Multiplex Optical Detection System
for Prosthetic Joint Infection Diagnosis
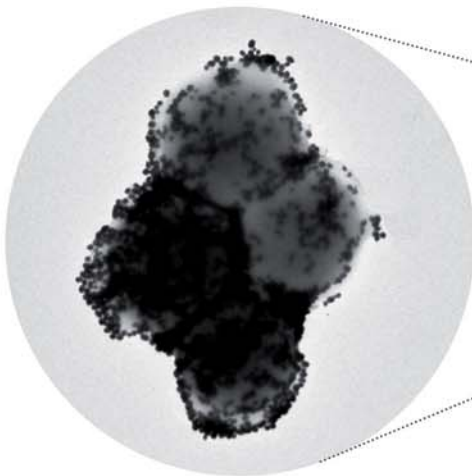
Bernat Mir de Simón

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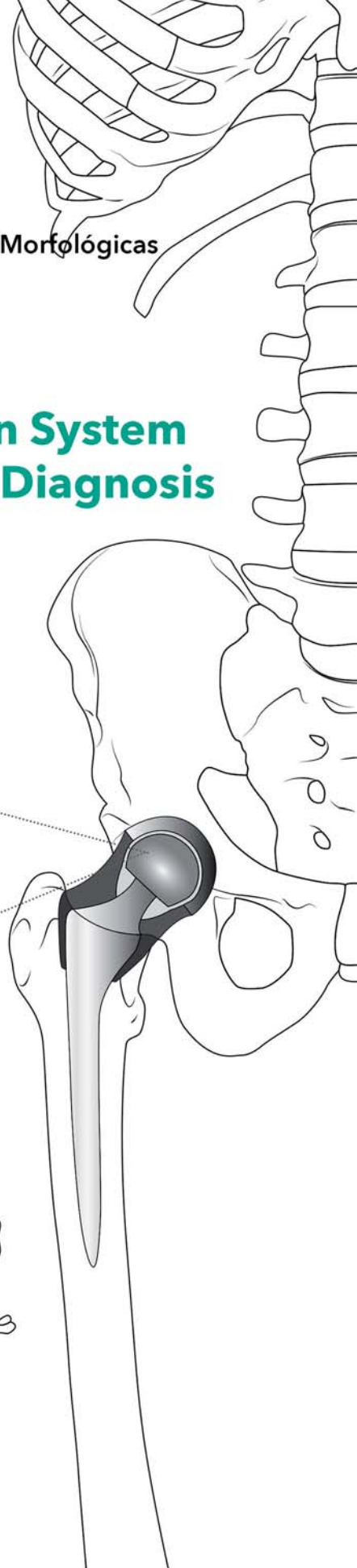
4 Synthesis and characterization of SERS encoded nanoparticles

4.1 Introduction

Encoded nanoparticles are among the most powerful alternatives for high-throughput multiplex screening[23,148] in microarray technology,[149] diagnosis[146] and bioimaging.[150] These materials are simple and cost-effective platforms which allow for fast, sensitive and reliable analysis.[23,151–156] Over the last decade, several encoded particles were prepared[157–159] using codification strategies based on changes in particle shape,[160] composition,[161] physical marks[159] or spectroscopic properties (e.g. luminescence or vibrational fingerprints).[150,162–164] Among all of them, those based on surface-enhanced Raman scattering (SERS) are gaining importance.[165] That gain is due to the virtually unlimited multiplexing capability associated with the unique vibrational fingerprint of the different codes, the short detection times (milliseconds) thanks to the intrinsic sensitivity of the SERS phenomena,[166] the small size that allows for bioimaging[167–169] as well as photostability and low toxicity (as compared with those of dyes or quantum dots).[170]

In essence, a SERS-encoded nanoparticle comprises a plasmonic nucleus that generates the electric field necessary for the Raman amplification, a Raman probe (i.e. code) that has the unique vibrational fingerprint of the encoded particle and a coating layer. This coating is of key importance as it protects the plasmonic particle from contaminations by the medium that may give rise to vibrational noise hindering the particle readout, increases the colloidal stability of the particle and provides a convenient surface for further chemical functionalization.

