Hz, 1H), 8.63 (t, J = 7.8 Hz, 2H), 8.55 (d, J = 7.9 Hz, 1H), 8.11 (t, J = 8.0 Hz, 2H), 7.58 (d, J = 7.3 Hz, 1H), 4.77 (s, 2H), 4.12 (t, J = 7.9 Hz, 1H), 1.95 (td, J = 12.8, 5.5 Hz, 1H), 1.68 (dd, J = 32.1, 12.6 Hz, 4H), 1.53 (d, J = 13.4 Hz, 1H), 1.34 – 1.06 (m, 6H), 0.86 (d, J = 6.7 Hz, 6H). ^{13}C NMR (126 MHz, d_6 -DMSO) δ (ppm): 170.1, 166.6, 150.1, 132.6, 130.8, 130.4, 129.5, 124.6, 58.7, 48.1, 42.9, 32.5, 31.0, 25.5, 25.0, 19.2, 18.6. HRMS (ESI-TOF, positive mode): m/z calcd. $C_{25}H_{29}N_4O_6^+$ 503.1907; found 503.1898 $[M+H]^+$ (Δ = 1.8 ppm).

Compound 7.14

1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.75 (d, J = 8.7 Hz, 1H), 8.70 – 8.53 (m, 3H), 8.13 (t, J = 8.0 Hz, 1H), 7.80 (t, J = 5.5 Hz, 1H), 5.01 (d, J = 9.1 Hz, 1H), 3.09 – 2.82 (m, 2H), 2.81 – 2.63 (m, 1H), 1.39 – 1.24 (m, 2H), 1.18 (d, J = 6.4 Hz, 3H), 0.78 – 0.62 (m, 6H).

Compound 7.4

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.63 (d, J = 8.4 Hz, 1H), 8.42 (d, J = 7.3 Hz, 1H), 8.18 (d, J = 8.4 Hz, 1H), 7.73 – 7.56 (m, 2H), 7.41 (s, 2H), 6.86 (d, J = 8.3 Hz, 1H), 5.02 (d, J = 9.2 Hz, 1H), 3.03 – 2.83 (m, 2H), 2.73 (dd, J = 15.4, 6.8 Hz, 1H), 1.40 – 1.24 (m, 2H), 1.15 (d, J = 6.3 Hz, 3H), 0.73 (t, J = 7.3 Hz, 3H), 0.59 (d, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 169.3, 164.5, 163.5, 153.1, 134.5, 131.5, 130.5, 129.6, 124.4, 122.6, 119.8, 108.6, 108.3, 58.8, 41.0, 26.7, 23.3, 22.6, 19.3, 11.8. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{20}\text{H}_{23}\text{N}_3\text{O}_3\text{Na}^+$ 376.1637; found 376.1638 $[\text{M}+\text{H}]^+$ (Δ =0.3 ppm).

Compound 7.5

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 8.63 (d, J = 8.4 Hz, 1H), 8.41 (d, J = 7.3 Hz, 1H), 8.21 – 8.13 (m, 2H), 7.76 (d, J = 7.9 Hz, 1H), 7.66 (t, J = 7.7 Hz, 1H), 7.45 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.69 (s, 2H), 4.12 (dd, J = 8.9, 7.0 Hz, 1H), 3.60 – 3.49 (m, 1H), 1.97 – 1.89 (m, 1H), 1.77 – 1.60 (m, 4H), 1.59 – 1.51 (m, 1H), 1.31 – 1.08 (m, 5H), 0.85 (d, J = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 170.1, 167.5, 164.2, 163.2, 153.4, 134.5, 131.6, 130.3, 130.1, 124.4, 122.1, 119.9, 108.7, 107.8, 58.2, 47.9, 42.4, 32.9, 32.6, 31.5, 25.7, 25.0, 24.9, 19.6, 18.7. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{25}\text{H}_{30}\text{N}_4\text{O}_4\text{Na}^+$ 473.2165; found 473.2168 $[\text{M}+\text{Na}]^+$ (Δ =0.6 ppm).

Compound 7.6 = 3.1 (see section 8.2) **3.1 = 7.6**

Compound **7.6** is the same as **3.1**. The compound has been renumbered for coherence with the chapter numeration.

7.6.2 Synthesis of no amino acid ANI derivatives

Scheme 7. Synthetic route for compound **1** and **3**; **v)** 4-nitro-1,8-naphthalic anhydride, CH_3OH , 75°C , 8 h.; **vj)** H_2 , Pd/C, MeOH, 12 h.

Compound 7.15

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 8.70 (d, $J = 8.7$ Hz, 1H), 8.63 (dd, $J = 10.5, 7.8$ Hz, 2H), 8.54 (d, $J = 8.0$ Hz, 1H), 8.10 (t, $J = 8.0$ Hz, 1H), 4.06 (t, $J = 7.3$ Hz, 2H), 1.72 – 1.60 (m, 2H), 1.40 – 1.30 (m, 4H), 1.29 – 1.16 (m, 14H), 0.85 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ

(ppm): 164.3, 163.3, 162.5, 149.7, 132.1, 130.5, 130.0, 129.0, 128.9, 127.1, 124.5, 123.3, 42.2, 40.5, 31.7, 29.4, 29.3, 29.3, 29.2, 29.1, 29.0, 29.0, 22.4, 14.2.

Compound 7.16

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 8.69 (d, J = 8.7 Hz, 1H), 8.60 (dd, J = 12.1, 7.6 Hz, 2H), 8.54 (d, J = 8.0 Hz, 1H), 8.08 (t, J = 8.0 Hz, 1H), 4.01 (d, J = 8.0 Hz, 2H), 1.72 – 1.62 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, d_6 -DMSO) δ (ppm): 163.4, 162.6, 149.6, 132.1, 130.6, 130.1, 129.2, 128.8, 127.1, 124.7, 123.2, 123.2, 42.0, 21.2, 11.8.

Compound 7.1

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.59 (d, J = 8.2 Hz, 1H), 8.40 (d, J = 7.1 Hz, 1H), 8.17 (d, J = 8.3 Hz, 1H), 7.62 (t, J = 7.7 Hz, 1H), 7.41 (s, 2H), 6.83 (d, J = 8.3 Hz, 1H), 3.97 (t, J = 6.9 Hz, 2H), 1.57 (s, 2H), 1.21 (d, J = 38.2 Hz, 19H), 0.81 (s, 3H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 164.2, 163.4, 153.1, 134.3, 131.4, 130.1, 129.7, 124.4, 122.3, 119.8, 108.6, 108.1, 31.8, 29.5, 29.5, 29.4, 29.4, 29.2, 29.2, 28.1, 27.0, 22.5, 14.4. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{24}\text{H}_{32}\text{N}_2\text{O}_2\text{Na}^+$ 403.2361; found 403.2364 [$\text{M}+\text{H}$] $^+$ (Δ = 0.7 ppm).

Compound 7.3

^1H NMR (500 MHz, d_6 -DMSO in presence of CH_3OH) δ (ppm): 8.60 (d, J = 8.3 Hz, 1H), 8.42 (d, J = 7.2 Hz, 1H), 8.19 (d, J = 8.4 Hz, 1H), 7.65 (t, J = 7.3 Hz, 1H), 7.40 (s, 2H), 6.84 (d, J = 8.4 Hz, 1H), 3.97 (t, J = 7.3 Hz, 2H), 1.68 – 1.55 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 163.8, 162.9, 133.9, 129.7, 129.2, 123.9, 121.8, 119.4, 108.2, 107.6, 40.7, 21.0, 11.4. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}^{15}\text{H}^{15}\text{N}^2\text{O}^+$ 255,2839; found 255.1133 $[\text{M}+\text{H}]^+$ (Δ = 0.4 ppm).

7.6.3 Synthesis of Glycine ANI derivatives

Scheme 8. Synthetic route for compound **7.2** *ii*) DMAP, 2-methoxyethan-1-ol, THF, r.t., 12h; *iv*) MeOH, Pd/C, H_2 , r.t., 10h; *v*) 4-Nitro-naphthalic anhydride, MeOH, 70°C, 8h; *vi*) MeOH, Pd/C, H_2 , r.t., 6h, *vii*) LiOH, $\text{CH}_3\text{OH}/\text{H}_2\text{O}$.

Compound 7.17

^1H NMR (300 MHz, CDCl_3) δ 7.40 – 7.28 (m, 1H), 5.25 (s, 1H), 5.13 (s, 1H), 4.41 – 4.24 (m, 1H), 4.03 (d, J = 5.6 Hz, 1H), 3.69 – 3.51 (m, 1H), 3.38 (s, 1H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 139.5, 136.1, 128.5, 128.2, 128.1, 118.1, 70.2, 67.2, 64.4, 59.0, 42.7.

Compound 7.18

^1H NMR (300 MHz, CDCl_3) δ (ppm): 4.29 (d, J = 4.6 Hz, 2H), 3.61 (d, J = 4.8 Hz, 2H), 3.48 (s, 2H), 3.39 (s, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ (ppm): 170.7, 70.4, 63.8, 59.0, 43.9. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_5\text{H}_{11}\text{NO}_3\text{Na}^+$ 157.071; found 157.0360 $[\text{M}+\text{Na}]^+$ (Δ = 0.8 ppm).

Compound 7.20

^1H NMR (500 MHz, CD_3OD) δ (ppm): 8.03 – 7.98 (m, 2H), 7.76 (d, J = 8.4 Hz, 1H), 7.14 (d, J = 7.2 Hz, 1H), 6.38 (d, J = 8.4 Hz, 1H), 3.87 – 3.83 (m, 2H), 3.19 – 3.14 (m, 2H), 2.89 (s, 3H), 2.87 – 2.84 (m, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ (ppm): 168.9, 164.6, 163.7, 153.40, 134.3, 131.4, 130.2, 129.1, 123.8, 121.5, 119.7, 108.3, 107.6, 69.9, 64.1, 57.7, 40.7. HRMS (ESI-TOF, positive mode): m/z calcd. For $\text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_5\text{Na}^+$ 351.0958; found 351.0960 $[\text{M}+\text{Na}]^+$ (Δ = 0.9 ppm).

Compound 7.2

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.67 (dd, J = 8.4, 1.0 Hz, 1H), 8.44 (dd, J = 7.3, 1.0 Hz, 1H), 8.20 (d, J = 8.4 Hz, 1H), 7.68 (dd, J = 8.3, 7.4 Hz, 1H), 7.55 (s, 2H), 6.87 (d, J = 8.4 Hz, 1H), 4.67 (s, 2H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 170.2, 164.0, 163.0, 159.8, 153.6, 134.7, 131.8, 130.2, 124.5, 121.9, 119.9, 108.8, 107.4, 41.4. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{14}\text{H}_{10}\text{N}_2\text{O}_4\text{Na}^+$ 293.0538; found 293.0536 [$\text{M}+\text{Na}$] $^+$ (Δ = 0.7 ppm).

7.6.4 Synthesis of Succinic acid-valine ANI derivatives.

Scheme 9. Synthetic route for compound **7.7** and **7.8**; *iii_a*) Cbz-Val-O-Suc, THF, r.t., 12h; *a*) TFA Seq., CH_2Cl_2 , r.t.; *v*) 4-Nitro-naphthalic anhydride, MeOH, 70°C , 8h; *iv*) MeOH, Pd/C, H_2 , r.t., 6h; *b*) Succinic anhydride, K_2CO_3 .

Compound 7.21

^1H NMR (500 MHz, CDCl_3) δ (ppm): 7.39 – 7.28 (m, 5H), 6.70 (s, 1H), 5.43 (d, J = 5.9 Hz, 1H), 5.10 (q, J = 12.2 Hz, 2H), 4.97 (s, 1H), 3.97 (dd, J = 8.5, 6.2 Hz, 1H), 3.42 – 3.31 (m, 2H), 3.25 (s, 2H), 2.65 (s, 2H), 2.19 – 2.07 (m, 1H), 0.93 (dd, J = 22.5, 6.8 Hz, 6H).

Compound 7.22

^1H NMR (500 MHz, CDCl_3) δ (ppm): 7.38 – 7.24 (m, 6H), 6.60 (s, 1H), 5.75 (d, J = 8.7 Hz, 1H), 5.08 (d, J = 13.7 Hz, 2H), 4.66 (s, 1H), 3.92 (dd, J = 8.7, 7.2 Hz, 1H), 3.32 – 3.21 (m, 1H), 3.13 – 2.94 (m, 2H), 2.64 (s, 4H), 2.14 – 2.01 (m, 1H), 1.53 – 1.37 (m, 14H), 1.29 (s, J = 20.7 Hz, 4H), 0.92 (dd, J = 10.1, 6.8 Hz, 6H).

Compound 7.23

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 7.89 (s, 1H), 7.44 – 7.17 (m, 6H), 5.02 (s, 2H), 3.78 (t, J = 7.7 Hz, 1H), 3.12 – 2.96 (m, 3H), 2.61 – 2.52 (m, 2H), 2.02 – 1.80 (m, 1H), 0.83 (d, J = 6.0 Hz, 7H). ^{13}C NMR (126 MHz, d_6 -DMSO) δ (ppm): 171.6, 156.6, 137.6, 128.8, 128.2, 128.1, 65.8, 61.0, 60.9, 42.8, 41.8, 30.6, 19.7, 18.7.

Compound 7.24

^1H NMR (500 MHz, d_6 -DMSO) δ (ppm): 7.96 (s, 1H), 7.44 – 7.24 (m, 5H), 7.22 (d, J = 8.1 Hz, 1H), 5.03 (d, J = 13.5 Hz, 2H), 3.83 – 3.76 (m, 1H), 3.14 – 2.92 (m, 2H), 2.54 (s, 2H), 1.92 (d, J = 6.4 Hz, 1H), 1.36 (s, 4H), 1.23 (s, 4H), 0.83 (s, 6H).

Compound 7.25

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.69 (d, J = 8.6 Hz, 1H), 8.64 – 8.50 (m, 3H), 8.08 (t, J = 7.9 Hz, 2H), 7.40 – 7.23 (m, 5H), 7.06 (d, J = 8.9 Hz, 1H), 4.95 (q, J = 12.6 Hz, 2H), 4.20 – 4.09 (m, 2H), 3.75 – 3.67 (m, 1H), 3.60 – 3.43 (m, 2H), 1.91 – 1.75 (m, 1H), 0.72 (dd, J = 19.6, 6.7 Hz, 6H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 171.9, 163.6, 162.8, 156.5, 149.6, 137.5, 132.1, 130.6, 130.0, 129.1, 128.9, 128.8, 128.2, 128.0, 127.3, 124.7, 123.4, 123.2, 65.7, 60.6, 40.5, 36.5, 30.5, 19.8, 18.3. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{27}\text{H}_{26}\text{N}_4\text{O}_7\text{Na}^+$ 541.1699; found 541.1708 [$\text{M}+\text{Na}$] $^+$ (Δ = 1.5 ppm).

Compound 7.26

^1H NMR (400 MHz, d_6 -DMSO) δ (ppm): 8.74 – 8.68 (m, 5H), 8.64 (t, J = 7.7 Hz, 2H), 8.52 (d, J = 8.0 Hz, 1H), 8.08 (t, J = 9.1 Hz, 1H), 7.64 (s, 1H), 7.40 – 7.22 (m, 5H), 6.78 (s, 1H), 5.04 (s, 2H), 4.09 (t, J = 7.4 Hz, 2H), 3.84 (dd, J = 8.8, 6.9 Hz, 1H), 3.08 (ddd, J = 25.6, 13.0, 6.8 Hz, 2H), 2.03 – 1.90 (m, 1H), 1.75 – 1.63 (m, 2H), 1.51 – 1.31 (m, 6H), 0.87 (t, J = 6.2 Hz, 6H). ^{13}C NMR (101 MHz, d_6 -DMSO) δ (ppm): 171.3, 163.4, 162.6, 149.7, 137.6, 132.1, 130.4, 130.0, 129.0, 128.6, 128.0, 127.9, 127.1, 127.0, 124.4, 124.1, 123.4, 121.3, 118.5, 65.9, 61.0, 40.5, 38.9, 30.8, 29.3, 27.8, 26.6, 26.5, 19.4, 18.4.

Compound 7.7

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.61 (dd, J = 8.4, 0.9 Hz, 1H), 8.43 (dd, J = 7.3, 0.9 Hz, 1H), 8.19 (d, J = 8.4 Hz, 1H), 7.85 (dd, J = 10.2, 7.3 Hz, 2H), 7.65 (dd, J = 8.3, 7.4 Hz, 1H), 7.43 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.08 (dd, J = 8.9, 6.9 Hz, 1H), 4.04 – 3.92 (m, 2H), 3.15 – 2.91 (m, 2H), 2.41 (s, 4H), 1.93 (dq, J = 13.5, 6.7 Hz, 1H), 1.64 – 1.51 (m, 2H), 1.45 – 1.23 (m, 6H), 0.82 (dd, J = 6.8, 1.3 Hz, 6H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 174.4, 171.6, 171.2, 164.2, 163.4, 153.2, 134.4, 131.5, 130.1, 129.7, 124.4, 122.3, 119.8, 108.6, 108.1, 58.2, 38.8, 30.9, 30.4, 29.8, 29.4, 28.1, 26.8, 26.6, 19.7, 18.6. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{27}\text{H}_{35}\text{N}_4\text{O}_6^+$ 511.2557; found 511.2549 $[\text{M}+\text{H}]^+$ (Δ = 1.6 ppm).

Compound 7.8

^1H NMR (300 MHz, d_6 -DMSO) δ (ppm): 8.61 (d, J = 8.4 Hz, 1H), 8.41 (d, J = 7.1 Hz, 1H), 8.18 (d, J = 8.4 Hz, 1H), 7.98 (t, J = 5.8 Hz, 1H), 7.74 (d, J = 9.0 Hz, 1H), 7.70 – 7.59 (m, 1H), 7.42 (s, 2H), 6.85 (d, J = 8.4 Hz, 1H), 4.13 (dd, J = 12.1, 4.7 Hz, 2H), 4.06 (dd, J = 9.0, 6.5 Hz, 1H), 3.57 – 3.20 (m, 2H), 2.40 (s, 4H), 1.96 – 1.77 (m, 1H), 0.85 – 0.64 (m, 6H). ^{13}C NMR (75 MHz, d_6 -DMSO) δ (ppm): 174.4, 171.5, 171.5, 164.4, 163.5, 153.1, 134.4, 131.4, 130.3, 129.7, 124.4, 122.4, 119.8, 108.6, 108.2, 59.6, 58.1, 31.0, 30.7, 30.4, 29.8, 19.8, 18.3. HRMS (ESI-TOF, positive mode): m/z calcd. $\text{C}_{23}\text{H}_{26}\text{N}_4\text{O}_6\text{Na}^+$ 477.1750; found 477.1745 $[\text{M}+\text{Na}]^+$ (Δ = 1.0 ppm).

7.6.5 Materials

Commercial reagents 1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium (NONOate), NaNO_2 and all solvents have been used as received. NMR spectra were recorded at 400 MHz (^1H NMR) and 101 MHz (^{13}C NMR) at 30 °C. Mass spectra were run in QTOF electrospray (ESMS) mode.

7.6.6 Fluorescence and UV-Vis spectroscopy

Solutions and suspensions were measured in a JASCO FP-8300 spectrofluorometer using 3 mL quartz cuvettes with 10 mm path length at 25 °C. For UV-Vis measurements, JASCO V-630 was used.

7.6.7 NO/ NO_2^- Sensing

The procedure to determine the sensitivity of the synthesized products against NO and NO_2^- was carried out by adding different amounts of concentrated stock solutions of 4-amino-1,8-naphthalimide derivatives in DMSO (1mM) to water (or aqueous solution of $\text{H}_2\text{O}/\text{DMSO}$ 9:1), in order to achieve a final concentration of 10 μM of sensors in aqueous solution. Stock solutions of NONOate and NaNO_2 (1.5 mM) in water were added to 3 mL of the previous sensor solution, finally obtaining different NO concentrations ($[\text{NO}] = 0, 0.37, 0.74, 1.65, 3.7, 7.5, 18.5, 37.5, 73.5$ and $111 \mu\text{M}$). The mixtures were left to stabilize for 8 hours and then the reactivity followed by fluorescence spectroscopy ($\lambda_{\text{ex}} = 420 \text{ nm}$, $\lambda_{\text{em}} = 540 \text{ nm}$). By plotting the fluorescence intensity vs NO concentration and fitting to a sigmoid curve (equation 1), the inflection (x_0) point can be taken as $[\text{NO}]_{50}$ that is the concentration at which the fluorescence is half of the initial. LOD was taken on the decrease onset of this sigmoid curves.

$$y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/dx}} - A_2$$

Sigmoid fit equation

7.6.8 Transmission electron microscopy (TEM)

TEM micrographs were taken on JEOL 2100 microscope with thermionic gun LaB₆ 200KV equipped with GatanOrion high resolution CCD camera. TEM samples were prepared over carbon formvar copper grids by putting a drop of suspensions and incubating for 5 minutes. The suspension excess was removed with filter paper and left overnight in a glass desiccator.

7.7 References

- (1) Koshland, D. E. *Science* **1992**, 258 (5090), 1861.
- (2) Furchgott, R. F. *Biosci. Reports Nobel Lect.* **1999**, 19 (4), 235.
- (3) Ignarro, L. J. *Angew. Chemie Int. Ed.* **1999**, 38 (13–14), 1882.
- (4) Murad, F. *Angew. Chemie Int. Ed.* **1999**, 38 (13–14), 1856.
- (5) Ignarro, L. J. *Nitric oxide : biology and pathobiology*; Academic Press, 2000.
- (6) Yuan, L.; Lin, W.; Zheng, K.; Zhu, S. *Acc. Chem. Res.* **2013**, 46 (7), 1462.
- (7) Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U. S. A.* **1987**, 84 (24), 9265.
- (8) McMullin, B. B.; Chittock, D. R.; Roscoe, D. L.; Garcha, H.; Wang, L.; Miller, C. C. *Respir. Care* **2005**, 50 (11), 1451.
- (9) Schairer, D. O.; Chouake, J. S.; Nosanchuk, J. D.; Friedman, A. J. *Virulence* **2012**, 3 (3), 271.
- (10) Feelisch, M.; Martin, J. F. *Trends Ecol. Evol.* **1995**, 10 (12), 496.
- (11) Xu, W.; Liu, L. Z.; Loizidou, M.; Ahmed, M.; Charles, I. G. *Cell Res.* **2002**, 12 (5–6), 311.
- (12) de la Torre, J. C.; Stefano, G. B. *Brain Res. Rev.* **2000**, 34 (3), 119.
- (13) Hogg, N. *Free Radic. Biol. Med.* **2010**, 49 (2), 122.
- (14) Privett, B. J.; Shin, J. H.; Schoenfisch, M. H. *Chem. Soc. Rev.* **2010**, 39 (6), 1925.
- (15) Nagano, T.; Yoshimura, T. *Chem. Rev.* **2002**, 102 (4), 1235.
- (16) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Springer US: Boston, MA, 2006.
- (17) Kojima, H.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Hirata, Y.; Nagano, T. *Angew. Chem. Int. Ed. Engl.* **1999**, 38 (21), 3209.
- (18) Kojima, H.; Hirotsu, M.; Nakatsubo, N.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Hirata, Y.; Nagano, T. *Anal. Chem.* **2001**, 73 (9), 1967.
- (19) Sasaki, E.; Kojima, H.; Nishimatsu, H.; Urano, Y.; Kikuchi, K.; Hirata, Y.; Nagano, T. *J. Am. Chem. Soc.* **2005**, 127 (11), 3684.
- (20) Galindo, F.; Kabir, N.; Gavrilovic, J.; Russell, D. A. *Photochem. Photobiol. Sci.* **2008**, 7 (1), 126.
- (21) Chen, Y.; Guo, W.; Ye, Z.; Wang, G.; Yuan, J. *Chem. Commun.* **2011**, 47 (22), 6266.
- (22) Yuan, L.; Lin, W.; Xie, Y.; Chen, B.; Zhu, S. *J. Am. Chem. Soc.* **2012**, 134 (2), 1305.