Although polymers have been reported as particles coatings,[169,171,172] the unique properties of silica (i.e. known surface chemistry, biocompatibility, optical transparency and colloidal stability) make this material the most used protective layer for nanoparticles.[173] Even so, silica coating requires the colloidal stabilization of the particles in an ethanolic solution prior to the hydrolysis/condensation of tetraethyl orthosilicate (TEOS). Though a range of polymers have been described for this task,[173] the most common is still polyvinylpyrrolidone (PVP).[173] Surfactants such as cetyltrimethyl

ammonium bromide (CTAB) are also commonly used for this reaction.[173] Besides, silica coating is also used to protect the plasmonic core from interacting with other plasmonic particles, avoiding plasmon coupling and thus the generation of hot-spots.

On the other hand, with the exception of nanogapped core-shell nanoparticles,[174,175] the most important factor for the generation of active SERS-encoded particles relies in the intimate contact between the Raman code and the plasmonic structure. This requirement introduces further complexity to the coating process associated with the surface chemistry properties. Both PVP and CTAB form solid layers of coating on the surface of the particles limiting or even prevent the interaction of the encoding agent with the metallic particle when added to the solution.[145,176] Therefore, to increase code adsorption efficiency on the plasmonic structure, and thus the SERS signal, PVP and CTAB species need to be removed from the metallic surfaces. This usually results in a drastic reduction in the colloidal stability, which is further aggravated by the non-polar nature of most of the codes, leading to uncontrolled particle agglomeration[166,177,178] or even to irreversible precipitation. Aggregation of labelled-nanoparticles into clusters of different size and geometry does generate very active SERS structures but with highly inhomogeneous SERS response. Moreover, these fabrication methods normally work for a very limited number of encoding molecules as, in many cases, precipitation of the whole colloids occurs upon addition of the code (unless for very low label amounts). In fact, this explains why examples of SERS encoded particles in most of the literature include a small number of codes, usually just three or four.

As an alternative to the conventional polymers or surfactants, thiolated poly(ethylene glycol) had been employed as a coating layer. The high polarity and porosity of this polymer efficiently stabilize particles in alcohol and water while allowing for the, at least, partial diffusion of the code to the metallic surface.[179]

Importantly, polymers commonly suffer from large fluctuations in size distribution from batch-to-batch. This is even true for the same commercial brand. As a result, the synthetic protocol to encode particles using these

materials often needs to be retuned when a new polymer is purchased. Additionally, the high price of the thiolated poly(ethylene glycol) hinders its use in the large-scale preparation of encoded particles as required for real life applications. In contrast to most of the reported procedures which typically involve complex steps, herein an easy and fast one-pot approach for the production of SERS-encoded nanoparticles was demonstrated. Several studies in SERS-encoded particles are carried out on spherical gold nanoparticles.[169,180] Therefore, citrate-capped gold nanoparticles were selected as the initial plasmonic material in this work. However, the encoding protocol can be readily applied to other metals like silver colloids or nanoparticle shapes such as gold nanostars.

This versatile strategy allows for the SERS codification of particles with a huge number of molecules that has affinity to the metal surface. To demonstrate this, 31 molecules with different chemical nature were choosen for the fabrication of encoded nanoparticles using exactly the same standard procedure. The method relies on the controlled co-absorption of 11-mercaptoundecanoic acid (MUA) and the Raman code on the metallic surfaces. In addition to the easiness of preparation, scalability to the liter regime, and stability in aqueous solutions, the MUA stabilized SERS-encoded particles show considerably higher optical efficiency than those fabricated by using HS-PEG$_{5000}$ or PVP polymers.

4.2   Experimental section

4.2.1 Materials and methods

Gold(III) chloride trihydrate (99.9 %, HAuCl$_4$·3H$_2$O), trisodium citrate dehydrated (≥99.5 %, C$_6$H$_5$Na$_3$O$_7$·2H$_2$O), ammonia solution (29%, NH$_4$OH), L-ascorbic acid (≥99.0 % , AA), tetraethoxy silane (99.999 %, TEOS), ethanol (99.5 %, EtOH), polyvinylpyrrolidone (average M.W. 58000, PVP$_{k58}$), polyvinylpyrrolidone (average M.W. 8000, PVP$_{k8}$), cetyltrimethylammonium bromide (99.72%, CTAB), *o*-[2-(3-mercaptopropionylamino)ethyl]-*o′*-methylpolyethylene glycol (M.W. 5000, HS-PEG$_{5000}$), 11-mercapto undecanoic acid (95 %, MUA), 2-mercaptopyridine (97%, MPy), 4-