- (23) Marín, M. J.; Thomas, P.; Fabregat, V.; Luis, S. V.; Russell, D. A.; Galindo, F. *ChemBioChem* **2011**, *12* (16), 2471.
- (24) Beltrán, A.; Isabel Burguete, M.; Abánades, D. R.; Pérez-Sala, D.; Luis, S. V.; Galindo, F. *Chem. Commun.* **2014**, *50* (27), 3579.
- (25) Yu, H.; Xiao, Y.; Jin, L. *J. Am. Chem. Soc.* **2012**, *134* (42), 17486.
- (26) Lv, X.; Wang, Y.; Zhang, S.; Liu, Y.; Zhang, J.; Guo, W. *Chem. Commun.* **2014**, *50* (56), 7499.
- (27) Yu, H.; Jin, L.; Dai, Y.; Li, H.; Xiao, Y. *New J. Chem.* **2013**, *37* (6), 1688.
- (28) Sazinsky, M. H.; Lippard, S. J. *Acc. Chem. Res.* **2006**, *39*, 558.
- (29) Lim, M. H.; Wong, B. A.; Pitcock, W. H.; Mokshagundam, D.; Baik, M.-H.; Lippard, S. J. *J. Am. Chem. Soc.* **2006**, *128* (44), 14364.
- (30) Pluth, M. D.; Chan, M. R.; McQuade, L. E.; Lippard, S. J. *Inorg. Chem.* **2011**, *50* (19), 9385.
- (31) Mondal, B.; Kumar, P.; Ghosh, P.; Kalita, A. *Chem. Commun.* **2011**, *47* (10), 2964.
- (32) Desai, A. V.; Samanta, P.; Manna, B.; Ghosh, S. K. *Chem. Commun* **2015**, *51* (28), 6111.
- (33) Apfel, U.-P.; Buccella, D.; Wilson, J. J.; Lippard, S. J. *Inorg. Chem.* **2013**, *52* (6), 3285.
- (34) Yang, Y.; Seidlits, S. K.; Adams, M. M.; Lynch, V. M.; Schmidt, C. E.; Anslyn, E. V.; Shear, J. B. *J. Am. Chem. Soc.* **2010**, *132* (38), 13114.
- (35) Ye, X.; Rubakhin, S. S.; Sweedler, J. V. *Analyst* **2008**, *133* (4), 423.
- (36) McQuade, L. E.; Lippard, S. J. *Curr. Opin. Chem. Biol.* **2010**, *14* (1), 43.
- (37) Li, H.; Wan, A. *Analyst* **2015**, *140* (21), 7129.
- (38) Chen, X.; Wang, F.; Hyun, J. Y.; Wei, T.; Qiang, J.; Ren, X.; Shin, I.; Yoon, J. *Chem. Soc. Rev.* **2016**, *45* (10), 2976.
- (39) Ye, X.; Rubakhin, S. S.; Sweedler, J. V. *Analyst* **2008**, *133* (4), 423.
- (40) Kim, W.-S.; Xiaoying Ye; Rubakhin, S. S.; Sweedler, J. V. *Anal. Chem* **2006**, *78* (6), 1859.
- (41) Ye, X.; Kim, W.-S.; Rubakhin, S. S.; Sweedler, J. V. *Analyst* **2004**, *129* (12), 1200.
- (42) Zhang, X.; Kim, W.-S.; Hatcher, N.; Potgieter, K.; Moroz, L. L.; Gillette, R.; Sweedler, J. V. *J. Biol. Chem.* **2002**, *277* (50), 48472.
- (43) Shaheen, F.; Shmygol, A.; Rabbani, N.; Thornalley, P. J. *Biochem. Soc. Trans.* **2014**, *42* (2), 548.
- (44) Shiue, T.-W.; Chen, Y.-H.; Wu, C.-M.; Singh, G.; Chen, H.-Y.; Hung, C.-H.; Liaw, W.-F.;

- Wang, Y.-M. *Inorg. Chem.* **2012**, *51* (9), 5400.
- (45) Huo, Y.; Miao, J.; Li, Y.; Shi, Y.; Shi, H.; Guo, W. *J. Mater. Chem. B* **2017**, *5* (13), 2483.
- (46) Desai, A. V.; Samanta, P.; Manna, B.; Ghosh, S. K. *Chem. Commun.* **2015**, *51* (28), 6111.
- (47) Xiao, N.; Yu, C. *Anal. Chem.* **2010**, *82* (9), 3659.
- (48) Han, J.; Zhang, C.; Liu, F.; Liu, B.; Han, M.; Zou, W.; Yang, L.; Zhang, Z. *Analyst* **2014**, *139* (12), 3032.
- (49) Duke, R. M.; Veale, E. B.; Pfeffer, F. M.; Kruger, P. E.; Gunnlaugsson, T. *Chem. Soc. Rev.* **2010**, *39* (10), 3936.
- (50) Gunnlaugsson, T.; Lee, T. C.; Parkesh, R.; Kennedy, R.; Pfeffer, F. M.; Hussey, G. M.; Rice, T. E.; Fierke, C.; Herman, P. *Org. Biomol. Chem.* **2003**, *1* (19), 3265.
- (51) Ramachandram, B.; Saroja, G.; Sankaran, N. B.; Samanta, A. *J. Phys. Chem. B* **2000**, *104*, 11824.
- (52) Bonnet, C. S.; Devocelle, M.; Gunnlaugsson, T. *Chem. Commun.* **2008**, *0* (38), 4552.
- (53) Gunnlaugsson, T.; Kruger, P. E.; Lee, T. C.; Parkesh, R.; Pfeffer, F. M.; Hussey, G. M. *Tetrahedron Lett.* **2003**, *44*, 6575.
- (54) Ferreira, R.; Baleizão, C.; Muñoz-Molina, J. M.; Berberan-Santos, M. N.; Pischel, U. *J. Phys. Chem. A* **2011**, *115* (6), 1092.
- (55) Remón, P.; Parente Carvalho, C.; Baleizão, C.; Berberan-Santos, M. N.; Pischel, U. *ChemPhysChem* **2013**, *14* (12), 2717.
- (56) Abad, S.; Kluciar, M.; Miranda, M. A.; Pischel, U. *J. Org. Chem.* **2005**, *70* (25), 10565.
- (57) Koner, A. L.; Schatz, J.; Nau, W. M.; Pischel, U. *J. Org. Chem.* **2007**, *72*, 3889.
- (58) Kluciar, M.; Ferreira, R.; De Castro, B.; Pischel, U. *J. Org. Chem.* **2008**, *73*, 6079.
- (59) Felip-León, C.; Díaz-Oltra, S.; Galindo, F.; Miravet, J. F. *Chem. Mater.* **2016**, *28* (21), 7964.
- (60) Felip-León, C.; Cejudo-Marín, R.; Peris, M.; Galindo, F.; Miravet, J. F. *Langmuir* **2017**, *33* (39), 10322.
- (61) Burglova, K.; Okorochonkov, S.; Hlavac, J. *Org. Lett.* **2016**, *18* (14), 3342.
- (62) Itoh, T.; Matsuya, Y.; Nagata, K.; Ohsawa, A. *Tetrahedron Lett.* **1996**, *37* (24), 4165.
- (63) Nagano, T.; Takizawa, H.; Hirobe, M. *Tetrahedron Lett.* **1995**, *36* (45), 8239.
- (64) Hu, J.; Whittaker, M. R.; Quinn, J. F.; Davis, T. P. *Macromolecules* **2016**, *49* (7), 2741.
- (65) Adarsh, N.; Shanmugasundaram, M.; Ramaiah, D. *Anal. Chem.* **2013**, *85* (21), 10008.

- (66) Maragos, C. M.; Morley, D.; Wink, D. A.; Dunams, T. M.; Saavedra, J. E.; Hoffman, A.; Bove, A. A.; Isaac, L.; Hrabie, J. A.; Keefer, L. K. *J. Med. Chem.* **1991**, *34* (11), 3242.
- (67) Saura, A. V.; Marín, M. J.; Burguete, M. I.; Russell, D. A.; Galindo, F.; Luis, S. V. *Org. Biomol. Chem.* **2015**, *13* (28), 7736.
- (68) Saura, A. V.; Burguete, M. I.; Galindo, F.; Luis, S. V. *Org. Biomol. Chem.* **2017**, *15* (14), 3013.

Summary in catalan

1 Resum en català

1.1 Introducció general

Com hem arribat aquí? Com va aconseguir la matèria assolir una complexitat per arribar als nivells d'un organisme pensant? Com ha succeït això? Quines formes superiors de matèria complexa hi haurà en el futur? Per descomptat, no estem en disposició de respondre aquestes qüestions filosòfiques fonamentals perquè no s'han recollit dades suficients per omplir aquests buits crítics de coneixement. Tanmateix, diversos autors van coincidir en assenyalar un esdeveniment decisiu que podria haver portat al naixement d'aquest fascinant món biològic meravellosament organitzat: l'auto-assemblatge. Probablement, tot va sorgir arrel d'una rudimentària compartimentació auto-assemblada, és a dir, mitjançant la formació d'una membrana primitiva, vesícula o microsfera en un entorn hidròfob per aconseguir un medi extern i intern. Aquesta restricció física va ser clau per a la formació posterior de macromolècules cel·lulars.¹ L'auto-assemblatge molecular consisteix en l'associació espontània i autònoma d'un conjunt de molècules sense intervenció humana en estructures supramoleculares (més enllà de la molècula). Es tracta d'interaccions no covalents, com ara van der Waals, electrostàtiques, hidrofòbiques, de coordinació i d'enllaç d'hidrogen.² Es poden considerar dues etapes principals pel que fa a l'autoassemblatge i l'organització: (i) el reconeixement molecular per a l'unió selectiva dels components bàsics; (ii) disposició relativa correcta i creixement d'elongació cooperativa mitjançant unió seqüencial i eventualment jeràrquica de múltiples components.

Figura 1. Representació de l'auto-assemblatge molecular.

1.1.1 Gels moleculars

Els gels són un estat col·loïdal de la matèria en què una petita quantitat d'una microfase sòlida en forma de xarxa (gelant) és capaç d'immobilitzar el flux massiu d'una quantitat més gran de líquid. Aquesta separació de microfases és induïda per nucleació. Com a resultat, el material és comporta com un sòlid en la seva conducta reològica.^{3,4} En general, la singular organització d'autoensamblatge i la naturalesa de les forces cohesives mitjançant les quals es formen els gels moleculars, confereix a aquests materials propietats molt interessants, en contrast amb les organitzacions covalents: comportament dinàmic, alt grau d'ordre estructural, propietats d'autocuració i correcció d'errors, la capacitat de resposta a múltiples estímuls externs, fàcil síntesi de materials supramoleculars així com la fàcil funcionalització per introduir les funcionalitats desitjades a nivell molecular que es traduiran (o fins i tot milloraran) el gel.

Clàssicament, els gels s'han utilitzat en nombroses aplicacions industrials; són importants en àmbits com ara cosmètica, processament d'aliments, lubricació i hidrometal·lúrgia. Els gels també s'han utilitzat amb finalitats ambientals, per exemple, en el control i recuperació d'olis vessats o de cocció. A més, els gels s'han utilitzat en contextos militars com a combustibles aeronàutics i tecnologia d'explosius i s'utilitzen extensivament entre altres aplicacions.⁵

Una infinitat d'aplicacions interessants ha sorgit fruit dels extensos estudis sobre gels moleculars, que els revelen com a materials molt interessants. Per exemple, els gels moleculars s'han estudiat en una gran varietat de camps, incloent-hi plantilles per a materials durs,⁶ fotòniques, ferromagnetisme magnètic (basat en molècules) i electrònica (conducció electrònica o semiconducció de sistemes conjugats donant-acceptor),⁷ catàlisis,⁸ sensors (temperatura, humitat i (bio) agents químics),⁹ reconeixements moleculars i quirals,^{10,11} càrrega controlada i alliberament¹² i enginyeria tissular.¹³

Figura 2. Descripció general de les aplicacions per a gels moleculars.¹⁴

1.2 Plantejament, metodologia y objectius

L'objectiu general de la tesi és l'estudi i desenvolupament de molècules de baix pes molecular que s'auto-assemblen per a ser utilitzades en aplicacions fotòniques i biomèdiques.

Els objectius concrets es tracten en cada capítol de la següent manera:

1. Es sintetitzaran i caracteritzaran noves famílies de gelants de baix pes molecular derivats d'aminoàcids que tenen fraccions fluorescents (principalment 4-amino-1,8-naftalimida, 1,8-naftalimida i acridina).
2. S'avaluarà el procés de transferència d'energia que es duu a terme en gels moleculars reversibles auto-assemblats com a possible símil de sistemes naturals. Construcció de xarxes auto-assemblades ortogonals de molècules donant-acceptor que són capaces de respondre a estímuls i actuar com a fotocaptadors. Aquestes xarxes pretenen unificar de manera senzilla i com a prova de concepte, la funció de les bastides de proteïnes (fotoprotecció, orientació i

aproximació) i els pigments (absorció lumínica i transferència d'energia) presents en sistemes fotosintètics en un material intel·ligent. El sistema a estudiar difereix dels casos relacionats anteriorment, ja que la fibril·lació del donant i l'acceptor és un requisit per a la manipulació de la llum. Aquest fet juntament amb la naturalesa reversible dels gels moleculars podrien permetre crear materials mols fotònics i modulables (Capítol 3).

3. S'estudia la construcció d'un nou gel versàtil que respongui als estímuls i que sofreix fenòmens d'emissió induïts per agregació degut a l'auto-organització en fibres. La formació de gels auto-assemblats de gelant derivat de 1,8-naftalimida en mitjans aquosos no té precedents. S'investigarà la millora de l'emissió de fluorescència, la capacitat de formar pel·lícules i gels forts, el comportament tixòtrop i les característiques de curació pròpia. Addicionalment, s'investiga l'estudi fotofísic de diferents compostos derivats de 1,8-naftalimida que s'assemblen en diferents morfologies per assenyalar la importància dels arranjaments espacials de les unitats de 1,8-naftalimida per tal d'explicar el comportament d'emissió induïda per l'agregació. Finalment, s'estudiarà la possible aplicació d'aquest fenomen per a la detecció potencial de la polaritat del dissolvent i la temperatura (Capítol 4).

4. S'estudia la miniaturització de gels moleculars macroscòpics en nano i microgels moleculars. S'analitzaran dues estratègies diferents per a la síntesi de nanogels de 5 compostos diferents de diferents tipus moleculars: 2 derivats de 4-amino-1,8-naftalimides, 2 derivats de 1,8-naftalimida i 1 d'acridina. La capacitat de resposta a estímuls, la capacitat de càrrega i alliberament d'actius dels nano(micro) gels moleculars, així com la capacitat d'actuar com a actius de càrrega i distribució intracel·lular, serà estudiada com a prova de concepte. Els nanogels moleculars poden representar una alternativa interessant als seus anàlegs polimèrics en nanomedicina tenint en compte la seva reversibilitat intrínseca i la seva naturalesa sensible als estímuls. (Capítol 5).

5. En el sisè capítol es durà terme un estudi fonamental d'un nou material híbrid basat en les relacions estructurals i energètiques entre dues àrees d'interès: Els gels moleculars fluorescents i les nanopartícules de conversió ascendent (UCNP). Aquest nou sistema té com a objectiu manipular la llum de forma reversible com a conseqüència d'una comunicació òptica entre les UCNPs i la xarxes fibril·lars de components fotoactius mitjançant una transferència

energètica. Es preveu que aquesta transferència energètica permeti una transformació de la llum emesa per les nanopartícules. Els sistemes híbrids UCNPs-gel podrien ser usats en aplicacions relacionades amb la detecció basada en IR o la manipulació de la llum en general. Anant un pas més enllà, es faran estudis sobre la miniaturització d'aquests materials híbrids. Es sintetitzaran nanomaterials de tres components formats per nanogels moleculars + fotosensibilitzadors + UCNPs. S'espera que aquests nous nanomaterials siguin estudiats i assajats per actuar potencialment com a agents de teràpia fotodinàmica en teixits profunds. El nanogel actuaria com a "cola molecular" per aproximar les nanopartícules de conversió ascendent i els fotosensibilizadors. Aquesta aproximació facilitarà la transferència d'energia entre ells per a la producció eficient d'oxigen singlet a partir de la irradiació IR. (Capítol 5)

7. S'estudia el comportament del cromòfor 4-amino-1,8-naftalimida en sistemes autoensamblats com a sensor d'òxid nítric (NO). A part, s'estudiarà la relació estructura-activitat relacionada amb l'organització supramolecular (Capítol 7).

Figura 3. Resum dels temes tractats en cada capítol.

1.3 Aportacions originals, conclusions i futures línies d'investigació

1. En el tercer capítol es presenta la formació d'un gel molecular ortogonal de dos components fotoactius. L'eficient transferència d'energia ha sigut demostrada entre fluoròfors complementaris, acridina i 4-amino-1,8-naftalimida. El sistema descrit aquí difereix dels casos relacionats anteriorment, ja que la fibril·lació del donant i l'acceptor és un requisit per a la manipulació de la llum. Aquest fet, juntament amb la naturalesa reversible dels gels moleculars, ofereix materials molls fotònics sintonitzables. En concret, quan aquests gels de dos components s'irradiaen a 360 nm, s'elimina l'emissió del residu d'acridina (donant) a 420 nm com a conseqüència de la transferència d'energia d'excitació i es potencia l'emissió de les unitats de naftalimida (acceptor) a 550 nm. L'acidificació del sistema provoca la formació d'espècies d'acridini, resultant en una reducció dramàtica de l'emissió de naftalimida i l'aparició d'una emissió a 490 nm degut a l'emissió dels residus d'acridini. L'EET s'observa tant per gels humits com per xerogels. Aquest procés és totalment reversible només en el cas de xerogels, els quals recuperen les seves propietats d'emissió inicial després de la seva basificació. Els fenòmens de fotocaptació es visualitzen a ull nu per xerogels que mostren una emissió de color groguenc sota irradiació a 365 nm. En general, el procés reversible d'EET entre fluoròfors auto-assemblats podria obrir noves maneres de desenvolupar sistemes intel·ligents amb una àmplia varietat d'aplicacions. També cal assenyalar que aquest tipus de materials podria ser d'interès pràctic per al desenvolupament de dispositius d'emissió de llum blanca, tenint en compte que l'emissió en una àmplia gamma de longituds d'ona (400-650 nm) es podria ajustar variant la proporció de concentracions de donants i acceptors.

2. L'emissió induïda per l'agregació (AIE) ha sigut estudiada per a diversos derivats de 1,8-naftalimida. Alguns dels derivats formen hidrogels moleculars, que formen xarxes auto-assemblades visualitzades per TEM. La formació de gels auto-assemblats de 1,8-naftalimida en medis aquosos no té precedents i obre el camí a aplicacions biomèdiques relacionades. S'observa que l'assemblatge fibril·lar està associat a AIE amb emissió a 405 nm. Notablement, els compostos que mostren agregació no fibril·lar també experimenten AIE, però l'emissió es desplaça a 460 nm, que es pot atribuir a la formació d'excimers. Per tant, el mode d'agregació

modula la longitud d'ona de la llum emesa. Els diferents patrons d'agregació dels derivats de 1,8-naftalimida també es reflecteixen en canvis notables en el temps de vida dels estats excitats. Addicionalment, la quantificació de l'emissió (rendiments quàntics) dels diferents col·loides indica que l'agregació fibrilar proporciona un efecte AIE més fort en comparació amb els agregats amorfs. Probablement, dos arranjaments espacials diferents de les unitats de 1,8-naftalimida produeixen els resultats observats. A més, s'ha trobat que l'agregació pot ser provocada per l'addició d'aigua a solucions de DMSO. D'aquesta manera, el llindar d'humitat que produeix AIE es pot modular per variació de la concentració del fluoròfor. Les capacitats d'aquests sistemes per actuar com a sensors de llindar d'humitat en DMSO es poden utilitzar com una eina senzilla per detectar un possible excés d'aigua en mostres utilitzades per a bioassatjos. Finalment, es considera que l'agregació és termoreversible en el rang de 25-80 °C, oferint fluorescència regulada per temperatura en funció de l'efecte AIE. La capacitat de resposta a l'estímul (transició de gel-sol a baixa temperatura i alta sensibilitat per a la presència d'aigua), un comportament viscoelàstic molt interessant (capacitat per construir pel·lícules primes tioxotrópiques o gels) i característiques emissives altament tunejables, fan que aquesta nova família de materials sigui molt atractiva per a futures aplicacions en optoelectrònica o com a sensors.

3. Es demostra la miniaturització de gels moleculars macroscòpics en nano i microgels moleculars i es demostra la capacitat d'aquestes partícules moleculars de carregar i alliberar actius, així com la capacitat d'actuar com a actius de càrrega i distribució intracel·lular com a prova de concepte. Es van explorar dues estratègies diferents per sintetitzar nanogels de cinc compostos diferents de diferents tipus moleculars: dos derivats de 4-amino-1,8-naftalimides, dos derivats de 1,8-naftalimida i un d'acridina. D'una banda, es va realitzar un mètode de precipitació i, per altra banda, un mètode de nanoemulsió. En tots dos casos, els nanogels es van sintetitzar amb èxit amb mides que van des de 60 a 300 nm. Aquestes mides són òptimes per a la circulació sistèmica i l'explotació passiva de l'efecte EPR per al tractament del càncer. A més, es va investigar la capacitat de resposta dels estímuls dels nanogels mostrant insensibilitat relativament gran a la temperatura, la dilució i el contingut de sal. També es va investigar l'estabilitat dels nanogels moleculars amb el temps mostrant un augment de la mida d'al voltant del 200% en un mes i l'agregació amb alta concentració de sals (0,3 M). Els

microgels moleculars també es van sintetitzar d'aquesta manera mitjançant un mètode de precipitació i es van investigar les seves habilitats de càrrega i alliberament. Els microgls van mostrar una elevada capacitat de càrrega de diferents colorants i fotosensibilitzadors com el Nile Red o l'hipericina, entre d'altres. Els microgels també van mostrar l'alliberament d'aquests diferents actius dins de cultius cel·lulars *in vitro*. Els microgels carregats amb hipericina van mostrar bons resultats quan es va analitzar la seva activitat en teràpia fotodinàmica (PDT). En un bon exemple de prova de concepte, la irradiació de les cèl·lules del càncer cervical epitelial HeLa en presència de microgels carregats d'hipericina mostra valors IC50 en el rang nanomolar respecte a la hipericina ($0.0042 \pm 0.005 \mu\text{M}$) i en el rang micromolar respecte a la concentració de microgel ($0.8 \pm 0.2 \mu\text{M}$) calculat per assaigs MTT. Aquest resultat preliminar s'ha de confirmar i investigar àmpliament per trobar i avaluar el seu possible ús com agent de teràpia fotodinàmica. Finalment, s'analitza una estratègia diferent de síntesi de nanogels per a la possible futura aplicació en teràpia fotodinàmica. En aquest cas, el fotosensibilitzador (acridina) és el gelant en si mateix, que constitueix un sistema on el propi gelant actua com a auto-transportador. S'han aconseguit bons resultats en la construcció del sistema nanoscòpic assolint nanopartícules d'uns 100 nm. No obstant això, es va descartar la seva eficàcia com a agent PDT ja que es va detectar que el procés d'agregació reduïa l'eficiència de la PDT. No obstant, els sistemes agregats van mostrar una notable activitat antiproliferativa, que obre el camí per a futurs estudis sobre aquesta classe de compostos. En conclusió, els nanogels poden representar una alternativa interessant als anàlegs polimèrics en el camp de la nanomedicina tenint en compte la seva reversibilitat intrínseca i la seva naturalesa sensible als estímuls. S'estan realitzant més treballs per avaluar la viabilitat de diferents estructures de nanogels i la seva capacitat d'actuar com agents per al seu ús en nanomedicina.

4. S'han preparat nous sistemes híbrids que combinen gels fotònics supramoleculars de derivats de naftalimida i nanopartícules de $\text{NaYF}_4: \text{Yb} / \text{Tm}$ (UCNPs). El sistema híbrid manipula la llum de forma reversible com a conseqüència d'una comunicació òptica entre les UCNPs i la xarxa de gel fotoactiu. La transferència d'energia d'excitació radiant es produeix de forma molt eficient amb una transformació de la llum emesa per les nanopartícules. La radiació emesa en el rang de l'absorció del cromòfor en gel és completament eliminada i una nova emissió de l'acceptor de 1,8-naftalimida (gelant) s'observa a 410 nm, resultant d'un procés de

fotocaptació i transferència energètica. Aquest procés només actua en forma de gel com a resultat de les propietats emissives induïdes per l'agregació del gelant supramolecular. El desmuntatge de gel reversible és promogut per canvis de temperatura que donen lloc a un material suau fotònic sintonitzable regulable per la temperatura. A més, la interacció de les UCNPs amb les fibres auto-assemblades fomenta les vies de relaxació emissives davant dels camins no radiatius, originant una notable millora de la seva eficiència en l'emissió. Aquest efecte es pot atribuir provisionalment a l'aïllament del dissolvent de les nanopartícules després de la interacció amb les fibres de gel juntament amb la dispersió de llum IR produïda per la xarxa de gel auto-assemblada. Els sistemes de gel UCNPs híbrids com els descrits aquí es preveuen que s'utilitzaran en aplicacions relacionades amb la detecció basada en IR o la manipulació de la llum en general, especialment tenint en compte la possibilitat de la seva miniaturització en forma de micro / nanògrafs per a aplicacions biomèdiques.

Prenent com a punt de partida l'enfocament anterior, es va preparar la síntesi d'un nou nanomaterial híbrid que es pot dispersar en aigua (colorant + UCNPs @ nanogel) amb la idea d'aplicar-lo a la teràpia fotodinàmica en teixits profunds. Es va aconseguir una bona dispersió i estabilització de les UCNPs de nucli-crosta recobertes d'àcid oleic incorporant-se a nanopartícules orgàniques (nanogels). Els nanomaterials híbrids van mostrar mides en el rang de 60 a 200 nm i una bona capacitat de càrrega del fotosensibilitzador hipericina (5% m/m) i el colorant Nile Red. La col·localització de nanopartícules dins de la nanopartícula de gel i el fotosensibilitzador (o tint) va ser detectat per microscòpia òptica de fluorescència i permeté una transferència energètica eficient després de la irradiació IR. Aquesta transferència d'energia s'ha detectat mitjançant espectroscòpia de fluorescència disminuint el senyal verd a 540 nm de les UCNPs i l'augment de la senyal a 600 nm a partir d'hipericina (o Nile Red). La producció d'oxigen de singlet després de la irradiació de l'IR confirma encara més el procés de transferència d'energia i revela els seus usos potencials com a agent fotodinàmic en teixits profunds després de la irradiació IR. Els estudis preliminars d'internalització cel·lular també van revelar l'absorció cel·lular del nanomaterial i actualment s'estan duent a terme experiments addicionals en la Universitat Nacional de Singapur (NUS) per tal de determinar el seu rendiment com agent de teràpia fotodinàmica amb la línia cel·lular de carcinoma urotelial de la bufeta de ratolí MB49.

5. S'ha estudiat l'agregació en mitjans aquosos de tres derivats de 4-amino-1,8-naftalimida (ANI) i la seva reactivitat davant l'òxid nítric dissolt en medi aquós. DLS, microscòpia electrònica i estudis de microscòpia confocal van revelar que alguns compostos s'agregaven per formar nanopartícules amb diàmetres d'aproximadament 130 i 420 nm, respectivament. S'ha trobat que l'ANI experimenta desaminació després de l'exposició a NO en sistemes aquosos airejats, amb la corresponent disminució de fluorescència. Ha sigut demostrat que la formació de nanopartícules redueix la reactivitat en comparació amb la de les espècies no agregades. Estudis addicionals de reactivitat front a l'òxid nítric de vuit compostos estructuralment diferents van demostrar que aquest mecanisme de desaminació depèn de l'arquitectura de la molècula i la seva organització supramolecular. D'aquesta manera, els sistemes que tendeixen a formar estructures auto-assemblades que orientin l'amina cap al dissolvent aquós presenten una reactivitat atenuada, o són completament insensibles quan l'amina reactiva està completament soterrada a les regions hidrofòbiques, mentre que aquelles molècules que són propenses a ser solvatades i independents d'altres molècules veïnes són més adequades per a la reacció de desaminació. La sensibilitat de les molècules estudiades es troba dins del rang micromolar, que podria millorar-se en futurs treballs mitjançant l'activació de l'anell 1,8-naftalimida que presenta un anell electrònicament deficient.

1.4 Referències

- (1) Trevors, J. T.; Psenner, R. *FEMS Microbiol. Rev.* **2001**, 25 (5), 573.
- (2) Lehn, J.-M. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, 99 (8), 4763.
- (3) Almdal, K.; Dyre, J.; Hvidt, S.; Kramer, O. *Polym. Gels Networks* **1993**, 1 (1), 5.
- (4) Flory, P. J. *Discuss Faraday* **1974**, 57 (7), 7.
- (5) Terech, P.; Weiss, R. G. *Chem. Rev.* **1997**, 97 (8), 3133.
- (6) Roy, G.; Miravet, J. F.; Escuder, B.; Sanchez, C.; Llusar, M.; Zhao, Y.; Wang, Z.; Kim, F.; Yan, H. *J. Mater. Chem.* **2006**, 16 (19), 1817.
- (7) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. *Chem. Rev.* **2014**, 114 (4), 1973.
- (8) Escuder, B.; Rodríguez-Llansola, F.; Miravet, J. F. *New J. Chem.* **2010**, 34 (6), 1044.
- (9) Wang, S.; Xue, P.; Wang, P.; Yao, B. *New J. Chem.* **2015**, 39 (9), 6874.
- (10) Dickert, F. L.; Haunschild, A. *Adv. Mater.* **1993**, 5 (12), 887.
- (11) James, T. D.; Kawabata, H.; Ludwig, R.; Murata, K.; Shinkai, S. *Tetrahedron* **1995**, 51 (2), 555.
- (12) Vintiloiu, A.; Leroux, J.-C. *J. Control. Release* **2008**, 125 (3), 179.
- (13) Hirst, A. R.; Escuder, B.; Miravet, J. F.; Smith, D. K. *Angew. Chemie - Int. Ed.* **2008**, 47 (42), 8002.
- (14) Draper, E. R.; Adams, D. J. *Chem* **2017**, 3 (3), 390.

Compound Chart

4-amino-1,8-naphthalimide derivatives

1,8-naphthalimide derivatives

Acridine derivatives

**Published papers
related with this thesis**

Chameleonic, Light Harvesting Photonic Gels Based on Orthogonal Molecular Fibrillization

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S Supporting Information

ABSTRACT: The preparation of smart photonic gels composed of two orthogonally self-assembled fibrillar networks is reported. The molecular gelators are a light acceptor derived from 4-amino-1,8-naphthalimide and an acridine-based energy donor. Fibrillar orthogonal assembly of both chromophores is a requisite for light harvesting to be observed which results in emission from the naphthalimide-based acceptor upon irradiation of the acridine-derived donor with a significant Stokes shift of ca. 200 nm. Energy transfer is present both in the form of molecular gels in acetonitrile and in the solid-like xerogels and efficiently visualized both by fluorescence spectroscopy and confocal laser scanning microscopy. The chameleonic, stimuli responsive behavior is linked to the fact that the gel network formed by the acridine derivative can be selectively disassembled by means of temperature changes or chemical species. For example, the light harvesting process can be switched off/on by addition of HCl or NaOH respectively, affording a new type of stimuli responsive photonic soft materials.

INTRODUCTION

Molecular gels consist of self-assembled fibrillar networks resulting from the aggregation of low molecular weight molecules. The interest in this type of material has increased enormously in the last two decades as a result of their interesting properties which endow them with applicability as new soft materials in areas such as molecular electronics, controlled release, tissue engineering, or catalysis, among others.^{1–7} The intrinsic reversibility of molecular gels allows for the preparation of stimuli responsive, smart materials.⁸ The preparation of multicomponent molecular gels expands tremendously their tunable properties.^{9,10} Especially interesting is the case of gels composed of orthogonal fibrillary networks as a result of a self-sorting process. For example, Smith et al. have shown self-sorting of molecular gelators in several examples.^{11–13} Noticeably, self-sorting was reported by Adams et al. in aqueous environment by means of a controlled pH change of the system.^{14,15} Related to this, self-sorting between molecular gelators and surfactants was analyzed.^{16–18} One step forward has been described recently in separate reports from Smith et al. and Adams et al., achieving photopatterned gels from orthogonal two-component aqueous systems.^{19,20}

Here, orthogonal molecular gelation is exploited to prepare new photoactive, stimuli responsive soft materials comprised by two separate self-assembled fibers. The studied systems present efficient and tunable excitation energy transfer (EET). EET plays a crucial role in photosynthesis. In photosynthetic centers, the proper spatial disposition of donor and acceptor chromophores provokes energy funneling toward the reaction center. Inspired by the natural systems, there is a growing interest in the development of materials for the capture and storage of solar energy. Such materials are constituted by the appropriate arrangement of energy donors and acceptors.^{21–23} For this

purpose, supramolecular assemblies have been studied extensively.^{24,25} Photoinduced energy transfer processes are at the front-line of research, provided their potential applications in diverse cutting-edge fields ranging from optoelectronics to tailored nanomedicine.^{26–31}

A number of reports have described the incorporation of photonic functionalities in molecular gels.³² For example, gels with chromophore units have been used as photocatalyst,^{33,34} and long-range energy transfer has been reported in single supramolecular nanofibers.³⁵ Energy transfer has been studied in molecular gels formed by photoactive fibers which contained entrapped dyes. In particular, the emission of white light using a cascade of energy transfer processes in molecular gels has received special attention.³⁶ For this purpose oligo(*p*-phenylenevinylene)s and related compounds have been used by Ajayaghosh et al.^{32,37–40} EET was demonstrated also in self-assembled [*n*]-acene fibers doped with tetracene derivatives.^{41,42} A 1,3,5-cyclohexyltricarboxamide-based low-molecular-weight hydrogelator derivatized with naphthyl units showed energy transfer properties when doped with propyldansylamide.⁴³ The adsorption of dyes in molecular gels fibers has been exploited to prepare photon upconversion matrices.⁴⁴ In a different approach, photoactive components are mixed in the gel fibers, (nonorthogonal fibrillization) producing efficient EET.^{45–48} Only a few reports have addressed the preparation of self-sorted photoactive fibers, and all of them are aimed to develop photoinduced electron transfer. For this purpose, electron deficient (n-type) and electron rich (p-type) units are present in the gelators.^{49–56}

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Figure 1. Schematic representation of the tunable emission properties of the studied systems. For the structure of **1** and **2** see Figure 2.

Distinctly from the precedents cited above, here, for the first time, a new photoactive multicomponent material comprised by two separate self-assembled fibers showing efficient and stimuli receptive excitation energy transfer is presented. Tunability is linked to the selective disassembly of one of the orthogonal fibrillar networks (Figure 1). Moreover, the system exhibits a notable Stokes-Shift of ca. 200 nm, taking into account the excitation and emission wavelength maxima of the hybrid material: 350 and 540 nm, respectively.

RESULTS AND DISCUSSION

Compounds **1** and **2** (Figure 2) contain amino acid or peptide moieties which confer them with the capability of gel

Figure 2. Structure of the studied compounds and overlap of their absorption and emission spectra in acetonitrile. Inset: picture of a molecular gel of compound **1** under UV light.

formation. The 4-amino-1,8-naphthalimide moiety present in **1** is widely used in photonic systems because its stability and high fluorescence quantum yield.⁵⁷ Additionally, the acridine unit introduced in compound **2** is a fluorophore with elevated emission quantum yields used for a variety of applications.⁵⁸ These compounds were designed taking into account their capability to self-sort into orthogonal fibrillar networks and the required spectral overlap for the occurrence of Förster's Resonance Energy Transfer (FRET),^{59–62} being the acridine

unit the light absorbing chromophore and energy donor (emission $\lambda = 400\text{--}600\text{ nm}$) and the 4-amino-1,8-naphthalimide unit the energy acceptor moiety (absorption $\lambda = 350\text{--}500\text{ nm}$) (see Figure 2). The spectral overlap integral is calculated to be $J(\lambda) = 1.55 \times 10^{14}\text{ M}^{-1}\text{ cm}^{-1}\text{ nm}^4$ which predicts a Förster's distance of 21 Å for a Coulombic mechanism of energy transfer.

Acetonitrile was found to be a suitable solvent for this study because both compounds formed optically transparent/translucent gels in this solvent. Tentative models for the self-assembly of the molecular gelators are proposed in Figure 3 based on our experience and the literature on related molecules.^{63–65} Those models are feasible according to energy minimized molecular mechanics calculations of oligomeric assemblies carried out (see the Supporting Information, SI).

Hybrid gel formation with mixtures of **1** and **2** could be efficiently carried out yielding transparent gels in acetonitrile. Several evidence point to the expected orthogonal self-assembly which is predicted from the structural differences of the compounds: (i) the gelation capability is not altered in the mixtures. Minimum gelation concentration values (mgc) of **1** and **2** are respectively 1.4 and 2.9 mM. Gels were formed for mixtures of both gelators containing half of their mgc values (0.7 and 1.4 mM); (ii) Raman spectra of the hybrid gel corresponds to the sum of that of isolated fibrillary networks with no new signals assignable to a new type of fibrillary assemblies (see the SI); (iii) XRD of the two component xerogel gel can be correlated to a sum of the separated xerogels, although in all cases the samples presented broad diffraction patterns indicating poor crystallinity (see the SI); (iv) No significant differences could be observed when comparing electron microscopy images of single- and two-component xerogels, which in all cases showed fibrillary structures, as is commonly observed in many cases of molecular gelators (Figure 4).

Additionally, further proof of orthogonality was obtained by selective disassembly of the fibrillary network formed by **2** from a two-component gel. This process was monitored by NMR and occurs following a temperature increase or acidification of the medium. As seen in Figure 5, the two-component gel releases selectively compound **2** upon heating from 30 to 70 °C, affording, according to NMR data, full solubilization of the network formed by **2** and only partial solubilization of that formed by **1**. Indeed, in the experiment represented in Figure 5 at 70 °C, a self-supported gel formed exclusively by **1** was obtained.

Figure 3. Pictures of vials containing gels formed by compounds 1 and 2 and schematic representation of the self-assembled structures proposed.

Figure 4. Electron microscopy images of xerogels from 1, 2, and their mixture. Top row: TEM, bottom row: SEM.

Figure 5. Left: Variation of the solubility with the temperature, as determined by ^1H NMR, of a two-component gel formed by a equimolar mixture of 1 and 2 (3 mM each) in CD_3CN . Right: Partial ^1H NMR spectra of a two-component gel formed by 1 + 2 (3 mM each) in CD_3CN , before and after addition of acetic acid (its final concentration is 1.8 M). The signals for compounds 1 and 2 correspond, respectively, to the protons attached to the chiral center of the glycine and valine amino acid moieties.

Also, addition of acetic acid to a two-component gel disassembles preferentially the aggregates formed by compound 2, which is transformed in its soluble, easily detected by NMR, acridinium derivative (it should be noted that the pK_a values of

acridine derivatives⁶⁶ and carboxylic acids⁶⁷ in acetonitrile do not predict full proton transfer, therefore, rather than protonation of acridine it is more accurate to use the term acridine–acetic acid complex).⁶⁸ As seen in Figure 5, the NMR spectrum

Figure 6. VT emission spectra for gels formed by compounds **1** and **2** in acetonitrile. Concentration: 3 mM. $\lambda_{\text{ex}} = 360$ nm.

of a gel formed by **1** + **2** shows only a minimal amount of soluble, nonfibrillized, compound **2**. Addition of acetic acid produced full solubilization of **2**, as calculated using an internal standard for integration, but only partial solubilization (most likely due to polarity increase) of compound **1**. Again, full removal of compound **2** from the two-component gel produced in this case a self-sustained molecular gel formed exclusively by compound **1**. Finally, rheological measurements revealed that the mixed gels present similar viscoelastic properties than the single component gel of **2**, and improved storage and loss moduli in comparison to the gel of **1** (see the SI) but these results cannot be unambiguously ascribed to the mentioned orthogonality.

In the systems studied here, fibrillar self-assembly affects noticeably the fluorescence efficiency of both chromophores but in opposite tendencies. After a critical aggregation concentration, fluorescence of compound **1** is significantly reduced. It must be noted that the long wavelength emission of 4-amino-1,8-naphthalimide chromophore⁵⁷ is due to an internal charge-transfer process (ICT) process⁵⁷ which would be favored under solvating conditions (in acetonitrile) but not in the fibers. Similar effects have been described in the literature. For instance, Zhong et al. report a derivative of 4-amino-1,8-naphthalimide which displays very low emissive properties when encapsulated in the cavity of a cyclodextrin. However, the emission of such a compound is boosted upon decomplexation and exposure to the solvating environment.⁶⁹ Contrarily, emission of compound **2** is increased approximately twice in the form of fibers, resulting an example of moderate aggregation induced emission.⁷⁰ These effects are clearly observed in variable temperature experiments. In the case of **1**, an important increase in the emission is observed upon heating and progressive disassembly of the fibrillar networks (see Figure 6). It has to be noted that the disassembly of the gel formed by **1** does not result in a shift of the maximum emission wavelength upon going from 25 to 75 °C. This effect is explained taking into account that the emission of the gel at 25 °C predominantly comes from the small fraction of free, nonaggregated molecules that coexist with the gel fibers, as a result of the above-mentioned aggregation induced emission quenching experienced by **1**. However, for compound **2** the fluorescence diminishes upon dissolution of the aggregates as a result of its properties as a fluorophore with aggregation induced emission.

The hybrid gels were studied as light harvesting systems measuring the energy transfer from the acridine units to the naphthalimide ones. For this purpose, the emission spectra of samples containing only **2** and a mixture of **1** and **2** were compared using a spectrofluorimeter with a front-face measurement setup and confocal laser scanning microscopy (CLSM).

Figure 7. Top: graph representing the FRET efficiency of systems formed by a gel of donor **2** (2.5 mM) and variable amounts of acceptor **1** in acetonitrile. Bottom left: overlap of emission spectra of diluted, nonaggregated samples of donor **2** and equimolar mixture of **1** and **2** ($c = 0.2$ mM). Bottom right: overlap of emission spectra of gel samples of donor **2** and equimolar mixture of **1** and **2** ($c = 3$ mM; $\lambda_{\text{ex}} = 360$ nm).

As shown in Figure 7, a significant energy transfer process is detected for two-component samples. Notably, the graph in Figure 7 reveals that when a gel of acridine derivative **2** is mixed with increasing amounts of **1** a critical point is reached where energy transfer takes place. The critical point is coincident with the aggregation onset of **1**, revealing that only fibrillary aggregates of **1** can act efficiently as acceptors. For diluted concentrations of donor **1** no energy transfer at all is observed. This fact should be ascribed to the lack of interaction of free molecules of **1** with the fibers formed by **2**. However, fibrillization of **1** must provoke intimate fibrillary entanglement with fibers of **2**, affording a quite efficient energy transfer process (see schematic representation in Figure 1). Most likely solvophobic interactions together with π -stacking between electron-rich naphthalimide and electron-poor acridine units could be responsible of intimate contact between fibers formed by **1** and **2**. A simplified energy minimized semiempirical (AM1) model of such stacking is shown at the SI supporting the feasibility of such structural arrangement. Very importantly, this process does not take place at all for systems with both components in solution (Figure 7, bottom left). Similarly, quite poor or null

Figure 8. Top: Emission spectra obtained by confocal microscopy for gels formed by **1**, **2** and their mixture in acetonitrile, $\lambda_{\text{ex}} = 405$ nm. Bottom: Pictures of the gels obtained by confocal microscopy; $\lambda_{\text{ex}} = 405$ nm, $\lambda_{\text{obs}} = 550$ nm.

energy transfer is measured in the presence of one of the fibrillary networks being the complementary fluorophore in solution.