nitrobenzenethiol (80%, 4NBT), 4-mercaptophenol (97%, 4MP), 4-mercaptobenzoic acid (99%, 4MBA), 3,5-bis(trifluoromethyl) benzenethiol (97%, 35BTFMBT), 4-fluorothiophenol (98%, 4FTP), 2,3,5,6-tetrafluoro benzenethiol (97%, 2356TFBT), 2-(trifluoromethyl) benzenethiol (96%, 2TFMBT), 3-fluorothiophenol (95%, 3FTP), nile blue A (95%, NBA), 2-fluorothiophenol (97%, 2FTP), toluidine blue O (≥84%, TB), benzenethiol (97%, BT), 4-(((3-mercapto-5-(2-methoxyphenyl)-4H-1,2,4-triazol-4-yl)imino)methyl)phenol (97%, MMPHTYIMP), n,n-dimethylformamide (≥90%, DMF) 4-(((3-mercapto-5-(2-pyridinyl)-4H-1,2,4-triazol-4-yl)imino)methyl)benzoic acid (MPHTYIMBA), 4-(((3-mercapto-5-(2-pyridinyl)-4H-1,2,4-triazol-4-yl)imino)methyl)-1,2-benzenediol acid (MPHTYIMBDO), 1-(4-hydroxyphenyl)-1H-tetrazole-5-thiol (97%, HPHTT), 1,1′,4′,1″-terphenyl-4-thiol (97%, TPT), 1-naphtalenethiol (99%, 1NT), 2-naphtalenethiol (99%, 2NT), 5-(4-methoxyphenil)-1,3,4-oxidazole-2-thiol (97%, MPOT), methylene blue (≥82%, MB), 3,4-dichlorobenzenethiol (97%, DCBT), biphenyl-4-thiol (97%, BPT), 7-mercapto-4-methylcoumarin (≥97%, MMC), biphenyl-4-4′-dithiol (95%, BPDT), thiosalicylic acid (97%, TSA), 5-amino-1,3,4-thiadiazole-2-thiol (87%, ATT), 4-aminothiophenol (97%, 4ATP), 2-phenylethanethiol (98%, 2PET), crystal violet (≥90%, CV) and rhodamine 6G (≥90%, R6G) were purchased from Sigma-Aldrich (Germany). All reactants were used without further purification. Mili-Q water (18 MΩ cm^{-1}) was used in all aqueous solutions, and all the glassware was cleaned with aqua regia before the experiments.

4.2.2 Synthesis of citrate-stabilized spherical gold nanoparticles

Spherical gold nanoparticles of approx. 50 nm in diameter were produced by a modification of the well-known Turkevich method.[77] Briefly, a 308 µL aqueous solution of HAuCl$_4$ (0.081 M) was added to a boiling aqueous solution of sodium citrate (100 mL, 0.27 mM) under vigorous stirring. Heating and stirring were continued for 30 min. A condenser was utilized in order to prevent evaporation of the solvent. During this time, the color of the solution gradually changes from colorless to purple to finally

become deep red. After this time, heating was continued and the condenser removed in order to allow the evaporation of the solvent to the half of its initial volume, to achieve a final $[Au] = 5 \times 10^{-4}$ M.

4.2.3 Mercaptoundecanoic acid funcionalization of gold nanoparticles

In order to provide colloidal stability to the Au nanoparticles during the encoding process and to later on promote silica growing, 50 mL of the as produced spherical gold nanoparticles were functionalized with a small amount of MUA (0.8 molecules nm^{-2}). To this end, a solution containing NH_4OH (879 µL, 29% aqueous solution) and MUA (1 mL, 3.99×10^{-5} M in EtOH) was prepared. This solution was then rapidly added under vigorous stirring to the gold nanoparticles sol (50 mL). Agitation was continued for 30 min to assure MUA functionalization on the Au surface.