Additional experiments with confocal microscopy allowed visualization of the energy transfer described above. As shown in Figure 8, the mixed gel shows a much brighter emission of yellowish light when compared with a single component of **1** under the microscope under the same conditions. Although the evidence shown above points to orthogonal fibrillary assembly, one might wonder if the observed excitation energy transfer could result from the incorporation of some units of **1** in the fibers of **2**. In this respect, in some areas of the confocal images corresponding to the interface of gel and solution, isolated domains of compound **2** could be observed (Figure 8, bottom right) which present no excitation transfer affording the original emission of **2**. This observation discards that the light harvesting process arises from fibers of **2** doped with compound **1**. The light harvesting phenomena is quantified in the emission spectrum recorded with the confocal microscope upon irradiation at 405 nm. As shown in the spectra at Figure 8, in the mixed gel the emission of acridine units at 450 nm is fully suppressed and that of the naphthalimide moieties is boosted in agreement with the results obtained by the in-cuvette results described above.

The interplay of aggregation induced emission of **2** and energy transfer from **2** to **1** in the mixed gels provides an interesting behavior in the study of VT emission of the gels. As illustrated in Figure 9, the emission of a single component gel of **2** is reduced upon heating, as shown previously, but for a mixed gel with **1** the intensity of emission is weakly affected in the range 25–75 °C. At 25 °C, the reduced emission of **2** in the two-component gel is ascribed to the EET process. Upon heating and progressive gel disassembly, the EET is less efficient, however, the molecules of **2** in solution are less emissive than in the gel form. The balance of these two opposite tendencies

Figure 9. VT emission intensity at 550 nm for a gel of **2** and a two-component gel of **1** + **2** in acetonitrile. Concentration = 3 mM. $\lambda_{\text{ex}} = 360$ nm.

affords the reduced sensibility of the emission to temperature changes in the mixed system. This example highlights how two-component gels can provide with novel properties such as lower sensibility of emission intensity to temperature changes when compared to single-component ones.

The EET process is also preserved and nicely visualized in the absence of solvent when hybrid xerogels were studied. As seen in Figure 10, a 96% energy transfer from the acridine to the naphthalimide moieties occur for a molar ratio 2:1, acridine:naphthalimide. Remarkably in this case, the EET process also produces a significant increase in the emission of naphthalimide units from **1**. Quantitative measurements of the energy transfer processes indicate that for an excitation at 360 nm, the hybrid xerogels present an emission intensity which is ca. 3 times that of the single component xerogel formed by acceptor **1**. This effect can be observed by the naked eye: the hybrid xerogel presents a bright yellowish color under UV light but a mechanically

Figure 10. Fluorescence emission of xerogels of 1, 2 and 1 + 2 mixtures in acetonitrile. Excitation: 360 nm. Left: Mixed xerogel at a 2:1 acridine/naphthalimide ratio. Middle: Mixed xerogel at a 5:1 acridine/naphthalimide ratio. Right: Overlap of normalized emission spectra of xerogel and ground solid samples from equimolar mixtures of 1 and 2 ($\lambda_{\text{ex}} = 360$ nm). Inset: pictures of the samples under UV light (365 nm).

Figure 11. Variation of fluorescence emission spectra of mixed xerogels of 1 + 2 in acetonitrile obtained by confocal microscopy upon acid/base addition ($\lambda_{\text{ex}} = 405$ nm). Picture shows the image obtained by confocal microscopy of the gel; circles indicate the areas where the emission spectra were collected ($\lambda_{\text{obs}} = 550$ nm).

grounded mixture of both solids (no fibrillization process) presents a completely different appearance with a bluish emission (Figure 10).

Furthermore, an interesting fact is that the basic nature of compound 2 permits disruption of the energy transfer by protonation of acridine units, affording acridinium chromophores which lack the spectral overlap required for excitation energy transfer to take place efficiently. Confocal microscopy nicely visualizes the emission tuning achieved in this way. As shown in Figure 11, when a mixed gel was acidified with aqueous HCl the emission of acceptor 1 dropped dramatically as a result of acridine chromophores donors protonation. The emission is only restored partially upon subsequent basification because of leaching of acridinium units out of the gel network. As a result, the acidified mixed gel shows an intense emission at 500 nm from the acridinium units dissolved in the pools of solvent of the gel.

Notably, acid–base tuning of the emission is more efficient in the absence of solvent, namely in xerogels. As revealed in Figure 12, upon exposition of the mixed xerogel to acetic acid vapors emission of compound 1 is blocked to a large extent. Then, addition of NH_3 fumes deprotonate the acridinium derivative restoring the efficient energy transfer process. Presumably in this case, the lack of solvent prevents migration of the acridinium and allows for a much more efficient recovery of the energy transfer. Also, rather than the acid-promoted fibrillary disassembly observed in gels, here most likely fibers of compound 2 become protonated or complexed

Figure 12. Fluorescence emission (533 nm, excitation 360 nm) of a xerogel formed by an equimolar mixture of 1 and 2. Upon treatment with acetic acid and ammonia vapors. Inset: images under UV light (365 nm) of the xerogels formed by 2 (left) and 1 + 2 (right).

with acetic but are preserved. As observed in the pictures of Figure 12, after restoring the energy transfer process by addition of ammonia, the material distribution on the slide is essentially the same, discarding fiber disassembly and reassembly. Therefore, these systems can be considered as stimuli responsive, namely, smart, soft photonic materials.

CONCLUSIONS

In summary, orthogonal fibrillization resulting in gel formation of photoactive compounds is reported. A very efficient EET between complementary fluorophores, acridine and 4-amino-1,8-naphthalimide has been shown. The system described here differs from previously reported related cases in that fibrillization of both donor and acceptor is a requisite for the light manipulation. This fact together with the reversible nature of molecular gels affords tunable photonic soft materials. As schematized in Figure 1, in two component gels when the system is irradiated at 360 nm acridine moieties emission at 420 nm is eliminated and, as a result of excitation energy transfer, the emission of naphthalimide acceptor units at 550 nm is boosted compared to that obtained irradiating at 420 nm (big and small sun cartoons in Figure 1). Acidification provokes the formation of acridinium species resulting in a dramatic reduction of naphthalimide emission and appearance of an emission at 490 nm from acridinium moieties. EET is observed both for wet gels and xerogels but the tuning is fully reversible only in the case of xerogels which recover their initial emission properties after basification. Light harvesting phenomena are nicely visualized even with the naked eye for xerogels which show a yellowish emission under a lamp irradiating at 365 nm.

Overall, the efficient EET process among reversibly self-assembled fluorophores could open new ways for the development of smart systems with a wide variety of envisaged applications. It must be also noted that this kind of materials could be of practical interest for the development of white light emitting devices,³⁶ taking into account that the emission over a wide range of wavelengths (400–650 nm) could be tuned by adjusting the proportion of donor and acceptor concentrations.

EXPERIMENTAL SECTION

Synthesis. The preparation and characterization of compounds 1 and 2 is described in the SI.

Gel Preparation. The required amount of compounds 1 and 2 and 1 mL of acetonitrile were heated (hot air at 240 °C from a heat-gun) in a screw-capped vial (8 mL, diameter 1.5 cm) until complete solubilization. The vial was cooled down at ambient temperature, and gel formation was observed after several minutes (upon vial inversion, all the solvent remains entrapped). The standard waiting time before manipulation or measurement of the gel was 30 min.

For xerogel preparation, the gel was extended on a black glass slide, and then it was dried off by simple evaporation waiting for 30 min.

Fluorescence. Solutions were measured in a JASCO FP-8300 spectrofluorimeter using 3 mL quartz cuvettes with 10 mm pathlength. In the case of gels or optically dense solutions triangular quartz cuvettes were employed. For xerogels samples were measured in a Spex Fluorolog 3–11 spectrofluorimeter with a “front face” setup. In order to get reliable results, ten measurements were carried out for each sample in different areas of the xerogel, and the results were averaged.

Confocal Laser Scanning Microscopy (CLSM). Experiments were performed on inverted confocal microscope Leica TCS SP8. Images were obtained with HC PL APO CS2 63x/1.40 OIL immersion objective. Excitation of samples were performed with diode laser (excitation 405 nm) and were acquired with PMT detector. Microspectroscopy was performed with $\Delta\lambda = 6$ nm. Samples were prepared on a glass slide and covered with 0.17 mm #1.5 coverslip. Fresh gels were measured as prepared, xerogels were covered and measured after evaporation of solvent. For experiments which acid or base additions were needed, a peristaltic pump with 0.17 mm tubing thickness was used. To acidify the sample 10 microlitres of aqueous HCl 0.01 M were added. For basification 5 microlitres of NaOH 0.1 M were added.

SEM. Field-emission scanning electron micrographs were taken on a JEOL 7001F microscope equipped with a digital camera.

The corresponding xerogels were placed on top of an aluminum specimen mount stub and sputtered with Pt (Baltec SCD500).

Raman Spectra. Raman spectra were recorded on a JASCO-NRS-3100 device with resolution of 1 cm^{-1} .

Rheology. Measurements were carried out on a TA AR-G2 rheometer equipped with a Peltier temperature control accessory, using steel parallel plate–plate geometry (40 mm diameter). The gap distance was fixed at 1000 nm. The gels were prepared under the desired conditions and aged for 24 h. A homogeneous layer of gel was placed between the two plates. Frequency and stress sweep steps were performed at 25 °C. Viscoelastic properties were studied under oscillatory experiments. All the measurements were carried out within the linear viscoelastic regime. For this purpose, the experimental conditions to achieve a linear viscoelastic regime (LVR) were determined by running a stress sweep step (oscillatory Stress 0.01–1000 Pa at 1 Hz) and a frequency sweep step (0.1–10 Hz at 0.1 Pa). The storage and loss modulus independence with frequency and oscillatory stress applied defined the linear viscoelastic regime.

Solubility. For the determination of solubility the gels in CD_3CN were prepared inside of an NMR tube, using hydroquinone as internal standard for integration. VT- ^1H NMR spectra (500 MHz) were recorded previous stabilization for 10 min at the desired temperature.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemmater.6b03137.

Synthesis of compounds 1 and 2, additional UV and fluorescence spectra, rheological measurements, and XRD and Raman spectroscopy data (PDF)

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Notes

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REFERENCES

- (1) Terech, P.; Weiss, R. G. Low Molecular Mass Gelators of Organic Liquids and the Properties of Their Gels. *Chem. Rev.* **1997**, *97*, 3133–3159.
- (2) Weiss, R. G. The Past, Present, and Future of Molecular Gels. What Is the Status of the Field, and Where Is It Going? *J. Am. Chem. Soc.* **2014**, *136*, 7519–7530.
- (3) Hirst, A. R.; Escuder, B.; Miravet, J. F.; Smith, D. K. High-Tech Applications of Self-Assembling Supramolecular Nanostructured Gel-Phase Materials: From Regenerative Medicine to Electronic Devices. *Angew. Chem., Int. Ed.* **2008**, *47*, 8002–8018.
- (4) Banerjee, S.; Das, R. K.; Maitra, U. Supramolecular Gels 'in Action'. *J. Mater. Chem.* **2009**, *19*, 6649–6687.
- (5) Steed, J. W. Supramolecular Gel Chemistry: Developments over the Last Decade. *Chem. Commun.* **2011**, *47*, 1379–1383.
- (6) Escuder, B.; Rodríguez-Llansola, F.; Miravet, J. F. Supramolecular Gels as Active Media for Organic Reactions and Catalysis. *New J. Chem.* **2010**, *34*, 1044–1054.

- (7) Dawn, A.; Shiraki, T.; Haraguchi, S.; Tamaru, S.-i.; Shinkai, S. What Kind of "Soft Materials" Can We Design from Molecular Gels? *Chem. - Asian J.* **2011**, *6*, 266–282.
- (8) Segarra-Maset, M. D.; Nebot, V. J.; Miravet, J. F.; Escuder, B. Control of Molecular Gelation by Chemical Stimuli. *Chem. Soc. Rev.* **2013**, *42*, 7086–7098.
- (9) Raeburn, J.; Adams, D. J. Multicomponent Low Molecular Weight Gelators. *Chem. Commun.* **2015**, *51*, 5170–5180.
- (10) Buerkle, L. E.; Rowan, S. J. Supramolecular Gels Formed from Multi-Component Low Molecular Weight Species. *Chem. Soc. Rev.* **2012**, *41*, 6089–6102.
- (11) Moffat, J. R.; Smith, D. K. Controlled Self-Sorting in the Assembly of 'Multi-Gelator' Gels. *Chem. Commun.* **2009**, 316–318.
- (12) Smith, M. M.; Smith, D. K. Self-Sorting Multi-Gelator Gels-Mixing and Ageing Effects in Thermally Addressable Supramolecular Soft Nanomaterials. *Soft Matter* **2011**, *7*, 4856–4860.
- (13) Garcia Velazquez, D.; Luque, R. Spontaneous Orthogonal Self-Assembly of a Synergetic Gelator System. *Chem. - Eur. J.* **2011**, *17*, 3847–3849.
- (14) Morris, K. L.; Chen, L.; Raeburn, J.; Sellick, O. R.; Cotanda, P.; Paul, A.; Griffiths, P. C.; King, S. M.; O'Reilly, R. K.; Serpell, L. C.; Adams, D. J. Chemically Programmed Self-Sorting of Gelator Networks. *Nat. Commun.* **2013**, *4*, 1480.
- (15) Colquhoun, C.; Draper, E. R.; Eden, E. G. B.; Cattoz, B. N.; Morris, K. L.; Chen, L.; McDonald, T. O.; Terry, A. E.; Griffiths, P. C.; Serpell, L. C.; Adams, D. J. The Effect of Self-Sorting and Co-Assembly on the Mechanical Properties of Low Molecular Weight Hydrogels. *Nanoscale* **2014**, *6*, 13719–13725.
- (16) Fleming, S.; Debnath, S.; Frederix, P. W. J. M.; Hunt, N. T.; Ulijn, R. V. Insights into the Coassembly of Hydrogelators and Surfactants Based on Aromatic Peptide Amphiphiles. *Biomacromolecules* **2014**, *15*, 1171–1184.
- (17) Heeres, A.; van der Pol, C.; Stuart, M. C. A.; Friggeri, A.; Feringa, B. L.; van Esch, J. Orthogonal Self-Assembly of Low Molecular Weight Hydrogelators and Surfactants. *J. Am. Chem. Soc.* **2003**, *125*, 14252–14253.
- (18) Brizard, A.; Stuart, M.; van Bommel, K.; Friggeri, A.; de Jong, M.; van Esch, J. Preparation of Nanostructures by Orthogonal Self-Assembly of Hydrogelators and Surfactants. *Angew. Chem., Int. Ed.* **2008**, *47*, 2063–2066.
- (19) Draper, E. R.; Eden, E. G. B.; McDonald, T. O.; Adams, D. J. Spatially Resolved Multicomponent Gels. *Nat. Chem.* **2015**, *7*, 848–852.
- (20) Cornwell, D. J.; Daubney, O. J.; Smith, D. K. Photopatterned Multidomain Gels: Multi-Component Self-Assembled Hydrogels Based on Partially Self-Sorting 1,3:2,4-Dibenzylidene-D-Sorbitol Derivatives. *J. Am. Chem. Soc.* **2015**, *137*, 15486–15492.
- (21) Scholes, G. D.; Fleming, G. R.; Olaya-Castro, A.; van Grondelle, R. Lessons from Nature About Solar Light Harvesting. *Nat. Chem.* **2011**, *3*, 763–774.
- (22) Fukuzumi, S. Development of Bioinspired Artificial Photosynthetic Systems. *Phys. Chem. Chem. Phys.* **2008**, *10*, 2283–2297.
- (23) Gilbert, M.; Albinsson, B. Photoinduced Charge and Energy Transfer in Molecular Wires. *Chem. Soc. Rev.* **2015**, *44*, 845–862.
- (24) Peng, H.-Q.; Niu, L.-Y.; Chen, Y.-Z.; Wu, L.-Z.; Tung, C.-H.; Yang, Q.-Z. Biological Applications of Supramolecular Assemblies Designed for Excitation Energy Transfer. *Chem. Rev.* **2015**, *115*, 7502–7542.
- (25) Calzaferri, G.; Huber, S.; Maas, H.; Minkowski, C. Host-Guest Antenna Materials. *Angew. Chem., Int. Ed.* **2003**, *42*, 3732–3758.
- (26) Cheng, Y. J.; Yang, S. H.; Hsu, C. S. Synthesis of Conjugated Polymers for Organic Solar Cell Applications. *Chem. Rev.* **2009**, *109*, 5868–5923.
- (27) Semonin, O. E.; Luther, J. M.; Choi, S.; Chen, H.-Y.; Gao, J.; Nozik, A. J.; Beard, M. C. Peak External Photocurrent Quantum Efficiency Exceeding 100% Via Meg in a Quantum Dot Solar Cell. *Science* **2011**, *334*, 1530–1533.
- (28) Wong, K.-T.; Bassani, D. M. Energy Transfer in Supramolecular Materials for New Applications in Photonics and Electronics. *NPG Asia Mater.* **2014**, *6*, e116.
- (29) Wang, J.-Y.; Hsu, F.-C.; Huang, J.-Y.; Wang, L.; Chen, Y.-F. Bifunctional Polymer Nanocomposites as Hole-Transport Layers for Efficient Light Harvesting: Application to Perovskite Solar Cells. *ACS Appl. Mater. Interfaces* **2015**, *7*, 27676–27684.
- (30) Lee, H.; Jeong, Y.-H.; Kim, J.-H.; Kim, I.; Lee, E.; Jang, W.-D. Supramolecular Coordination Polymer Formed from Artificial Light-Harvesting Dendrimer. *J. Am. Chem. Soc.* **2015**, *137*, 12394–12399.
- (31) Harriman, A. Artificial Light-Harvesting Arrays for Solar Energy Conversion. *Chem. Commun.* **2015**, *51*, 11745–11756.
- (32) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. Functional Π -Gelators and Their Applications. *Chem. Rev.* **2014**, *114*, 1973–2129.
- (33) Weingarten, A. S.; Kazantsev, R. V.; Palmer, L. C.; McClendon, M.; Koltonow, A. R.; Samuel, A. P. S.; Kiebal, D. J.; Wasielewski, M. R.; Stupp, S. I. Self-Assembling Hydrogel Scaffolds for Photocatalytic Hydrogen Production. *Nat. Chem.* **2014**, *6*, 964–970.
- (34) Weingarten, A. S.; Kazantsev, R. V.; Palmer, L. C.; Fairfield, D. J.; Koltonow, A. R.; Stupp, S. I. Supramolecular Packing Controls H-2 Photocatalysis in Chromophore Amphiphile Hydrogels. *J. Am. Chem. Soc.* **2015**, *137*, 15241–15246.
- (35) Haedler, A. T.; Kreger, K.; Issac, A.; Wittmann, B.; Kivala, M.; Hammer, N.; Koehler, J.; Schmidt, H.-W.; Hildner, R. Long-Range Energy Transport in Single Supramolecular Nanofibres at Room Temperature. *Nature* **2015**, *523*, 196–U127.
- (36) Praveen, V. K.; Ranjith, C.; Armaroli, N. White-Light-Emitting Supramolecular Gels. *Angew. Chem., Int. Ed.* **2014**, *53*, 365–368.
- (37) Ajayaghosh, A.; Praveen, V. K. Pi-Organogels of Self-Assembled P-Phenylenevinyls: Soft Materials with Distinct Size, Shape, and Functions. *Acc. Chem. Res.* **2007**, *40*, 644–656.
- (38) Ajayaghosh, A.; Praveen, V. K.; Vijayakumar, C.; George, S. J. Molecular Wire Encapsulated into Pi Organogels: Efficient Supramolecular Light-Harvesting Antennae with Color-Tunable Emission. *Angew. Chem., Int. Ed.* **2007**, *46*, 6260–6265.
- (39) Vijayakumar, C.; Praveen, V. K.; Ajayaghosh, A. Rgb Emission through Controlled Donor Self-Assemble and Modulation of Excitation Energy Transfer: A Novel Strategy to White-Light-Emitting Organogels. *Adv. Mater.* **2009**, *21*, 2059–2063.
- (40) Praveen, V. K.; Ranjith, C.; Bandini, E.; Ajayaghosh, A.; Armaroli, N. Oligo(Phenylenevinylene) Hybrids and Self-Assemblies: Versatile Materials for Excitation Energy Transfer. *Chem. Soc. Rev.* **2014**, *43*, 4222–4242.
- (41) Desvergne, J.-P.; Olive, A. G. L.; Sangeetha, N. M.; Reichwagen, J.; Hopf, H.; Del Guerso, A. Self-Assembling and Light-Harvesting Properties of Fluorescent Linear Condensed Aromatic Gelators. *Pure Appl. Chem.* **2006**, *78*, 2333–2339.
- (42) Del Guerso, A.; Olive, A. G. L.; Reichwagen, J.; Hopf, H.; Desvergne, J. P. Energy Transfer in Self-Assembled N -Acene Fibers Involving ≥ 100 Donors Per Acceptor. *J. Am. Chem. Soc.* **2005**, *127*, 17984–17985.
- (43) Montalti, M.; Dolci, L. S.; Prodi, L.; Zaccheroni, N.; Stuart, M. C. A.; van Bommel, K. J. C.; Friggeri, A. Energy Transfer from a Fluorescent Hydrogel to a Hosted Fluorophore. *Langmuir* **2006**, *22*, 2299–2303.
- (44) Duan, P.; Yanai, N.; Nagatomi, H.; Kimizuka, N. Photon Upconversion in Supramolecular Gel Matrixes: Spontaneous Accumulation of Light-Harvesting Donor-Acceptor Arrays in Nanofibers and Acquired Air Stability. *J. Am. Chem. Soc.* **2015**, *137*, 1887–1894.
- (45) Shu, T.; Wu, J.; Lu, M.; Chen, L.; Yi, T.; Li, F.; Huang, C. Tunable Red-Green-Blue Fluorescent Organogels on the Basis of Intermolecular Energy Transfer. *J. Mater. Chem.* **2008**, *18*, 886–893.
- (46) Sugiyasu, K.; Fujita, N.; Shinkai, S. Visible-Light-Harvesting Organogel Composed of Cholesterol-Based Perylene Derivatives. *Angew. Chem., Int. Ed.* **2004**, *43*, 1229–1233.
- (47) Ardon, H. A. M.; Tovar, J. D. Energy Transfer within Responsive Pi-Conjugated Coassembled Peptide-Based Nanostructures in Aqueous Environments. *Chem. Sci.* **2015**, *6*, 1474–1484.