4.2.4 Gold nanoparticles codification

With the aim to prove the versatility of the presented method, 31 different SERS active molecules were used MPy, 4NBT, 4MP, 4MBA, 35BTFMB, 4FTP, 2356TFBT, 2TFMBT, 3FTP, NBA, 2FTP, TB, BT, MMPHTYIMP, HPHTT, TPT, 1NT, 2NT, MPOT, MB, DCBT, BPT, MMC, BPDT, CV, 2PET, 4ATP, ATT, TSA, MPHTYIMBDO, MPHTYIMBA. The exact same procedure was used for each molecule. Briefly, a solution containing EtOH (324.89 mL) and NH_4OH (5.73 mL, 29% aqueous solution) was prepared. This solution was then rapidly added under vigorous stirring to the MUA stabilized gold nanoparticles (51.88 mL). Next, a stock solution of the SERS active molecule was prepared (10^{-2} M, in EtOH) and 74.8 µL of this solution was added to the Au MUA functionalized particles (382 mL) under strong magnetic stirring for 30 min (to assure proper Au functionalization). The amount of SERS active molecules added was calculated to be 15 molecules nm^{-2}.

4.2.5 Synthesis of citrate-stabilized spherical silver nanoparticles

Spherical silver nanoparticles (Ag NPs) of approx. 50 nm in diameter were produced by adding AA (50 µL, 0.10 mM) to 47.5 mL of boiling water. 1 min after the addition, a previously 5 min incubated solution of AgNO$_3$ (0.25 mL, 1 wt %), sodium citrate (1 mL, 1 wt %) and 1.25 mL Milli Q water was injected into the boiling aqueous solution. The color of the reaction solution quickly changes from colorless to yellow. The solution was further boiled for 1 h under stirring to guarantee the formation of uniform quasi-spherical silver nanoparticles.[181]

Then, the synthesized Ag NPs were cleaned by centrifugation (5400 rpm, 30 min) and redispersed in a volume of Milli Q water to achieve a silver concentration of 2.5×10^{-4} M (Figure 18A).

4.2.6 Mercaptoundecanoic acid funcionalization and codification of silver nanoparticles

In order to provide colloidal stability during the encoding process Ag NPs were then functionalized with MUA (1.8 molecules nm^{-2}). Specifically, 5 ml of Ag NPs ([Ag]= 2.5×10^{-4} M) were rapidly added to a solution containing NH$_4$OH (662 µL, 29% aqueous solution), MUA (41.6 µL, 1.0×10^{-4} M in EtOH) and EtOH (32,50 mL) under vigorous stirring. Agitation was continued for 1 h to assure the complete MUA functionalization on the Ag surface. Then, 3.8 µL of a 10^{-2} M stock solution of different SERS probes (4MBA, DBT and 35BTFMBT) were added under strong magnetic stirring. Once more, stirring was continued for another 1 h. The amount of SERS active molecules added was calculated to be 15 molecules nm^{-2}.

4.2.7 Silica encapsulation of gold nanoparticles

The silica encapsulation of the encoded nanoparticles was achieved through a modified-Stöber method using the MUA carboxylic group to promote silica growth as follows: The proper concentrations of H$_2$O,

NH_4OH and EtOH for the silica growth of the MUA-SERS code encoded particles solution was previously adjusted, during the codification step, to yield final concentrations of 7.94 M, 0.128 M and 14.60 M, respectively (the molar ratio $EtOH/H_2O$ = 1.84). Then, TEOS (13.2 µL) was added, the solution was energetically shaken and left undisturbed at room temperature 14 h. Finally, The resulting core-shell NPs were cleaned to remove excess reactants by means of centrifugation (3 × 6000 rpm, 20 min), and then re-dispersed in ethanol. In order to concentrate the solution (10^{-3} M) to perform the SERS characterization, 15.3 mL of this solution were centrifuged again (2 × 6000 rpm, 20 min) and re-dispersed in water. After the final centrifugation step everything was re-suspended in a final volume of 1 mL.

4.2.8 Silica encapsulation of silver nanoparticles

As for the gold NPs, the silica encapsulation of the silver encoded nanoparticles was achieved through a modified-Stöber method using the MUA carboxylic group to promote silica growth. TEOS (12 µL, 10% v/v) was added to a 5 mL of the as produced encoded silver NPs. Then, the solution was energetically shaken and left undisturbed at room temperature 14 h. The solution was centrifuged and redispersed in water following the same procedure as for spherical gold nanoparticles.