- (48) Channon, K. J.; Devlin, G. L.; MacPhee, C. E. Efficient Energy Transfer within Self-Assembling Peptide Fibers: A Route to Light-Harvesting Nanomaterials. *J. Am. Chem. Soc.* **2009**, *131*, 12520–12521.
- (49) Sugiyasu, K.; Kawano, S.-i.; Fujita, N.; Shinkai, S. Self-Sorting Organogels with P-N Heterojunction Points. *Chem. Mater.* **2008**, *20*, 2863–2865.
- (50) van Herrikhuyzen, J.; Syamakumari, A.; Schenning, A.; Meijer, E. W. Synthesis of N-Type Perylene Bisimide Derivatives and Their Orthogonal Self-Assembly with P-Type Oligo(P-Phenylene Vinylene)-S. *J. Am. Chem. Soc.* **2004**, *126*, 10021–10027.
- (51) Xue, P.; Wang, P.; Yao, B.; Sun, J.; Gong, P.; Zhang, Z.; Qian, C.; Lu, R. Nanofibers of Hydrogen-Bonded Two-Component Gel with Closely Connected P- and N-Channels and Photoinduced Electron Transfer. *ACS Appl. Mater. Interfaces* **2014**, *6*, 21426–21434.
- (52) Prasanthkumar, S.; Ghosh, S.; Nair, V. C.; Saeki, A.; Seki, S.; Ajayaghosh, A. Organic Donor-Acceptor Assemblies Form Coaxial P-N Heterojunctions with High Photoconductivity. *Angew. Chem., Int. Ed.* **2015**, *54*, 946–950.
- (53) López-Andarias, J.; Rodríguez, M. J.; Atienza, C.; López, J. L.; Mikie, T.; Casado, S.; Seki, S.; Carrascosa, J. L.; Martín, N. Highly Ordered N/P-Co-Assembled Materials with Remarkable Charge Mobilities. *J. Am. Chem. Soc.* **2015**, *137*, 893–897.
- (54) Marty, R.; Szilluweit, R.; Sánchez-Ferrer, A.; Bolisetty, S.; Adamcik, J.; Mezzenga, R.; Spitzner, E.-C.; Feifer, M.; Steinmann, S. N.; Corminboeuf, C.; Frauenrath, H. Hierarchically Structured Microfibers of “Single Stack” Perylene Bisimide and Quaterthiophene Nanowires. *ACS Nano* **2013**, *7*, 8498–8508.
- (55) Che, Y.; Huang, H.; Xu, M.; Zhang, C.; Bunes, B. R.; Yang, X.; Zang, L. Interfacial Engineering of Organic Nanofibril Heterojunctions into Highly Photoconductive Materials. *J. Am. Chem. Soc.* **2011**, *133*, 1087–1091.
- (56) Draper, E. R.; Lee, J. R.; Wallace, M.; Jackel, F.; Cowan, A. J.; Adams, D. J. Self-Sorted Photoconductive Xerogels. *Chem. Sci.* **2016**, *7*, 6499–6505.
- (57) Duke, R. M.; Veale, E. B.; Pfeffer, F. M.; Kruger, P. E.; Gunnlaugsson, T. Colorimetric and Fluorescent Anion Sensors: An Overview of Recent Developments in the Use of 1,8-Naphthalimide-Based Chemosensors. *Chem. Soc. Rev.* **2010**, *39*, 3936–3953.
- (58) Martí-Centelles, V.; Angeles Izquierdo, M.; Isabel Burguete, M.; Galindo, F.; Luis, S. V. Recognition of Free Tryptophan in Water by Synthetic Pseudopeptides: Fluorescence and Thermodynamic Studies. *Chem. - Eur. J.* **2014**, *20*, 7465–7478.
- (59) Forster, T. Experimentelle Und Theoretische Untersuchung Des Zwischenmolekularen Übergangs Von Elektronenanregungsenergie. *Zeitschrift Fur Naturforschung Section a-a Journal of Physical Sciences* **1949**, *4*, 321–327.
- (60) Braslavsky, S. E.; Fron, E.; Rodríguez, H. B.; Roman, E. S.; Scholes, G. D.; Schweitzer, G.; Valeur, B.; Wirz, J. Pitfalls and Limitations in the Practical Use of Forster's Theory of Resonance Energy Transfer. *Photochem. Photobiol. Sci.* **2008**, *7*, 1444–1448.
- (61) Yuan, L.; Lin, W.; Zheng, K.; Zhu, S. FRET-Based Small-Molecule Fluorescent Probes: Rational Design and Bioimaging Applications. *Acc. Chem. Res.* **2013**, *46*, 1462–1473.
- (62) Saura, A. V.; Marin, M. J.; Burguete, M. I.; Russell, D. A.; Galindo, F.; Luis, S. V. The Synthesis of New Fluorescent Bichromophoric Compounds as Ratiometric Ph Probes for Intracellular Measurements. *Org. Biomol. Chem.* **2015**, *13*, 7736–7749.
- (63) Hanabusa, K.; Tanaka, R.; Suzuki, M.; Kimura, M.; Shirai, H. Excellent Gelators for Organic Fluids: Simple Bolaform Amides Derived from Amino Acids. *Adv. Mater.* **1997**, *9*, 1095–1097.
- (64) Escuder, B.; Martí, S.; Miravet, J. F. Organogel Formation by Coaggregation of Adaptable Amidocarbamates and Their Tetraamide Analogues. *Langmuir* **2005**, *21*, 6776–6787.
- (65) Doi, M.; Asano, A.; Yoshida, H.; Inoueguchi, M.; Iwanaga, K.; Sasaki, M.; Katsuya, Y.; Taniguchi, T.; Yamamoto, D. Structure and Property of Self-Assemble Valinyl Bolaform Amides Having Different Chirality. *J. Pept. Res.* **2005**, *66*, 181–189.
- (66) Kaljurand, I.; Kütt, A.; Sooväli, L.; Rodima, T.; Mäemets, V.; Leito, I.; Koppel, I. A. Extension of the Self-Consistent Spectrophotometric Basicity Scale in Acetonitrile to a Full Span of 28 Pka Units: Unification of Different Basicity Scales. *J. Org. Chem.* **2005**, *70*, 1019–1028.
- (67) Kütt, A.; Leito, I.; Kaljurand, I.; Sooväli, L.; Vlasov, V. M.; Yagupolskii, L. M.; Koppel, I. A. A Comprehensive Self-Consistent Spectrophotometric Acidity Scale of Neutral Brønsted Acids in Acetonitrile. *J. Org. Chem.* **2006**, *71*, 2829–2838.
- (68) Akiyama, Y.; Wakisaka, A.; Mizukami, F.; Sakaguchi, K. Solvent Effect on Acid-Base Clustering between Acetic Acid and Pyridine. *J. Chem. Soc., Perkin Trans. 2* **1998**, 95–99.
- (69) Zhong, C.; Mu, T.; Wang, L.; Fu, E.; Qin, J. Unexpected Fluorescent Behavior of a 4-Amino-1,8-Naphthalimide Derived B-Cyclodextrin: Conformation Analysis and Sensing Properties. *Chem. Commun.* **2009**, 4091–4093.
- (70) Mei, J.; Leung, N. L. C.; Kwok, R. T. K.; Lam, J. W. Y.; Tang, B. Z. Aggregation-Induced Emission: Together We Shine, United We Soar! *Chem. Rev.* **2015**, *115*, 11718–11940.

Sizing Down a Supramolecular Gel into Micro- and Nanoparticles

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S Supporting Information

ABSTRACT: A low molecular weight gelator with a fluorescent 1,8-naphthalimide unit forms micro- and nanoparticles in aqueous media. Slow addition of a DMSO solution of the gelator into water affords either a self-assembled fibrillar network, sheaf-like microparticles, or nanoparticles depending to the concentration used in the experiment. The micro- and nanoparticles were characterized by dynamic light scattering (DLS), electron microscopy, and fluorescence measurements. In an initial assay of particle loading, Rose Bengal and Rhodamine 123 were shown to be incorporated in the particles. Light-promoted singlet oxygen generation capabilities of Rose Bengal were modulated by its incorporation in the particles. Additionally, the particles were found to promote the transport of Rhodamine 123 into human lung carcinoma live cells. These results indicate that nanoparticles arising from low molecular weight gelators may represent a new type of nanocarriers, being a potential alternative to polymeric nanogels used in nanomedicine.

INTRODUCTION

Supramolecular gels have emerged in the last decades as versatile soft materials. They are constituted from self-assembled fibrillar networks formed from the supramolecular aggregation of low molecular weight organic compounds.¹ Therefore, their molecular nature differentiates this type of gels from the conventional polymeric gel materials. Molecular gels present characteristic properties that provide them with an extraordinary added value in comparison to polymeric analogues: easy structural tuning, superior degradability/biocompatibility, and stimuli responsive disassembly, all of which are associated with their intrinsic noncovalent nature and the use of low molecular weight building blocks. The study of molecular gels has received extensive attention during the last two decades.^{2–4} Our group has studied the fundamentals of molecular gel formation, their use in catalysis and as photonic materials.^{5–7} Aside from that, molecular gels have been studied in a wide variety of fields including photonic and electronic materials,⁸ controlled release,⁹ and tissue engineering.¹⁰ All these applications are mainly based on the use of bulk, macroscopic gels. In the case of controlled release applications, the self-assembled fibrillar network from molecular gels can entrap bioactive substances that are liberated progressively. This approach is generally limited to topic or subcutaneous application.⁹

Increasing interest is devoted lately to the preparation and study of polymeric nanogels as delivery vehicles in the context of nanomedicine.^{11–15} Some drawbacks in the biomedical use of polymeric nanogels may arise from biodegradation or biocompatibility issues. Alternatively, nonpolymeric nanocarriers include liposomes,¹⁶ solid-lipid nanoparticles,¹⁷ and supramolecular systems based on host–guest interactions.^{18,19} Here, to the best of our knowledge, for the first time

miniaturization of molecular gels to nanometric dimensions is assessed. This challenging task raises questions such as if nanometer sized porous particles can be prepared at all from molecular gels, their thermodynamic and kinetic stability, and, very importantly, if the particles are able to incorporate and release active moieties.

EXPERIMENTAL SECTION

Synthesis and characterization of compound **1** is described in the SI.

Nanoparticles were prepared by syringe pump addition (0.6 mL/min) of 1 mL of **1** 0.1 mM (0.04 mg/mL) in DMSO to 9 mL of milli-Q water filtered over polyvinylidene difluoride (PVDF, 0.22 μ m mesh) to achieve a 10 μ M dispersion of **1**. The addition was performed with vigorous stirring using cylindrical (1.5 $\times$ 0.5 cm) magnetic Teflon-coated bar, at 800 rpm in a cylindrical vial (height 3.5 cm, width 2 cm).

Microparticles were prepared in a similar way but using a higher concentration of **1** and in the presence of gelatin as stabilizer. For example, 1 mL of stock solution of **1** 1 mM (0.4 mg/mL) in DMSO was added through a syringe pump (0.6 mL/min) to 9 mL of filtered milli-Q water containing 0.02% w/w gelatin. The addition was carried out with vigorous stirring to achieve a 100 μ M dispersion of **1**. A purification step is performed by centrifugation at 6000 rpm for 60 min ($\times$ 3) adding 10 mL of water after each centrifugation step. A white pellet composed of microparticles is formed which can be dispersed in pure water, PBS, or culture media prior to use.

Loading of dyes in the microparticles was performed by a coprecipitation–entrapment method.²⁰ 200 μ L of dye solution (Rose Bengal or Rhodamine 123) 1 mM in DMSO was mixed with 1 mL of the stock solution of **1** 1 mM (0.4 mg/mL) and then microparticles were prepared as described above.

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Scheme 1. Structure and Preparation of Compound 1

Field-emission scanning electron micrographs were taken on a JEOL 7001F microscope equipped with a digital camera. The corresponding particle dispersions were placed on top of an aluminum specimen mount stub, fast frozen with liquid N_2 , and lyophilized overnight. The sample was then sputtered with Pt (Baltec SCD500) for 30 s.

Transmission electron micrographs were taken on JEOL 2100 microscope with thermionic gun LaB6 200 kV equipped with Gatan Orius high resolution CCD camera. TEM samples were prepared over Carbon Formvar copper grids by placing a drop of nano/microparticle suspension and incubating for 5 min. The solvent was removed with filter paper by capillarity.

UV-vis and fluorescence of solutions and suspensions were measured, respectively, in a JASCO V-630 and a JASCO FP-8300 spectrofluorimeter using 3 mL quartz cuvettes with 10 mm path length.

Minimum gelator concentration of **1** was determined by inverse tube method (8 mL screw-capped vial, diameter = 2 cm) by adding the corresponding amount of **1** and 2 mL of solvent. The closed system was heated until complete solubilization. Finally, the system was rested at room temperature for 10 min.

DLS measurements were performed with Malvern Zetasizer nano ZS instrument. 3 mL aliquots of nanoparticle/microparticle suspensions were measured in 1 cm optical path PMMA cuvettes. Z-potential measurements were carried out with Malvern Zetasizer nano ZS instrument. 1 mL of nanoparticle/microparticle suspension were measured in disposable folded capillary cells.

Singlet oxygen generation experiments in the presence of Rose Bengal were performed inside a 1×1 cm² fluorescence quartz cuvette with 3 mL of microparticle suspension ($\approx 100 \mu\text{M}$) loaded with Rose Bengal ($\approx 6 \mu\text{M}$). ABDA (9,10-anthracenediyl-bis(methylene)-dimalonic acid) $50 \mu\text{M}$ was used as singlet oxygen probe.²¹ The irradiation was carried out in a photochemical reactor equipped with white light LEDs (400–700 nm, 110 W) and a heating stir plate. The cuvette was placed at 15 cm from the light source and illuminated homogeneously. The decrease on the ABDA absorption was fitted to a pseudo-first-order model.

CLSM experiments were performed on inverted confocal microscope Leica TCS SP8. Images were obtained with HC PL APO CS2 63 $\times$ /1.40 oil immersion objective. Excitation of samples was performed with sequential diode laser excitation 405 and 514 nm and images were acquired with a PMT detector. Microspectroscopy was performed with $\Delta\lambda = 5$ nm. Samples were prepared on sterilized Ibidi μ -Slide 8 Well Glass Bottom: # 1.5H ($170 \mu\text{m} \pm 5 \mu\text{m}$) Schott glass.

To evaluate the use of the particles as transporters of Rhodamine 123, 500 μL of Rhodamine-loaded nano/microparticle suspensions ($[1] \approx 40 \mu\text{M}$, $[\text{Rhod123}] = 0.1 \mu\text{M}$) were mixed with 800 μL cell culture media (DMEM 1 $\times$ no glucose, no glutamine, no red phenol (Gibco, Grand Island, NY, USA)). After 48 h, cells were washed and treated with 300 μL of the previous solution and incubated for 12 h at 37 $^\circ\text{C}$. Human lung carcinoma cells (A549) were obtained from American Type Culture Collection and were grown at 37 $^\circ\text{C}$ in a humidified 5% CO_2 , 95% air incubator. A549 cells were grown in Dulbecco's modified Eagle's medium, DMEM/high glucose modified (+4500 mg/L glucose, +110 mg/L sodium pyruvate w/o L-glutamine) (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cell culture media was purchased from Hyclone (South

Logan, Utah, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, UK). Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St Louis, MO, USA). Plastics for cell culture were supplied by Thermo Scientific BioLite. A549 cells at 70–80% confluence were collected and 2.5×10^4 cells were placed in a Ibidi μ -slide 8 well glass bottom well plate in 300 μL of medium.

Citotoxicity assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) dye reduction assay in 96-well microplates. Some 2.5×10^3 HeLa cells in a total volume of 100 μL of their respective growth media were incubated with serial dilutions of the **1**. After 2 days of incubation (37 $^\circ\text{C}$, 5% CO_2 in a humid atmosphere), 10 μL of MTT (5 mg mL^{-1} in PBS) were added to each well and the plate was incubated for a further 4 h (37 $^\circ\text{C}$). The resulting formazan was dissolved in 150 μL of 0.04 N HCl/2-propanol and read at 550 nm. All determinations were carried out in triplicate.

Photodynamic therapy studies using microparticles of **1** loaded with Rose Bengal were carried out in a 96-well plate using 10 different concentrations of the particles (up to 10 μM) in the presence of HeLa cells (30 000 each). The system was allowed to rest for 24 h, and after this time, removal of noninternalized particles was carried out and new culture medium was added. Finally, irradiation of the plates was carried out for 2 and 5 min by a two white LED module 2×11 W located at 5 cm of the plates. The study was carried out for six samples at every concentration value. After the irradiation process, cells were left at 37 $^\circ\text{C}$ for 24 h prior to analyzing the cell mortality by MTT assay.

RESULTS AND DISCUSSION

Among a variety of supramolecular gelators available in our laboratories, we chose compound **1** (Scheme 1) to assay the preparation of nano- and microparticles because its fluorescence properties, which facilitate monitoring of aggregation and, importantly, visualization of the particles by confocal laser scanning microscopy (CLSM). Compound **1** is constituted by a 1,8-naphthalimide unit linked to a dipeptidic, Gly-Val, unit. This type of structure was designed for the study of photoactive supramolecular gels, and excitation energy transfer of a close derivative containing a 4-amino-1,8-naphthalimide unit was reported recently.⁷ Synthesis of **1** was achieved by reaction of the propylamide of glycyl-valine with 1,8-naphthalic anhydride (Scheme 1).

After heating until complete dissolution and posterior cooling down to room temperature, **1** formed supramolecular gels in a variety of organic solvents. Self-sustained gels were prepared with minimum gelator concentrations values (mgc) ranging from 4 to 15 mM in all the assayed common organic solvents except hexane where compound **1** is very insoluble. Additionally, gels could be formed in aqueous media by addition of a DMSO solution of **1** into water (see Table S1 at SI).

Dropwise addition of dissolved substrates into a solvent where the species are insoluble represents a simple approach for the preparation of micro- and nanosized aggregates.^{22,23} In our case, controlled slow addition (syringe pump) of compound **1** dissolved in DMSO to water afforded particles

Figure 1. Top row: From left to right, TEM images of the macroscopic gel network, micro- and nanoparticles formed by compound **1**. Bottom row: SEM image of nanoparticles formed by **1** and their DLS analysis.

whose size is regulated by the concentration used in the experiment. For the sake of reproducibility, all the experiments were carried out under similar stirring conditions (see [Experimental Section](#)). A final solvent composition of H₂O/DMSO 9/1 was desired for the sake of biocompatibility. [Figure 1](#) compares transmission electron microscopy (TEM) images for samples prepared at final concentrations of gelator **1** of 2 mM, 0.1 mM, and 10 μM. It can be observed that for the most concentrated sample macroscopic gels containing elongated fibers of monodisperse width (20 ± 3 nm) were formed. Interestingly, a 20-fold reduction of the concentration affords discrete objects, microparticles, composed of several short fibers with a sheaf-like arrangement. These particles would probably result from truncated growth of the larger fibers. Finally, at the lowest concentration, 10 μM, a rather monodisperse distribution nanosized spherical objects (diameter of ca. 20 nm) was observed by TEM. We ascribe these structures to particles formed in the initial stages of nucleation of the self-assembly process. Similar nucleation and growth processes have been reported leading to the formation of spherulitic structures.²⁴

In the experiment carried out to achieve a final concentration of 5 μM, the nanoparticles showed a number-averaged diameter (D_n) by dynamic light scattering (DLS) of (87 ± 3) nm with an intensity averaged diameter (D_i) of (133 ± 3) nm, indicating moderate polydispersity ([Figure 1](#)). Variation of the final concentration of nanoparticles in the range 0.1–26 μM showed no significant trend in the variation of particle size which should diameters by DLS around 100 nm in most of the cases (see [Figure S12](#)). Scanning electron microscopy (SEM) images corroborate the presence of spherical nanoparticles with similar dimensions to those obtained by TEM ([Figure 1](#)).

It was observed that nanoparticles could be prepared reliably in the range of concentrations 0.1–25 μM. For higher concentration values microparticles were predominant. Monitoring the aggregation of **1** by fluorescence reveals a change in

the emission maxima which was used to estimate a critical aggregation concentration of 0.1 μM ([Figure S5](#)). Therefore, this value represents the bottom concentration limit of thermodynamic stability of the self-assembled nanoparticles. The colloid showed excellent temporal stability toward further aggregation/precipitation, being the DLS analysis reproducible for several days. Even after one month storage of the suspension, nanoparticles were still present although with higher average diameter (D_n increased to 343 ± 27 nm, [Figure S10](#)). This increase of diameter should most likely be ascribed to particle aggregation (see discussion about Z-potential and aggregation below). It must be stressed that this stability is enough for many biological applications, especially biomaging of live cells with CLSM. Additionally, the particles showed thermal stability in the temperature range 25–85 °C ([Figure S11](#)).

Next, the preparation of a stable dispersion of sheaf-like microparticles required the presence of gelatin which is described to avoid premature aging into macroscopic aggregates by means of surface adsorption.²² In relation to this, ¹H NMR experiments on centrifuged particles revealed that gelatin is not detectable in the material ([Figure S14](#)). In this way stable 0.1 mM samples containing microparticles could be reproducible prepared with $D_n = 920 \pm 198$ nm and moderate polydispersity measured by DLS ([Figure 2](#)). It has to be recalled that DLS data correspond to the fit of scattering data to spherical objects, but as shown by CLSM and electron microscopy, this is not the case and DLS diameter represents only a very rough indication of the size of the aggregates. These samples also contain a population of nanoparticles according to DLS ($D_n = 84 \pm 2$ nm). The sheaf-like assembly of short fibers mentioned above, ca. 2 μm long and ca. 1 μm width, is clearly visualized by SEM together with spherical nanoparticles ([Figure 2](#)). Nicely, the morphology observed for the dry samples used for electron microscopy is in accordance with that observed by CLSM in wet samples ([Figure 2](#)).

Figure 2. Top: SEM images of microparticle and nanoparticle particles formed by **1**. Bottom left: CLSM image of sheaf-like microparticles formed by **1** in water (image converted to gray scale for clarity). Bottom right: DLS analysis of the system.

An important consideration is if the described nano- and microparticles are gel-like, in the sense that they constitute particles mainly composed of solvent retained in the microstructure, or, on the contrary, are solid-like, nonporous objects. Evidence on this respect comes from the important size reduction detected in the comparison of wet samples of nanoparticles (87 ± 3 nm width) and dried ones (ca. 20 ± 5 nm width), suggesting that the nanoparticles are swollen in the solvent. However, no significant change in the diameter of the nanoparticles was observed by DLS in the presence of up to 16 mM NaCl (Figure S13). This insensitivity to ionic strength seems to discard a gel-like nature of the nanoparticles, although the fact that compound **1** is neutral, with no ionizable groups, could make the particles insensitive to ionic strength. In relation to this, Z-potential measurements revealed that the nanoparticles are negatively charged (potential = -22 mV) despite the absence of ionizable groups, a common feature, not fully understood in the literature, of neutral organic nanoparticles in water.^{25,26} The negative Z-potential of the nanoparticles would explain their stability toward flocculation. Indeed, according to DLS measurements addition of high concentrations of sodium chloride, up to 0.3 M, provoked

particle aggregation that should be ascribed to charge screening effects (Figure S13).

In relation with this, the capability of the particles to be loaded with organic molecules was studied. For this purpose, Rose Bengal (RB) and Rhodamine 123 (Rh123) were loaded into the microparticles. Unfortunately, studies of loading were carried only for microparticles but not for nanoparticles, because in the latter case it was not possible to isolate loaded particles from free dye, which was achieved by centrifugation in the case of microparticles. These dyes are widely used in CLSM. Additionally, RB has been used in photodynamic and antibacterial therapy²⁷ and Rhodamine 123 as mitochondria marker.²⁸

As seen in Figure 3, a strong bathochromic shift in the UV-vis spectrum (27 nm) clearly demonstrates incorporation of RB, being then in a quite less polar environment, in agreement with the literature.²¹ The loading can be visualized by CLSM (Figure 4). It can be observed that the images using sequential detection at 520–660 nm range (RB) and 410–496 nm range (gelator **1** fluorescence) colocalize, indicating incorporation of the dye into the microparticle. The dual emission of the ensemble is an attractive feature because it is uncommon to

Figure 3. UV-vis absorption spectra of aqueous systems containing microparticle particles of **1** and RB.

find in the literature systems in which both carrier and cargo can be visualized by fluorescence spectroscopy. Loading efficiency for this dye was determined by UV-vis spectroscopy to be 60 mmol/mol (15% w/w). Noteworthy, the loaded material can be released to the medium in response to a temperature increase. Upon progressive heating RB is released to the medium (Figure S15). Very importantly, the loading is reversible and upon cooling down the dye is again trapped in the microparticle, although with a moderate decrease in loading efficiency. As a proof of concept, the well-known capabilities of RB as photosensitizer were modulated by its incorporation into the particles. The production of singlet oxygen, $^1\text{O}_2$, was notably slowed down for RB trapped in the microparticle as revealed by the kinetic constants of the process. Partial release of RB by heating to 80 °C or complete release by addition of DMSO restored the singlet oxygen photogeneration capabilities (Figures S16–S19). In vitro studies of the RB-loaded particles as photodynamic therapy agents were carried out using HeLa cells revealing no significant influence of the concentration of particles in the cell viability. This fact confirms that the photogeneration of singlet oxygen is inhibited when RB is incorporated into the particles (Figure S19).

Finally, an initial assay revealed that the particles could act as vectors for the introduction of fluorescent probes into cells. For this purpose Rhodamine 123, a well-known mitochondrial marker was loaded into the microparticles and free dye removed by centrifugation. Incorporation of Rh123 into the centrifuged particles was confirmed by UV-vis spectroscopy with a loading efficiency of 2 mmol/mol (0.2% w/w). The microparticle dispersion was loaded to a culture of human lung carcinoma live cells (A549) and the system was compared by

CLSM with a control sample using the same amount of free Rh123 (0.1 μM) as that calculated to be into the microparticle sample using UV-vis measurements. Dramatic differences between both samples can be seen in Figure 5. Rh123-

Figure 5. CLSM images of a culture of human lung carcinoma live cells (A549) in the presence of Rh123-loaded microparticle of **1** (left) and the control experiment in the absence of microparticle (right). In the left image green spots correspond to Rhodamine in the cells and blue spots correspond to particles of **1**. In the right image, bluish color corresponds to the fluorescent background of the cells.

microparticle sample showed a noticeable transport into the cells, visualized as green spots at Figure 5, left (fluorescence detection at 520–660 nm). No incorporation of Rh123 was visible in the control experiment (Figure 5, right; see further images at SI, Figures S20–S21; for a superimposition of bright field, red, and blue channels, see Figure S22). The detailed role played by the microparticle needs further evaluation in future work, but it seems that the nanoparticles that coexist with the microparticles may act as active carriers into the cell.²⁹

An important issue for potential future biomedical applications of particles of **1** is their biocompatibility. An MTT assay in HeLa cells revealed a IC_{50} value of 3 μM (see Experimental Section) revealing a moderate cytotoxicity in accordance with the anticancer properties associated with DNA targeting reported for naphthalimides.³⁰ Therefore, compound **1** shows potential as a self-delivered theranostic agent due to their anticancer and fluorescent properties.

CONCLUSIONS

To summarize, miniaturization of macroscopic molecular gels into nano- and microparticles is demonstrated. The rather different diameter observed for dry particles by TEM compared to the one obtained by DLS analysis of wet samples suggest

Figure 4. CLSM images of sheaf-like microparticles loaded with RB. From left to right: red channel $\lambda = 520\text{--}660$ nm, blue channel $\lambda = 410\text{--}496$ nm, and the merger of both.

that they are swollen, containing important amounts of water. On the other hand, the diameter is insensitive to the ionic strength. Unambiguous determination of the internal structure of the nanoparticles is rather challenging and further work will be required to evaluate if they are solid-like, highly solvated, or gel like. A high loading of Rose Bengal (15% w/w) was achieved pointing to the presence of highly hydrated particles. Interestingly, the dye is reversibly released to the medium upon heating. The capability of Rose Bengal to produce singlet oxygen upon irradiation is found to be blocked when entrapped in the particles. The particles showed enhanced intracellular transport of Rhodamine 123 as a proof of concept. Overall, the rather different chemical nature and properties molecular nanoparticles derived from a low molecular weight gelator when compared to liposomes, solid-lipid nanoparticles and polymeric gels could broaden the scope of available nanocarriers. Further work is in progress to assess the feasibility of different nanoparticles and their capability to act as carriers of actives for their use in nanomedicine.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.7b02440.

Synthesis and characterization of **1**. Further characterization of nano- and microparticles and experiments of singlet oxygen generation. (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Weiss, R. G. The Past, Present, and Future of Molecular Gels. What Is the Status of the Field, and Where Is It Going? *J. Am. Chem. Soc.* **2014**, *136*, 7519.
- (2) Steed, J. W. Supramolecular gel chemistry: Developments over the last decade. *Chem. Commun.* **2011**, *47*, 1379.
- (3) Du, X.; Zhou, J.; Shi, J.; Xu, B. Supramolecular Hydrogelators and Hydrogels: From Soft Matter to Molecular Biomaterials. *Chem. Rev.* **2015**, *115*, 13165.
- (4) Banerjee, S.; Das, R. K.; Maitra, U. Supramolecular gels 'in action'. *J. Mater. Chem.* **2009**, *19*, 6649.

- (5) Escuder, B.; Rodríguez-Llansola, F.; Miravet, J. F. Supramolecular gels as active media for organic reactions and catalysis. *New J. Chem.* **2010**, *34*, 1044.
- (6) Hirst, A. R.; Coates, I. A.; Boucheteau, T. R.; Miravet, J. F.; Escuder, B.; Castelletto, V.; Hamley, I. W.; Smith, D. K. Low-Molecular-Weight Gelators: Elucidating the Principles of Gelation Based on Gelator Solubility and a Cooperative Self-Assembly Model. *J. Am. Chem. Soc.* **2008**, *130*, 9113.
- (7) Felip-León, C.; Díaz-Oltra, S.; Galindo, F.; Miravet, J. F. Chameleonic, Light Harvesting Photonic Gels Based on Orthogonal Molecular Fibrillization. *Chem. Mater.* **2016**, *28*, 7964.
- (8) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. Functional π -gelators and their applications. *Chem. Rev.* **2014**, *114*, 1973.
- (9) Vintiloiu, A.; Leroux, J.-C. Organogels and their use in drug delivery — A review. *J. Controlled Release* **2008**, *125*, 179.
- (10) Webber, M. J.; Appel, E. A.; Meijer, E. W.; Langer, R. Supramolecular biomaterials. *Nat. Mater.* **2015**, *15*, 13.
- (11) Sharma, A.; Garg, T.; Aman, A.; Panchal, K.; Sharma, R.; Kumar, S.; Markandeywar, T. Nanogel - An advanced drug delivery tool: Current and future. *Artif. Cells, Nanomed., Biotechnol.* **2016**, *44*, 165.
- (12) Kabanov, A. V.; Vinogradov, S. V. Nanogels as pharmaceutical carriers: Finite networks of infinite capabilities. *Angew. Chem., Int. Ed.* **2009**, *48*, 5418.
- (13) Vinogradov, S. V.; Senanayake, T. Nanogel-drug conjugates: A step towards increasing the chemotherapeutic efficacy. *Nanomedicine* **2013**, *8*, 1229.
- (14) Chan, M.; Almutairi, A. Nanogels as imaging agents for modalities spanning the electromagnetic spectrum. *Mater. Horiz.* **2016**, *3*, 21.
- (15) Raemdonck, K.; Demeester, J.; De Smedt, S. Advanced nanogel engineering for drug delivery. *Soft Matter* **2009**, *5*, 707.
- (16) Aryasomayajula, B.; Salzano, G.; Torchilin, V. P. *Methods Mol. Biol.* **2017**, *1530*, 41.
- (17) Geszke-Moritz, M.; Moritz, M. Solid lipid nanoparticles as attractive drug vehicles: Composition, properties and therapeutic strategies. *Mater. Sci. Eng., C* **2016**, *68*, 982.
- (18) Ma, X.; Zhao, Y. Biomedical Applications of Supramolecular Systems Based on Host-Guest Interactions. *Chem. Rev.* **2015**, *115*, 7794.
- (19) Liu, X.; Jia, K.; Wang, Y.; Shao, W.; Yao, C.; Peng, L.; Zhang, D.; Hu, X.-Y.; Wang, L. Dual-Responsive Bola-Type Supra-Amphiphile Constructed from Water-Soluble Pillar[5]arene and Naphthalimide-Containing Amphiphile for Intracellular Drug Delivery. *ACS Appl. Mater. Interfaces* **2017**, *9*, 4843.
- (20) An, B.-K.; Kwon, S.-K.; Jung, S.-D.; Park, S. Y. Enhanced Emission and Its Switching in Fluorescent Organic Nanoparticles. *J. Am. Chem. Soc.* **2002**, *124*, 14410.
- (21) Fabregat, V.; Burguete, M. I.; Galindo, F.; Luis, S. V. Singlet oxygen generation by photoactive polymeric microparticles with enhanced aqueous compatibility. *Environ. Sci. Pollut. Res.* **2014**, *21*, 11884.
- (22) Auweter, H.; Haberkorn, H.; Heckmann, W.; Horn, D.; Lüddecke, E.; Rieger, J.; Weiss, H. Supramolecular structure of precipitated nanosize β -carotene particles. *Angew. Chem., Int. Ed.* **1999**, *38*, 2188.
- (23) Guo, Z.; Shao, A.; Zhu, W.-H. Long wavelength AIEgen of quinoline-malononitrile. *J. Mater. Chem. C* **2016**, *4*, 2640.
- (24) Li, L.; Chan, C.-M.; Li, J.-X.; Ng, K.-M.; Yeung, K.-L.; Weng, L.-T. A Direct Observation of the Formation of Nuclei and the Development of Lamellae in Polymer Spherulites. *Macromolecules* **1999**, *32*, 8240.
- (25) Hozumi, A.; Sugimura, H.; Yokogawa, Y.; Kameyama, T.; Takai, O. ζ -Potentials of planar silicon plates covered with alkyl- and fluoroalkylsilane self-assembled monolayers. *Colloids Surf., A* **2001**, *182*, 257.
- (26) Beattie, J. K.; Djerdjev, A. M.; Warr, G. G. The surface of neat water is basic. *Faraday Discuss.* **2009**, *141*, 31.
- (27) *Singlet Oxygen: Applications in Biosciences and Nanosciences*; Nonell, S.; Flors, C., Eds.; RSC, 2016.

(28) Schneckenburger, H.; Stock, K.; Lyttke, M.; Strauss, W. S. L.; Sailer, R. Fluorescence lifetime imaging (FLIM) of rhodamine 123 in living cells. *Photochem. Photobiol. Sci.* **2004**, *3*, 127.

(29) Polo, E.; Collado, M.; Pelaz, B.; del Pino, P. Advances toward More Efficient Targeted Delivery of Nanoparticles in Vivo: Understanding Interactions between Nanoparticles and Cells. *ACS Nano* **2017**, *11*, 2397.

(30) Banerjee, S.; Veale, E. B.; Phelan, C. M.; Murphy, S. A.; Tocci, G. M.; Gillespie, L. J.; Frimannsson, D. O.; Kelly, J. M.; Gunnlaugsson, T. Recent advances in the development of 1,8-naphthalimide based DNA targeting binders, anticancer and fluorescent cellular imaging agents. *Chem. Soc. Rev.* **2013**, *42*, 1601.

Synthesis, spectroscopic studies and biological evaluation of acridine derivatives: The role of aggregation on the photodynamic efficiency

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ABSTRACT

Two new photoactive compounds (**1** and **2**) derived from the 9-amidoacridine chromophore have been synthesized and fully characterized. Their abilities to produce singlet oxygen upon irradiation have been compared. The synthesized compounds show very different self-aggregating properties since only **1** present a strong tendency to aggregate in water. Biological assays were conducted with two cell types: hepatoma cells (Hep3B) and human umbilical vein endothelial cells (HUVEC). Photodynamic therapy (PDT) studies carried out with Hep3B cells showed that non-aggregating compound **2** showed phototoxicity, ascribed to the production of singlet oxygen, being aggregating compound **1** photochemically inactive. On the other hand suspensions of **1**, characterized as nano-sized aggregates, have notable antiproliferative activity towards this cell line in the dark.

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Introduction

Acridine derivatives are well known biologically active compounds, widely used, for instance, as topical antibacterial and antiparasitic agents.¹ They have been used also as anticancer drugs since the planar structure of this chromophore permits the intercalation into the major groove of DNA and hence disruption of the replication process.² Further understanding of their molecular mode of action showed that this biological effect is not only due to their intercalating ability but also can be explained by targeting of overexpressed biomolecules in tumoral cells, such as telomerase, protein kinase and topoisomerases I and II.³ An abundant collection of acridine-based antiproliferative compounds can be found in the literature, with plethora of structural variants designed to enhance not only their binding abilities at the site of action but also the membrane crossing features and transportation properties of the molecules through the cellular milieu.⁴ For example, Delcros et al. have described a series of 9-substituted aminoacridines and amidoacridines linked to polyamine chains capable of inhibiting the growth of L1210 and CHO cells with IC₅₀ values in the micromolar range.⁵ One strategy followed to enhance the DNA binding efficiency is the introduction of a second

acridine moiety in the structure, which improves the stacking to the nucleic acids and hence potentiates the biological effect.⁶ Apart from applications in oncology, acridines have also found utility in other therapeutic fields, for instance as antimalarials⁷ and as cholinesterase inhibitors for Alzheimer's disease therapy.⁸

In parallel with this conventional approach there is another therapeutic strategy also using acridine-derived compounds and light to inhibit the proliferation of cancerous cells⁹ and also to kill microorganisms.¹⁰ Photodynamic therapy (PDT) is a clinical tool that uses a photosensitizer in combination with visible or UV light to produce cytotoxic reactive oxygen species (ROS) including superoxide radical anion (O₂^{•−}) and singlet oxygen (¹O₂). Numerous types of compounds have been employed so far for the generation of ¹O₂, not only for photobiological applications,^{11,12} but also for synthetic purposes.¹³

The use of acridines in PDT dates back to the very origin of this discipline¹⁴ and currently there is a renewed interest in the development of acridine derivatives for photodynamic applications. In principle, acridine chromophore is an excellent candidate to develop a bioactive photosensitizer, taking into account the very efficient population of the triplet excited state upon irradiation.¹⁵ Energy transfer to molecular triplet oxygen gives rise to very high yields of ¹O₂ (Φ_Δ) both in polar and apolar medium. For instance, Ogilby et al.¹⁶ have reported Φ_Δ (benzene) = 0.84 and Φ_Δ (acetonitrile) = 0.97. However, compared to the number of acridine derived compounds reported as DNA binding agents, the number of PDT active

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compounds based on this chromophore is relatively low. The aim of this study is to compare the ability of two new 9-amidoacridine compounds (**1** and **2** in Fig. 1) to generate $^1\text{O}_2$ upon irradiation. The hypothesis of this work is that the superstructure adopted by the photosensitizing molecules affects dramatically their photobiological efficiency, in such a way that aggregation can favour deactivation pathways of the excited states, leading to quenching of the photosensitizer (like molecule **1**) and hence their invalidation as PDT agents. In this regard, a simpler structure (like molecule **2**) avoiding this aggregation phenomenon would be more favourable for the photogeneration of $^1\text{O}_2$ in aqueous medium, and then their use for clinical application more recommendable.

Compounds **1** and **2** were synthesized by coupling carbobenzyloxy-L-valine with the corresponding amine or diamine, as depicted in Scheme 1, followed by deprotection of the Cbz group and reaction with acridine-9-carboxylic acid. Compounds were purified by subsequent filtration and washing steps, and were fully characterized by means of HRMS, ^1H and ^{13}C NMR spectroscopy (see Supporting information).

Fig. 1. Synthesized compounds based on the 9-amidoacridine chromophore.

In acetonitrile, compounds **1** and **2** show the typical absorption of the acridine chromophore at 360 nm, displaying optical features appropriate for UVA excitation and weak fluorescence at 419 nm (Fig. 2).¹⁷ In a polar solvent such as water, absorption maximum undergoes opposed shifts (365 nm for **1** and 357 nm for **2**) and fluorescence emission bathochromic shifts (439 nm for **1** and 435 nm for **2**). The emission quantum yield in water is notably different: very low for compound **1** and moderate for the mono-acridine derivative **2**. Summarized photophysical properties of the synthesized compounds are shown in Table 1.

The generation of $^1\text{O}_2$ upon irradiation was tested using a well-known benchmark reaction like the oxygenation of 1,5-dihydroxynaphthalene (DHN) to juglone, depicted in Scheme 2.¹⁸ Compounds **1** and **2** were irradiated in quartz cuvettes with light of 365 nm. The analysis of the reaction was performed by UV-vis absorption measurements following the decreasing absorption of DHN at 298 nm.

An illustrative example of the spectral changes occurring upon irradiation of acridine derivatives in the presence of DHN can be found in Fig. 3. As it can be seen, the absorption bands of DHN at 298 nm disappear when increasing the irradiation time and,

Fig. 2. Absorption and emission spectra of synthesized compounds in CH_3CN . **1** continuous line, **2** dashed line.

Scheme 1. Synthetic route for compounds **1** and **2** a) THF, Et_3N ClCOOEt , r.t., 12 h b) CH_3OH , Pd/C H_2 , r.t., 6 h c) for acridine-9-carboxylic acid activation: CH_2Cl_2 , oxalyl chloride, DMF(cat). For activated acid and amine coupling reaction: THF, Et_3N , 16 h.

Table 1
Photochemical properties of compounds **1** and **2**.

Compd.	Solvent	λ_{abs} (nm)	λ_{em} (nm)	Φ_F	Φ_A
1	CH ₃ CN	360	418	0.003 ± 0.001	0.96 ± 0.04
	H ₂ O ^a	365	439	0.04 ± 0.01	0.010 ± 0.008
2	CH ₃ CN	359	419	<0.001	0.97 ± 0.01
	H ₂ O ^a	357	435	0.32 ± 0.06	0.263 ± 0.004

^a Contains 1% CH₃CN from a concentrated stock solution used to prepare the aqueous sample.

Scheme 2. Photooxidation reaction of DHN. PS = Acridine photosensitizer; ISC = Intersystem crossing; DHN = 1,5-dihydroxynaphthalene.

Fig. 3. Photooxidation of DHN by $^1\text{O}_2$ induced by irradiation of **1** in CH₃CN, monitored by UV-Vis spectroscopy. [DHN] = 80 μM ; [**1**] = 33 μM .

concomitantly, the rise of a new absorption band takes place at 425 nm corresponding to the juglone photoproduct.

The initial kinetic points of the absorption bleaching of DHN were fitted to a pseudo-first order model ($\ln C/C_0 = -k_{\text{obs}} t$, where C is the concentration of DHN at a certain time t and C_0 is the initial concentration). The irradiations were performed in a series of media with different water content, from acetonitrile to water. The quantum yields for the $^1\text{O}_2$ generation induced by irradiation of **1** and **2** were determined by comparing the slopes of the above mentioned fittings to the same reaction photosensitized by phenone, as a well-known photosensitizer standard ($\Phi_A \sim 1$),¹⁹ in acetonitrile. All the fitted kinetics can be visualized in Fig. 4 (see details in the Supporting information file).

As shown in Fig. 5 both mono- (**2**) and bis-acridine (**1**) compounds generate very efficiently singlet oxygen upon irradiation at 365 nm in acetonitrile, as expected, with $\Phi_A = 0.97$ and 0.96 respectively. However, upon addition of water to the medium, this parameter falls dramatically, especially in the case of the bichromophoric compound **1** (in water $\Phi_A = 0.010$). However the decrease for monochromophoric derivative **2** is not so pronounced, affording a moderate yield (in water $\Phi_A = 0.263$).

This differential behaviour prompted us to investigate the reason for the absence of photo-reactivity in the case of bichromophoric compound **1**. Aqueous samples of **1** and **2** were analysed by dynamic light scattering (DLS) revealing a clear differ-

ence between them: whereas the larger compound **1** formed nanostructures in suspension with an average diameter of 122 ± 11 nm, monochromophoric acridine **2** did not form any detectable colloidal species (Fig. 6(a)). Further experiments confirmed that **1** undergoes self-aggregation in different polar and apolar solvents.