Nanoparticle concentration was calculated to be 0.26 nM with Lambert-Beer's law using the extinction coefficient for silver nanoparticles of 5.37 × 10^{10} M^{-1} cm^{-1}, derived from literature.[182]

4.2.9 Synthesis of PVP-based spherical encoded gold nanoparticles

The different SERS active molecules used were 1NT, MPy, 4NBT, 4MBA, NBA, TB and 2356TFBT. Spherical gold nanoparticles of approx. 51 nm in diameter were produced as previously described. To 75 mL of PVP_{k58} solution (0.69 mM), 50 mL of the citrate Au particles were added dropwise and left to react overnight under stirring.

Next, the solution was centrifuged (5400 rpm, 25 min) and redispersed in 50 mL EtOH ([Au] = 0.5 mM), to remove at maximum the excess of PVP_{k58}. This process was repeated four times. Then, the SERS code molecule (74.8 µL; 10^{-2} M) was added under stirring for 2 h. Finally, silica coating process was carried out through adjustment of the final concentrations as follows (in a 50 mL solution): [Au] =0.5 mM, [H_2O] = 10.55 M, [NH_3] = 0.2 M, [EtOH] = 13.4 M and [TEOS] = 1.12 mM. The reaction mixture was allowed to react for 24 h. When the reaction time was completed, the particles were centrifuged and washed with ethanol. In order to concentrate the solution (10^{-3} M) to perform the SERS measurements, 2 mL of this solution were centrifuged again (2 × 6000 rpm, 20 min) and redispersed in water. After the final centrifugation step everything was resuspended in a final volume of 1 mL.

4.2.10   Synthesis of HS-PEG$_{5000}$ based spherical encoded gold nanoparticles

The different SERS active molecules used were 1NT, MPy, 4NBT, 4MBA, NBA, TB and 2356TFBT. First, CTAB-stabilized gold nanospheres of 51 nm in diameter were prepared with the seeded growth approach previously described in the literature[183] as follows: A seed solution was made ready by preparing an aqueous solution (20 mL) containing $HAuCl_4$ (2.5×10^{-4} M) and sodium citrate (2.5×10^{-4} M). While the mixture was vigorously stirred, a $NaBH_4$ (600 µL, 0.1 M) solution was added, observing a fast color change into red which indicates the formation of the gold particles. The seeds were left under stirring in an open atmosphere for 1 h to allow the $NaBH_4$ to decompose. Next, a growth solution was prepared by dissolving CTAB (from Mercks, 100 mL, 0.1 M) and potassium iodide (0.3 mg/gram of CTAB) in Milli-Q water followed by the addition of $HAuCl_4$ (510 µL, 0.103 M) and ascorbic acid (735 µL, 0.1 M). After each addition, the bottles were vigorously shaken. Seeds in the amount of 187 µL were added, and the solution was again vigorously shaken. The flask was left undisturbed at 28 °C for 48 h. After this time, a small amount of gold particles is observed as sediment in the bottom of the flask. Since particles below 100 nm are

stable in solution, this precipitate must be composed of larger Au structures coming from seeds with a different crystallographic structure. Carefully, the supernatant is collected and the precipitate discarded in order to ensure the monodispersity of the particles. The second step involves the HS-PEG$_{5000}$ capping, ethanol transfer, and silica coating. To do this, 100 mL of the as-synthesized Au spheres ([Au] =0.25 mM, [CTAB] =0.1 M) were centrifuged for 20 min (6000 rpm). The precipitate was re-dispersed with a CTAB solution ([CTAB] =0.5 mM) in order to clean at much as possible the CTAB without compromising the colloidal stability of the particles. This process was repeated 3 times to finally be re-dispersed in a final volume of 50 mL to obtain [CTAB] ~0.5 mM and [Au] ~0.5 mM. Next, a stock solution of the HS-PEG$_{5000}$ was prepared and sonicated for 15 min (10^{-3} M, in H$_2$O) and 89.8 µL of this solution was added to the Au CTAB stabilized particles (50 mL) under strong magnetic stirring for 30 min (to assure proper Au functionalization).