Fig. 4. Comparison of pseudo-first order linear fitting for the production of $^1\text{O}_2$ by **1** and **2** in different media (CH₃CN/H₂O).

Fig. 5. $^1\text{O}_2$ generation quantum yield (ϕ_A) by compounds **2** and **1** in different media (CH₃CN/H₂O).

Fig. 6. (a) Representative DLS analysis of **1** aggregates; (b) SEM analysis of **1** aggregates; (c) TEM analysis of **1** aggregates; (d) SEM micrograph of **1**; (e) TEM micrograph of **1**.

Aggregates of bis-acridine compound **1** were also observed by electron microscopy (SEM and TEM) with average diameters of 93 ± 24 nm and 135 ± 40 nm, respectively (Fig. 6b–e).

This finding suggests that, upon aggregation, a competing pathway is favoured at the expense of energy transfer to triplet oxygen to yield $^1\text{O}_2$. This situation does not occur to the same extent in the non-aggregated photosensitizer **2**, which retains certain capacity to generate $^1\text{O}_2$ in aqueous medium.

Compounds **1** and **2** have also very different properties from the photobiological viewpoint. When Hep3B cells incubated with the compounds were irradiated with light of 365 nm, only **2** was capable of inducing some PDT effect. As it can be seen in Fig. 7, Hep3B cells were incubated for 1 h in the presence of **2** ($10 \mu\text{M}$) and then irradiated for 3 min. Propidium iodide (PI) was used to test the photodamage, monitored by means of confocal fluorescence laser scanning microscopy (CLSM). Cells stained with PI after irradiations would indicate dead or damaged cells. In the control cells (no photosensitizer added) the number of damaged or dead cells was very low (only several per field) meaning that the irradiation *per se* was not deleterious. However, the number of PI-positive cells was dramatically increased in the cell population that was irradiated after up-take of **2**, pointing to the existence of a particularly harmful effect on cell viability when the two stimuli (**2** + irradiation) were combined. The observed effect was confirmed by the quantitative assessment of PI fluorescence using fluores-

cence microscopy coupled with static cytometry (see Supporting information). While the presence of **2** without irradiation only slightly enhances the number of PI⁺ cells (3.38% of the cells were PI positive after exposure to $20 \mu\text{M}$ **2** in comparison to 1.25% of the untreated cells), this number is notably higher when both stimuli are present and reaches the highest value with $20 \mu\text{M}$ of **2** (8.7%). For compound **1**, no induced photodamage was detected under the same experimental conditions. This observation confirms that aggregation must be avoided for photosensitizers based on acridine, at the designing stage, since this process hampers the generation of $^1\text{O}_2$. The negative effect of aggregation on the PDT activity is already well known for porphyrins and phthalocyanines, and efforts are devoted to the design of photosensitizers circumventing this process.²⁰ According to the observations here reported, the same kind of considerations should be taken into account when dealing with acridine-based photosensitizers.

Despite the lack of PDT activity of **1**, this compound apparently retains the DNA binding ability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays showed that exposure to **1** diminished cell viability in a concentration-dependent manner while exposure of **2** had no effect. The primary cell line Human umbilical vein endothelial cells (HUVEC) was slightly more susceptible than hepatoma cells Hep3B which is in line with previous reports of pharmacological toxicities using both cell models.²¹ After 3 h exposure to the highest concentration of **1** employed,

Fig. 7. CLSM images of Hep3B cells (30.000 cell/well) stained with propidium iodide used to mark damaged or dead cells (visualized in red color) (1 μ M). Top row: Irradiated set of wells in absence of **2** (10 μ M); (a) bright field; (b) PI fluorescence, λ_{ex} = 488 nm, $\Delta\lambda_{\text{em}}$ = 500–700 nm; (c) merged channels. Bottom row: Irradiated control set of wells in presence of **2**; (d) bright field; (e) PI fluorescence, λ_{ex} = 488 nm, $\Delta\lambda_{\text{em}}$ = 500–700 nm; (f) merged channels. Irradiation performed with UV lamp at 365 nm, 6 W, for 3 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. CLSM images of Hep3B cells (30.000 cell/well) of **1** (10 μ M) incubated for 1 h stained with Draq5 (1 μ M). (a) Bright field; (b) Merged images of Draq5 fluorescence (nuclei stained in red) and **1** fluorescence (blue) sequentially acquired (**1** fluorescence, λ_{ex} = 405 nm, $\Delta\lambda_{\text{em}}$ = 410–550 nm, Draq5 fluorescence, λ_{ex} = 488 nm, $\Delta\lambda_{\text{em}}$ = 600–700 nm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the calculated IC_{50} was 29.5 μ M for Hep3B cells and 18.62 μ M for HUVEC. Of note, prolonged incubation (24 h) with **1** did not exacerbate the effect on cellular viability recorded after 3 h-exposure, in Hep3B cells. In HUVEC cells, the prolonged incubation did have a greater effect (46% and 27% vs control after 3 h- and 24 h-incubation respectively). Also, we observed the capacity of both cell lines to recover from exposure to **1** and **2** (21 h recovery after the 3 h-treatment, see Fig. S4 in the supporting information file). The calculated IC_{50} value lies within the range of reported toxicities of similar compounds. Thus, Kožurková et al. described antitumor and binding studies of acridine dialkylureas in HeLa and HCT-116 cell lines with an IC_{50} of 3.1 μ M.²² Analogously Arya et al. described several 9-aminoacridine derivatives showing IC_{50} on the 5–10 μ M range in different cell lines: breast T47D, lung NCI-H522, colon HCT-15, ovary PA-1, liver HEPG2 and COS-1 cells.²³ This fact could be related to the best binding ability of bichromophoric acridines as compared to the monochromophoric ones.⁶ Notably compound **2** is not toxic at concentrations lower than 20 μ M.

The cellular uptake of compound **1** was examined by means of CLSM. As it can be seen in Fig. 8, the uptake and subcellular localization of compound **1** was studied by live cell confocal fluorescence microscopy using Hep3B cells. These experiments revealed that after 1 h of incubation with 10 μ M of **1**, fluorescence can be visualized inside cells and tend to localize in the perinuclear area, often in association with the nuclear membrane (Fig. 8 inset). Colocalization experiments using organelle-specific fluorochromes failed to show correlation of **1** with any of the subcellular compartments studied: ER, mitochondria and lysosomes (data not shown).

In summary, the synthesis and chemical characterization of two new acridine derived compounds, one monochromophoric (**2**) and the other bichromophoric (**1**), has been presented. Photophysical and photobiological studies of these compounds have been carried out. Their ability to produce 1O_2 upon irradiation has been compared, focusing the attention on the influence of chemical structure. The synthesized compounds show very different self-aggregating properties since only the bichromophoric molecule (**1**) presents a strong tendency to aggregate in water, which blocks the ability to produce 1O_2 . On the contrary, the non-aggregating molecule (**2**) still retains the 1O_2 production capacity, even in water ($\Phi_A = 0.263$). In biological medium both compounds also show a distinct behaviour, probably due to their different self-aggregating properties. Thus, PDT studies were carried out with Hep3B cells which showed that only non-aggregated monochromophoric compound (**2**) has some efficiency after irradiation with UV light, probably due to production of 1O_2 . Hence, it can be stated that aggregation influence the dynamics of the excited states of **1**, discarding its use as a PDT agent. Nevertheless, despite this lack of photoactivity, suspensions of this molecule, characterized as nano-sized aggregates, have notable antiproliferative activity in the dark, which opens the way for future studies on this class of compounds.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.bmcl.2018.02.005>.

References

- Wainwright MJ. *Antimicrob Ther.* 2001;47:1.
- Cholewiński G, Dzierzbicka K, Kołodziejczyk AM. *Pharmacol Rep.* 2011;63:305.
- Denny WA. *Curr Med Chem.* 2002;9:1655;
- Belmont P, Bosson J, Godet T, Tiano M. *Anticancer Agents Med Chem.* 2007;7:139.
- Shinozuka K, Seto Y, Sawai H, et al. *J Chem Soc Chem Commun.* 1994;23:1377; Norman TJ, Parker D, Smith FC, et al. *J Chem Soc Chem Commun.* 1879;1995; Blagbrough IS, Taylor S, Taylor S, et al. *Chem Commun.* 1998;18:929; Li L, Kracht J, Peng S, et al. *Bioorg Med Chem Lett.* 2003;13:1717; Takashima H, Tara C, Namikawa S, et al. *J Phys Chem B.* 2006;110:26413; Farrera-Sinfreu J, Aviñó A, Navarro I, et al. *Bioorg Med Chem Lett.* 2008;18:2440; Janovec L, Kožurková M, Sabolová D, et al. *Bioorg Med Chem.* 2011;19:1790.
- Delcros JG, Tomasi S, Carrington S, et al. *J Med Chem.* 2002;45:5098.
- Far S, Kossanyi A, Verchère-Béaur C, Gresh N, Taillandier E, Perrée-Fauvet M. *J Org Chem.* 2004;1781; Borgions F, Ghysels D, Van Aerschot A, Rozenski J, Herdewijn P. *Helv Chim Acta.* 2006;89:1194.
- Guetzoyan L, Ramandrasoa F, Dorizon H, et al. *Bioorg Med Chem Lett.* 2007;15:3278.
- Csuk R, Barthel A, Raschke C, et al. *Arch Pharm.* 2009;342:699.
- Yang P, Yang Q, Qian X, Tong L, Li X. *J Photochem Photobiol B.* 2006;84:221; Benhabane Y, Di Giorgio C, Boyer G, et al. *Eur J Med Chem.* 2009;44:2459; Satonaka H, Kusuzaki K, Matsubara T, et al. *Oncol Lett.* 2010;1:69; Pitchaimani A, Renganathan A, Cinthalkinian S, Premkumar K. *RSC Adv.* 2014;4:22123; Čížková L, Grolmusová A, Ipóthová Z, et al. *Bioorg Med Chem.* 2014;22:4684; Cisaríková A, Barbieriková Z, Janovec L, et al. *Bioorg Med Chem.* 2011;2016:24; Mukherjee NN, Podder S, Banerjee S, Majumdar S, Nandi D, Chakravarty AR. *Eur J Med Chem.* 2016;122:497.
- Iwamoto Y, Yoshioka H, Yanagihara Y. *Chem Pharm Bull.* 1987;35:2478.
- Nonell S, Flors C. *Singlet Oxygen: Applications in Biosciences and Nanosciences.* Cambridge: RSC; 2016; Ogilby PR. *Chem Soc Rev.* 2010;39:3181; Huang Y-Y, Sharma SK, Dai T, et al. *Nanotechnol Rev.* 2012;1:111; Dolmans DEJGJ, Fukumura D, Jain RK. *Nat Rev Cancer.* 2003;3:380; Macdonald IJ, Dougherty TJ. *Porphyryns Phthalocyanines.* 2001;5:105; Galindo F, Kabir N, Gavrilovic J, Russell DA. *Photochem Photobiol Sci.* 2008;7:126; Burguete MI, Galindo F, Gavaara R, Luis SV, Moreno M, Thomas P, Russell DA. *Photochem Photobiol Sci.* 2009;8:37; Beltrán A, Mikhailov M, Sokolov MN, Pérez-Laguna V, Rezusta A, Revillo MJ, Galindo F. *J Mater Chem B.* 2016;4:5975; Felip-León C, Arnau del Valle C, Pérez-Laguna V, et al. *J Mater Chem B.* 2017;5:6058.
- Luan L, Ding L, Zhang W, Shi J, Yu X, Liu W. *Bioorg Med Chem Lett.* 2013;23:3775; Verwilt P, David CC, Leen V, Hofkens J, de Witte PAM, De Borggraeve WM. *Bioorg Med Chem Lett.* 2013;23:3204; Yanik H, Göksel M, Yeşilot S, Durmuş M. *Tetrahedron Lett.* 2016;57:2922; Durmuş S, Bulut M. *M Tetrahedron Lett.* 2016;57:1124; Zhou L, Ge X, Liu J, et al. *Bioorg Med Chem Lett.* 2013;23:5317; Li X-S, Guo J, Zhuang J-J, Zheng B-Y, Ke M-R, Huang J-D. *Bioorg Med Chem Lett.* 2015;25:2386; Guo XX, Wang L, Wang S, et al. *Bioorg Med Chem Lett.* 2015;25:4078; Bio M, Rajaputra P, You Y. *Bioorg Med Chem Lett.* 2016;26:145; Slomp AM, Barreira SMW, Carrenho LZB, Vandresen CC, Zattoni IF, Ló SMS, Dallagnol JCC, Ducatti DRB, Orsato A, Duarte MER, Nosedá MD, Otuki MF, Gonçalves AG. *Bioorg Med Chem Lett.* 2017;27:156.
- Ghogare AA, Greer A. *Chem Rev.* 2016;116:9994; Burguete MI, Gavaara R, Galindo F, Luis SV. *Catal Commun.* 2010;11:1081; Burguete MI, Gavaara R, Galindo F, Luis SV. *Tetrahedron Lett.* 2010;51:3360; Fabregat V, Burguete MI, Galindo F, Luis SV. *Environ Sci Pollut Res.* 2014;21:11884; Fabregat V, Burguete MI, Luis SV, Galindo F. *RSC Adv.* 2017;7:35154.
- Raab O. *Z Biol.* 1900;39:524.
- Komorowski SJ, Grabowski ZR, Zielenkiewicz W. *J Photochem.* 1985;30:141; Eberhard J, Peuntinger K, Rath S, et al. *Photochem Photobiol Sci.* 2014;13:380; Zhao J, Wu W, Sun J, Guo S. *Chem Soc Rev.* 2013;42:5323.
- Scurlock RD, Ogilby P. *J Photochem Photobiol A.* 1993;72:1.
- Martí-Centelles V, Izquierdo MA, Burguete MI, Galindo F, Luis SV. *Chem A Eur J.* 2014;20:7465.
- Takizawa S, Aboshi R, Murata S. *Photochem Photobiol Sci.* 2011;10:895; Wu W, Geng Y, Fan W, Li Z, Zhan L, Wu X, Zheng J, Zhao J, Wu M. *RSC Adv.* 2014;4:51349.
- Schmidt R, Tanielian C, Dunsbach R, Wolff C. *J Photochem Photobiol A Chem.* 1994;79:11.
- Bennett LE, Ghigino KP, Henderson RW. *J Photochem Photobiol B.* 1989;3:81; Rossetti FC, Lopes LB, Carollo ARH, Thomazini JA, Tedesco AC, Bentley MVLB. *J Contr Release.* 2011;155:400; Siraj N, Kolic PE, Regmi BP, Warner IM. *Chem Eur J.* 2015;21:14440.
- Apostolova N, Gomez-Sucerquia LJ, Moran A, Alvarez A, Blas-Garcia A, Esplugues JV. *J Pharmacol.* 2010;160:2069.
- Kožurková M, Sabolová D, Janovec L, Mikeš J, Koval J, Ungvárský J, Štefanišinová P, Fedoročko P, Kristian P. *Imrich Bioorganic Med Chem.* 2008;16:3976–3984.
- Arya S, Kumar A, Kumar N, Roy P, Sondhi SM. *Med Chem Res.* 2015;24:1942; Gensicka-Kowalewska M, Cholewiński G, Dzierzbicka K. *RSC Adv.* 2017;7:15776.

Multimodal Light-Harvesting Soft Hybrid Materials: Assisted Energy Transfer upon Thermally Reversible Gelation

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S Supporting Information

ABSTRACT: Multimodal light-harvesting soft systems able to absorb UV-to-NIR radiations and convert into visible emissions have drawn much attention in the last years in order to explore new areas of application in energy, photonics, photocatalysis, sensors, and so forth. Here, we present a new hybrid system combining a supramolecular photonic gel of naphthalimide-derived molecules self-assembled into fibers and upconverting NaYF₄:Yb/Tm nanoparticles (UCNPs). The hybrid system presented here manipulates light reversibly as a result of an optical communication between the UCNPs and the photoactive gel network. Upon UV irradiation, the system shows the characteristic emission at 410 nm from the photoactive organo-molecule. This emission is also activated upon 980 nm excitation thanks to an efficient energy transfer from the UCNPs to the fibrillary network. Interestingly, the intensity of this emission is thermally regulated during the reversible assembly or disassembly of the organogelator molecules, in such a way that gelator emission is only observed in the aggregated state. Additionally, the adsorption of the UCNPs with the supramolecular gel fibers enhances their emissive properties, a behavior ascribed to the isolation from solvent quenchers and surface defects, as well as an increased IR light scattering promoted by the fibrillary network. The reported system constitutes a unique case of a thermally regulated, reversible, dual UV and IR light-harvesting hybrid soft material.

INTRODUCTION

Supramolecular gels based on self-assembled fibrillary networks are soft materials with significant advantages when compared to polymeric analogues such as intrinsic reversibility, stimuli responsiveness, and superior biocompatibility.^{1–4} The increasing attention paid to supramolecular gels is related to their applicability as new soft materials in areas such as molecular electronics, controlled release, tissue engineering, or catalysis, among others.^{2–7} Several approaches have addressed the inclusion of photonic functionalities into supramolecular gels.⁸ For example, gels with chromophore units have been used as photocatalysts^{9,10} and excitation energy transfer has been studied in supramolecular gels formed by photoactive fibers which contained entrapped dyes.^{11–15} Recently, we reported orthogonal fibrillization of two fluorescent supramolecular gelators.¹⁶ Additionally, supramolecular gels have been used as photon upconversion matrixes based on the triplet–triplet-annihilation mechanism via organic molecules.^{17,18} Some studies have also been devoted to hybrid systems of noble (Ag and Au) metallic nanoparticles (NPs) and supramolecular networks.¹⁹ In this case, supramolecular gels act as scaffold to support the NPs,^{20–22} which in some of cases were prepared in situ,^{23–26} and can find application as antibacterial soft materials^{26–29} or catalysts.³⁰ Some studies

have evaluated the influence of the molecular gel network in the photoluminescence of NPs.^{31–34} Core/shell semiconductor nanocrystals of CdSe/ZnS, or quantum dots (QDs), have been incorporated into molecular gels, affording an improvement in emission quantum yields³⁵ and have been used as sensors of nitric oxide.³⁶

In this work, a soft hybrid system based on IR light upconverting NaYF₄:Yb/Tm nanoparticles (UCNPs) and a fluorescent supramolecular network containing naphthalimide-derived molecules is described. Bulk upconverting lanthanide-based crystals have been known for decades, but there has been a resurgence of their study associated with the preparation of upconverting nanoparticles (UCNPs) about one decade ago.^{37,38} Manipulation of IR radiation using UCNPs has been found to be of much interest because the UV–vis output can be used to promote several chemical processes or to develop a number of applications in the fields of lighting and displays, energy, photocatalysis, sensing, bioanalytics, and theranostics.^{39–45} To our knowledge, only two reports concerning the inclusion of UCNPs in self-assembled organogels are available

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in the literature. UCNP could be dispersed in a supramolecular gel matrix formed by a peptide derivative, reinforcing the gel structure.⁴⁶ However, the gel matrix was acting as a passive scaffold. In another report, a photoactive supramolecular gel formed by a derivative of *trans*-stilbene experienced energy transfer from UCNP.⁴⁷ Unlike the results reported here, the reversibility of the hybrid gel was not studied and the emission of gelator overlapped with that of the UCNP. In this work, there is an optical communication between the UCNP and the organic chromophore, and the light manipulation process is found to be fully reversible and associated with the assembly/disassembly of the gel. Furthermore, the gel provides much improved stability toward aggregation to the UCNP.

EXPERIMENTAL SECTION

The synthesis of the hybrid supramolecular gel is fully described in the [Supporting Information](#) file (SI). In a typical procedure, the organogelator molecule (labeled as compound 1) and the upconverting NaYF₄:Yb/Tm nanoparticles (20%-Yb and 0.5%-Tm molar ratio replacing yttrium ions in the lattice, labeled as UCNP) were first prepared. The hybrid system (UCNP-1gel) was prepared as follows: 20 mg of UCNP was suspended in 2 mL of butanol and sonicated for 5 min in a screw-capped vial. Then, 6×10^{-3} mmol of compound 1 were added and the closed system heated to 80 °C until it was completely solubilized. The system was left to cool down until room temperature for 10 min, and the hybrid gel was formed. The samples were prepared in cylindrical glass vials with different sizes, and the gel dimensions ranged from 10 to 15 mm diameter $\times$ 5–20 mm height.

The characterization by high-resolution transmission electronic microscopy (HRTEM) was carried out on a JEOL-2100 LaB₆ microscope, at an accelerating voltage of 200 kV, with an Inca Energy TEM 200 (Oxford) energy dispersive X-ray spectroscopy (XEDS). The gel samples were deposited over Ni grids coated with a carbon film before TEM observation. The absorption spectra were measured on a Cary 500 Scan UV–vis–NIR spectrophotometer (Varian) equipped with an integrating sphere. The upconversion spectra were measured using an infrared laser diode (model RLMDL-980-2W, Roithner LaserTechnik, 980 nm $\pm$ 5 nm, 2 W continuous waveform, stability <5%, laser head 141 $\times$ 46 $\times$ 73 nm) as the pump source. The emission spectra were measured at 1 W output with a focus lens, providing 105 W cm⁻² optical power density on the sample. A StellarNet EPP2000-UV–vis spectrometer was employed for the fluorescence detection in the visible region. Additional experiments on the hybrid system were carried out in a transparent cuvette using the setup shown in the [SI](#) using a spectrofluorimeter JASCO FP-8300.

RESULTS AND DISCUSSION

Compound 1 (see [Figure 1](#)) is a low molecular weight gelator derived from 1,8-naphthalimide chromophore. When compound 1 is dissolved in a hot solution of butanol and the clear solution is left to cool down to room temperature, supramolecular gels are formed. The minimum concentration required for gelation (mgc) was 5 mM with a transition from gel to solution (T_{gel}) taking place at 65 °C using a vial inversion test. Transmission electron microscopy of the xerogel revealed the usual entanglement of fibers observed commonly in supramolecular gels ([Figure S1](#), [Figure S1](#) from [Supporting Information](#) file). The 1,8-naphthalimide unit introduced in the

Figure 1. Overlaid absorption spectrum of compound 1 in butanol (dotted line) and emission spectrum of UCNP (λ_{exc} = 980 nm, 105 W cm⁻² optical power density).

gelator is a well-known fluorophore,⁴⁸ presenting light absorbance at λ_{max} = 335 nm and emission at λ_{max} = 410 nm (see [Figure 1](#) and [Figure S2](#)). NaYF₄:Yb³⁺/Tm³⁺ upconverting nanocrystals (UCNP) prepared following a simple solvothermal procedure (see [SI](#))⁴⁹ produce different emissions with maximum intensity at wavelengths of 345, 355, 450, 475, and 650 nm ([Figure 1](#)) upon excitation at 980 nm. The hybrid system UCNP-1gel was designed in such a way that the absorption of the gelator overlaps with the two emission bands of the UCNP located at ca. 350 nm ([Figure 1](#)), and an energy transfer from the UCNP to the chromophoric residue could take place (see adapted Jablonsky diagram in [Figure S8](#)).

Examination of the fluorescent properties of the pure gel formed by compound 1 revealed a strong aggregation induced emission effect upon excitation with 335 nm light.⁵⁰ This means that when the gel is heated from 20 to 80 °C a dramatic decrease of the 410 nm emission is observed, associated with a progressive gel disassembly ([Figure 2](#)). This behavior is

Figure 2. Variable temperature study of the emission spectra of the gel of compound 1 (λ_{exc} = 335 nm). Intensity at λ_{max} is normalized to 1 for the spectrum at 20 °C.

opposite to that observed by a 4-amino-1,8-naphthalimide analogue which was studied by us recently.¹⁶ Under 980 nm excitation, the pure gel did not exhibit any emission.

The hybrid system containing the UCNP within the fibrillary network has a good translucent/transparent (depending on the concentration of UCNP and the cooling rate of the gel) appearance. [Figure 3](#) left shows the picture of the hybrid UCNP-1gel under natural, UV, and NIR light. Electron

Figure 3. (Left) Pictures of the hybrid UCNP-1 gel under natural light, UV lamp excitation, and 980 nm laser irradiation. (Right) Transmission electron microscopy image of the hybrid UCNP-1 gel.

microscopy revealed that the hybrid material is formed by thin self-assembled fibers isolated and well-dispersed UCNP mostly stacked at the surface of the fibers (Figure 3 right). It could be argued that the solvophobic oleic acid units covering the UCNP provide anchorage points to the surface of the fibers as described, for example, for the interaction with polymers.^{51,52}

Next, the emission of UV-vis light upon irradiation of the UCNP-loaded gels with 980 nm IR light was studied. It was found that the emission of the UCNP at ca. 350 nm disappears and the emission band at 410 nm emerges (Figure 4). This is a consequence of the overlapped emission of the UCNP and the absorption of compound 1, as expected.

Figure 4. Overlay of the emission spectra of UCNP (dotted line) and hybrid UCNP-1 gel (upon 980 nm excitation). Intensity at λ_{max} for both systems is normalized to 1.

Interestingly, the hybrid system only originates IR-promoted emission at 410 nm in the gel state (Figure 5), with this process being canceled when the gel is disassembled at 80 °C, a behavior directly related to the aggregation induced emission properties of compound 1. The system showed good reversibility and several heating-cooling cycles could be performed, restoring the upconversion to 410 nm at low temperatures. Consequently, the system formed by UCNP-1gel constitutes a thermally regulated light upconverting soft material.

It is important to remark that the light emitted by UCNP at ca. 350 nm is absorbed by compound 1 when the hybrid system is either in the gel state (at 30 °C-assembled) or in the sol state (80 °C-disassembled) (see Figure 5). This fact has implications

Figure 5. Top: Variable temperature study of the emission spectra of the hybrid UCNP-1gel ($\lambda_{\text{exc}} = 980$ nm). Bottom: Variation of the emission intensity at 410 nm for the hybrid system UCNP-1 upon heating-cooling cycles. The dotted line is used as a guide to the eye ($\lambda_{\text{exc}} = 980$ nm).

for the mechanism of energy transfer taking place in the system. At 80 °C the fibers are disassembled and spatial proximity between UCNP and naphthalimide units is precluded, discarding a dipole-dipole energy transfer mechanism such as resonance energy transfer (RET).⁵³ Therefore, a photon reabsorption process, also known as the inner filter effect, emerges as the most plausible mechanism for energy transfer between UCNP in both gel and solution states although RET cannot be discarded to take place in the former case. The inner filter effect has been used in sensing applications based on UCNP.⁵⁴

Aside from the previous results, it is noteworthy that the emission intensity of the UCNP's in the presence of the supramolecular gel is clearly improved. Variable temperature studies were carried out for suspensions of the UCNP's in butanol in the presence and absence of compound 1. As can be seen in Figure 6, the emission of the UCNP's at 475 nm

Figure 6. Variable temperature study of the emission intensity at 475 nm of UCNP's (suspended in butanol) and hybrid UCNP's-1 gel. Values are normalized taking as reference the intensities measured at 90 °C for both systems ($\lambda_{\text{exc}} = 980$ nm).

measured in the range 20–90 °C varies weakly, being moderately higher at low temperatures. However, in the presence of gelator 1 a very notable dependence with temperature was detected. It is important to recall that in the range 20–90 °C a progressive thermal disassembly of the gel network takes place and the system is converted from a gel to a solution. For the sake of comparison, it was decided to analyze the behavior of both samples, with and without gelator, normalizing the results to the emission intensity measured at 90 °C for pure UCNP's. This seems reasonable because in both cases free and disperse UCNP's and no aggregates are present. In this way, the fluorescence intensity at 20 °C in the presence of the gelator is much higher than that observed in the presence of UCNP's alone, with a 6-fold increase (Figure 6).

The process was found to be reversible and after a heating–cooling cycle, the emission was restored to initial values. These results indicate an improvement of the lanthanide nanocrystals emission ascribable to their interaction with the fibrillar network. A plausible rationale for this behavior is based on the partial isolation from the solvent experienced by the UCNP's upon adsorption on the gel fibers, avoiding in this way strong quenching effects from the high vibrational states of the hydroxyl groups of butanol. Such quenching effects have been demonstrated unequivocally in the case of water molecules and other alcohols.^{55,56} It is also important to mention that the fibrillar network avoids the clustering of the UCNP's, leading to a more homogeneous and uniform luminescent material. Additionally, the scattering of IR light in the supramolecular gel could also enlarge the interaction of the laser with the UCNP's, contributing to the observed emission.⁴⁷

CONCLUSIONS

We present a new hybrid system combining a supramolecular photonic gel of naphthalimide-derived molecules self-assembled into fibers and upconverting $\text{NaYF}_4\text{:Yb/Tm}$ nanoparticles (UCNP's). The hybrid system presented here manipulates light reversibly as a result of an optical communication between

the UCNP's and the photoactive gel network. Radiative excitation energy transfer occurs very efficiently affording a transformation of the light emitted from the nanoparticles. Upon NIR radiation, the UCNP's emission overlapping the absorption of the gel chromophore is removed and a new emission from the 1,8-naphthalimide acceptor appears at 410 nm. This fact corresponds to a light-harvesting process. This process is only active in the form of gel as a result of the aggregation induced emissive properties of the supramolecular gelator. Reversible gel disassembly is promoted by temperature changes, giving place to a temperature regulated tunable photonic soft material. Additionally, the interaction of the UCNP's with the self-assembled fibers fosters the emissive relaxation pathways more than nonradiative decays. This fact is accompanied by a greater exposure of the NIR radiation from the gel scattering, originating an outstanding improvement of the emission intensity. This effect can also be ascribed to isolation from the solvent of the nanoparticles upon interaction with the gel fibers together with IR light scattering produced by the self-assembled gel network. Hybrid UCNP's-gel systems such as those described here are envisaged to be used in applications related to IR-based sensing or light manipulation in general, especially taking into account the possibility of their miniaturization in the form of micro-/nanogels for biomedical applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcc.7b06441.

Experimental details regarding UCNP's and gelator preparation as well as hybrid gel formation and its fluorescence studies (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Hirst, A. R.; Escuder, B.; Miravet, J. F.; Smith, D. K. High-tech applications of self-assembling supramolecular nanostructured gel-

phase materials: From regenerative medicine to electronic devices. *Angew. Chem., Int. Ed.* **2008**, *47*, 8002–8018.

(2) Banerjee, S.; Das, R. K.; Maitra, U. Supramolecular gels 'in action'. *J. Mater. Chem.* **2009**, *19*, 6649–6687.

(3) Steed, J. W. Supramolecular gel chemistry: developments over the last decade. *Chem. Commun.* **2011**, *47*, 1379–1383.

(4) Weiss, R. G. The past, present, and future of molecular gels: what is the status of the field, and where is it going. *J. Am. Chem. Soc.* **2014**, *136*, 7519–7530.

(5) Terech, P.; Weiss, R. G. Low molecular mass gelators of organic liquids and the properties of their gels. *Chem. Rev.* **1997**, *97*, 3133–3159.

(6) Dawn, A.; Shiraki, T.; Haraguchi, S.; Tamaru, S.-i.; Shinkai, S. What Kind of "Soft Materials" Can We Design from Molecular Gels? *Chem. - Asian J.* **2011**, *6*, 266–282.

(7) Escuder, B.; Rodriguez-Llansola, F.; Miravet, J. F. Supramolecular gels as active media for organic reactions and catalysis. *New J. Chem.* **2010**, *34*, 1044–1054.

(8) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. Functional π -gelators and their applications. *Chem. Rev.* **2014**, *114*, 1973–2129.

(9) Weingarten, A. S.; Kazantsev, R. V.; Palmer, L. C.; McClendon, M.; Koltonow, A. R.; Samuel, A. P. S.; Kiebal, D. J.; Wasielewski, M. R.; Stupp, S. I. Self-assembling hydrogel scaffolds for photocatalytic hydrogen production. *Nat. Chem.* **2014**, *6*, 964–970.

(10) Weingarten, A. S.; Kazantsev, R. V.; Palmer, L. C.; Fairfield, D. J.; Koltonow, A. R.; Stupp, S. I. Supramolecular packing controls H-2 photocatalysis in chromophore amphiphile hydrogels. *J. Am. Chem. Soc.* **2015**, *137*, 15241–15246.

(11) Ajayaghosh, A.; Praveen, V. K. π -organogels of self-assembled p-phenylenevinyls: Soft materials with distinct size, shape, and functions. *Acc. Chem. Res.* **2007**, *40*, 644–656.

(12) Vijayakumar, C.; Praveen, V. K.; Ajayaghosh, A. RGB emission through controlled donor self-assemble and modulation of excitation energy transfer: A novel strategy to white-light-emitting organogels. *Adv. Mater.* **2009**, *21*, 2059–2063.

(13) Babu, S. S.; Praveen, V. K.; Ajayaghosh, A. Functional π -gelators and their applications. *Chem. Rev.* **2014**, *114*, 1973–2129.

(14) Desvergne, J.-P.; Olive, A. G. L.; Sangeetha, N. M.; Reichwagen, J.; Hopf, H.; Del Guerso, A. Self-assembling and light-harvesting properties of fluorescent linear condensed aromatic gelators. *Pure Appl. Chem.* **2006**, *78*, 2333–2339.

(15) Del Guerso, A.; Olive, A. G. L.; Reichwagen, J.; Hopf, H.; Desvergne, J. P. Energy transfer in self-assembled n -acene fibers involving ≥ 100 donors per acceptor. *J. Am. Chem. Soc.* **2005**, *127*, 17984–17985.

(16) Felip-León, C.; Díaz-Oltra, S.; Galindo, F.; Miravet, J. F. Chameleonic, light harvesting photonic gels based on orthogonal molecular fibrillization. *Chem. Mater.* **2016**, *28*, 7964–7972.

(17) Duan, P.; Yanai, N.; Nagatomi, H.; Kimizuka, N. Photon upconversion in supramolecular gel matrixes: Spontaneous accumulation of light-harvesting donor-acceptor arrays in nanofibers and acquired air stability. *J. Am. Chem. Soc.* **2015**, *137*, 1887–1894.

(18) Duan, P.; Yanai, N.; Nagatomi, H.; Kimizuka, N. Photon upconversion in supramolecular gel matrixes: Spontaneous accumulation of light-harvesting donor-acceptor arrays in nanofibers and acquired air stability. *J. Am. Chem. Soc.* **2015**, *137*, 1887–1894.

(19) Cametti, M.; Džolić, Z. New frontiers in hybrid materials: Noble metal nanoparticles-supramolecular gel systems. *Chem. Commun.* **2014**, *50*, 8273–8286.

(20) Coates, I. A.; Smith, D. K. Hierarchical assembly - Dynamic gel-nanoparticle hybrid soft materials based on biologically derived building blocks. *J. Mater. Chem.* **2010**, *20*, 6696–6702.

(21) Nanda, J.; Adhikari, B.; Basak, S.; Banerjee, A. Formation of hybrid hydrogels consisting of tripeptide and different silver nanoparticle-capped ligands: Modulation of the mechanical strength of gel phase materials. *J. Phys. Chem. B* **2012**, *116*, 12235–12244.

(22) Chakrabarty, A.; Maitra, U.; Das, A. D. Metal cholate hydrogels: Versatile supramolecular systems for nanoparticle embedded soft hybrid materials. *J. Mater. Chem.* **2012**, *22*, 18268–18274.

(23) Chakrabarty, A.; Maitra, U. Organogels from dimeric bile acid esters: In situ formation of gold nanoparticles. *J. Phys. Chem. B* **2013**, *117*, 8039–8046.

(24) Lu, J.; Wu, J.; Ju, Y. Tuning the aggregation mode to induce different chiralities in organogels of mono- and bis-triterpenoid derivatives and the preparation of gold nanoparticles for use as a template. *New J. Chem.* **2014**, *38*, 6050–6056.

(25) He, H.; Chen, S.; Tong, X.; Chen, Y.; Wu, B.; Ma, M.; Wang, X.; Wang, X. Strong and fast-recovery organic/inorganic hybrid AuNPs-supramolecular gels based on loofah-like 3D networks. *Soft Matter* **2016**, *12*, 957–964.

(26) Das, D.; Maiti, S.; Brahmachari, S.; Das, P. K. Refining hydrogelator design: Soft materials with improved gelation ability, biocompatibility and matrix for in situ synthesis of specific shaped GNP. *Soft Matter* **2011**, *7*, 7291–7303.

(27) Dutta, S.; Kar, T.; Mandal, D.; Das, P. K. Structure and properties of cholesterol-based hydrogelators with varying hydrophilic terminals: Biocompatibility and development of antibacterial soft nanocomposites. *Langmuir* **2013**, *29*, 316–327.

(28) Mandal, S. K.; Brahmachari, S.; Das, P. K. In situ synthesised silver nanoparticle-infused L-lysine-based injectable hydrogel: Development of a biocompatible, antibacterial, soft nanocomposite. *ChemPlusChem* **2014**, *79*, 1733–1746.

(29) Shome, A.; Dutta, S.; Maiti, S.; Das, P. K. In situ synthesized Ag nanoparticle in self-assemblies of amino acid based amphiphilic hydrogelators: Development of antibacterial soft nanocomposites. *Soft Matter* **2011**, *7*, 3011–3022.

(30) Nanda, J.; Biswas, A.; Adhikari, B.; Banerjee, A. A gel-based trihybrid system containing nanofibers, nanosheets, and nanoparticles: Modulation of the rheological property and catalysis. *Angew. Chem., Int. Ed.* **2013**, *52*, 5041–5045.

(31) Sangeetha, N. M.; Bhat, S.; Raffy, G.; Belin, C.; Loppinet-Serani, A.; Aymonier, C.; Terech, P.; Maitra, U.; Desvergne, J. P.; Del Guerso, A. Hybrid materials combining photoactive 2,3-didecyloxyanthracene physical gels and gold nanoparticles. *Chem. Mater.* **2009**, *21*, 3424–3432.

(32) Das, R. K.; Bhat, S.; Banerjee, S.; Aymonier, C.; Loppinet-Serani, A.; Terech, P.; Maitra, U.; Raffy, G.; Desvergne, J. P.; Del Guerso, A. Self-assembled composite nano-materials exploiting a thermo reversible n-acene fibrillar scaffold and organic-capped ZnO nanoparticles. *J. Mater. Chem.* **2011**, *21*, 2740–2750.

(33) Roy, S.; Banerjee, A. Amino acid based smart hydrogel: Formation, characterization and fluorescence properties of silver nanoclusters within the hydrogel matrix. *Soft Matter* **2011**, *7*, 5300–5308.

(34) Yamamoto, K.; An, Z.; Saito, N.; Yamaguchi, M. Fluorescent gold nanoparticles: Synthesis of composite materials of two-component disulfide gels and gold nanoparticles. *Chem. - Eur. J.* **2013**, *19*, 10580–10588.

(35) Wadhavane, P. D.; Galian, R. E.; Izquierdo, M. A.; Aguilera-Sigalat, J.; Galindo, F.; Schmidt, L.; Burguete, M. I.; Pérez-Prieto, J.; Luis, S. V. Photoluminescence enhancement of CdSe quantum dots: A case of organogel-nanoparticle symbiosis. *J. Am. Chem. Soc.* **2012**, *134*, 20554–20563.

(36) Wadhavane, P. D.; Izquierdo, M. A.; Galindo, F.; Burguete, M. I.; Luis, S. V. Organogel-quantum dots hybrid materials displaying fluorescence sensitivity and structural stability towards nitric oxide. *Soft Matter* **2012**, *8*, 4373–4381.

(37) Heer, S.; Lehmann, O.; Haase, M.; Güdel, H. U. Blue, green, and red upconversion emission from lanthanide-doped LuPO₄ and YbPO₄ nanocrystals in a transparent colloidal solution. *Angew. Chem., Int. Ed.* **2003**, *42*, 3179–3182.

(38) Yan, C.; Zhao, H.; Perepichka, D. F.; Rosei, F. Lanthanide ion doped upconverting nanoparticles: synthesis, structure and properties. *Small* **2016**, *12*, 3888–3907.

(39) Zhou, B.; Shi, B.; Jin, D.; Liu, X. Controlling upconversion nanocrystals for emerging applications. *Nat. Nanotechnol.* **2015**, *10*, 924–936.

- (40) Gonell, F.; Haro, M.; Sánchez, R. S.; Negro, P.; Mora-Seró, I.; Bisquert, J.; Julián-López, B.; Gimenez, S. Photon up-conversion with lanthanide-doped oxide particles for solar H_2 generation. *J. Phys. Chem. C* **2014**, *118*, 11279–11284.
- (41) Tang, Y.; Di, W.; Zhai, X.; Yang, R.; Qin, W. NIR-responsive photocatalytic activity and mechanism of $\text{NaYF}_4\text{:Yb,Tm@TiO}_2$ core-shell nanoparticles. *ACS Catal.* **2013**, *3*, 405–412.
- (42) Sedlmeier, A.; Achatz, D. E.; Fischer, L. H.; Gorris, H. H.; Wolfbeis, O. S. Photon upconverting nanoparticles for luminescent sensing of temperature. *Nanoscale* **2012**, *4*, 7090–7096.
- (43) Sedlmeier, A.; Gorris, H. H. Surface modification and characterization of photon-upconverting nanoparticles for bioanalytical applications. *Chem. Soc. Rev.* **2015**, *44*, 1526–1560.
- (44) Chen, G.; Qiu, H.; Prasad, P. N.; Chen, X. Upconversion nanoparticles: Design, nanochemistry, and applications in theranostics. *Chem. Rev.* **2014**, *114*, 5161–5214.
- (45) Chatterjee, D. K.; Gnanasammandhan, M. K.; Zhang, Y. Small upconverting fluorescent nanoparticles for biomedical applications. *Small* **2010**, *6*, 2781–2795.
- (46) Wu, J.; Tian, Q.; Hu, H.; Xia, Q.; Zou, Y.; Li, F.; Yi, T.; Huang, C. Self-assembly of peptide-based multi-colour gels triggered by up-conversion rare earth nanoparticles. *Chem. Commun.* **2009**, 4100–4102.
- (47) Tong, X.; Xiang, J.; Shi, F.; Zhao, Y. Near-infrared light-sensitive supramolecular gel with enhanced visible light upconversion. *Adv. Opt. Mater.* **2016**, *4*, 1392–1396.
- (48) Duke, R. M.; Veale, E. B.; Pfeffer, F. M.; Kruger, P. E.; Gunnlaugsson, T. Colorimetric and fluorescent anion sensors: an overview of recent developments in the use of 1,8-naphthalimide-based chemosensors. *Chem. Soc. Rev.* **2010**, *39*, 3936–3953.
- (49) Wang, M.; Liu, J.-L.; Zhang, Y.-X.; Hou, W.; Wu, X.-L.; Xu, S.-K. Two-phase solvothermal synthesis of rare-earth doped NaYF_4 upconversion fluorescent nanocrystals. *Mater. Lett.* **2009**, *63*, 325–327.
- (50) Mei, J.; Leung, N. L. C.; Kwok, R. T. K.; Lam, J. W. Y.; Tang, B. Z. Aggregation-induced emission: Together we shine, united we soar! *Chem. Rev.* **2015**, *115*, 11718–11940.
- (51) Yi, G.-S.; Chow, G.-M. Water-soluble $\text{NaYF}_4\text{:Yb,Er(Tm)}/\text{NaYF}_4/\text{Polymer}$ core/shell/shell nanoparticles with significant enhancement of upconversion fluorescence. *Chem. Mater.* **2007**, *19*, 341–343.
- (52) Wu, T.; Boyer, J.-C.; Barker, M.; Wilson, D.; Branda, N. R. A “plug-and-play” method to prepare water-soluble photoresponsive encapsulated upconverting nanoparticles containing hydrophobic molecular switches. *Chem. Mater.* **2013**, *25*, 2495–2502.
- (53) Riuttamäki, T.; Hyppänen, I.; Kankare, J.; Soukka, T. Decrease in luminescence lifetime indicating nonradiative energy transfer from upconverting phosphors to fluorescent acceptors in aqueous suspensions. *J. Phys. Chem. C* **2011**, *115*, 17736–17742.
- (54) Sun, L.-N.; Peng, H.; Stich, M. I. J.; Achatz, D.; Wolfbeis, O. S. pH sensor based on upconverting luminescent lanthanide nanorods. *Chem. Commun.* **2009**, 5000–5002.
- (55) Liu, C.; Wang, H.; Li, X.; Chen, D. Monodisperse, size-tunable and highly efficient $\beta\text{-NaYF}_4\text{:Yb,Er(Tm)}$ up-conversion luminescent nanospheres: controllable synthesis and their surface modifications. *J. Mater. Chem.* **2009**, *19*, 3546–3553.
- (56) Boyer, J.-C.; Manseau, M.-P.; Murray, J. I.; van Veggel, F. C. J. M. Surface modification of upconverting NaYF_4 nanoparticles with PEG–phosphate ligands for NIR (800 nm) biolabeling within the biological window. *Langmuir* **2010**, *26*, 1157–1164.