The amount added of HS-PEG$_{5000}$ was calculated to be 1.8 molecules nm^{-2}. The HS-PEG$_{5000}$-modified particles were centrifuged twice to remove excess HS-PEG$_{5000}$ and redispersed in ethanol (50 mL). In the second centrifugation the particles were redispersed in a solution (50 mL) adjusting the following final concentrations: [Au] = 0.5 mM, [H$_2$O] = 10.55 M, [NH$_3$] = 0.2 M and [EtOH] = 13.39 M. The third step is the encoding of the nanoparticles. A stock solution of the SERS active molecule was prepared (10^{-2} M, in EtOH) and 74.8 µL of this solution was added to the Au HS-PEG$_{5000}$ functionalized particles (50 mL) under strong magnetic stirring for 2 h. The amount added of SERS active molecules was calculated to be 15 molecules nm^{-2}. Finally, TEOS (13.20 µL) was added and the solution was energetically shaken and left undisturbed at room temperature 14 h. In order to concentrate the solution ([Au] = 1 mM) to perform the SERS measurements, 2 mL of this solution were centrifuged again (2 × 6000 rpm, 20 min) and re-dispersed in water. After the final centrifugation step everything was re-suspended in a final volume of 1 mL. Nanoparticle concentration of ca. 0.24 nM was calculated by Lambert-Beer's law using the extinction coefficient for gold nanoparticles of 4.2 × 10^{10} M^{-1} cm^{-1}, derived from literature.[184]

4.2.11 Synthesis, codification and silica-coating of gold nanostars

The synthesis of gold nanostars (GNS) was performed using a modified PVP-based method.[185] In the first step, spherical gold seeds of approx. 10 nm in diameter were produced by a modification of the well-known Turkevich method.[77] Briefly, a solution of sodium citrate in MilliQ water (150 mL, 1.93 mM) was heated with a heating mantle in a 250 mL three-necked round bottom flask for 15 minutes under vigorous stirring. A condenser was utilized in order to prevent the evaporation of the solvent. After boiling had commenced, 308 μL of $HAuCl_4$ (0.081 M) were injected. The color of the solution changed from yellow to bluish grey and then to soft pink in 10 minutes and after 30 min to wine-red. The resulting particles were coated with negatively charged citrate ions and hence well suspended in H_2O. Then, seeds were concentrated in a 250 mL beaker to a [Au] = 9.45×10^{-4} M with a constant flow of N_2 during 120 min. In the second step, a solution of PVP_{k8} (15 mM) in DMF (35 mL) was sonicated during 15 min. Then, 216 μL of $HAuCl_4$ (0.081 M) was added to the mixture under rapid stirring at room temperature, followed by the addition of the concentrated seeds (1.108 mL, [Au] = 9.45×10^{-4} M). Within 5 min, the color of the solution changed from pink to blue, indicating the formation of gold nanostars. The solution was left under stirring overnight. Then, excess of PVP_{k8} was removed under a six-fold centrifugation (7000 rpm, 15 min) and redispersed in ethanol (particles solution was adjusted, to yield a final concentration of [Au] = 4.5×10^{-4} M) (Figure 18B).

The so-synthesized GNS were then functionalized with MUA (0.8 molecules nm^{-2}) following the previously described protocol. Specifically, a solution containing NH_4OH (87.9 μL, 29% aqueous solution) and MUA (31.6 μL, 1.0×10^{-4} M in EtOH) was rapidly added under vigorous stirring to 5 mL of GNS suspension ([Au] = 4.5×10^{-4} M). Agitation was continued for 3 h to assure complete MUA adhesion on the Au surface. Next, 5.9 μL of a 10^{-2} M stock solution of different SERS probes were added under strong magnetic

stirring. Once more, stirring was continued for another 3 h. The amount of SERS active molecules added was calculated to be 15 molecules nm^{-2}.

Finally, the same modified-Stöber method applied to spherical gold nanoparticles was followed to obtain a homogenous silica shell around the Raman-labelled GNS. The colloidal suspension concentrated to [Au] = 1 mM prior to the SERS measurements.
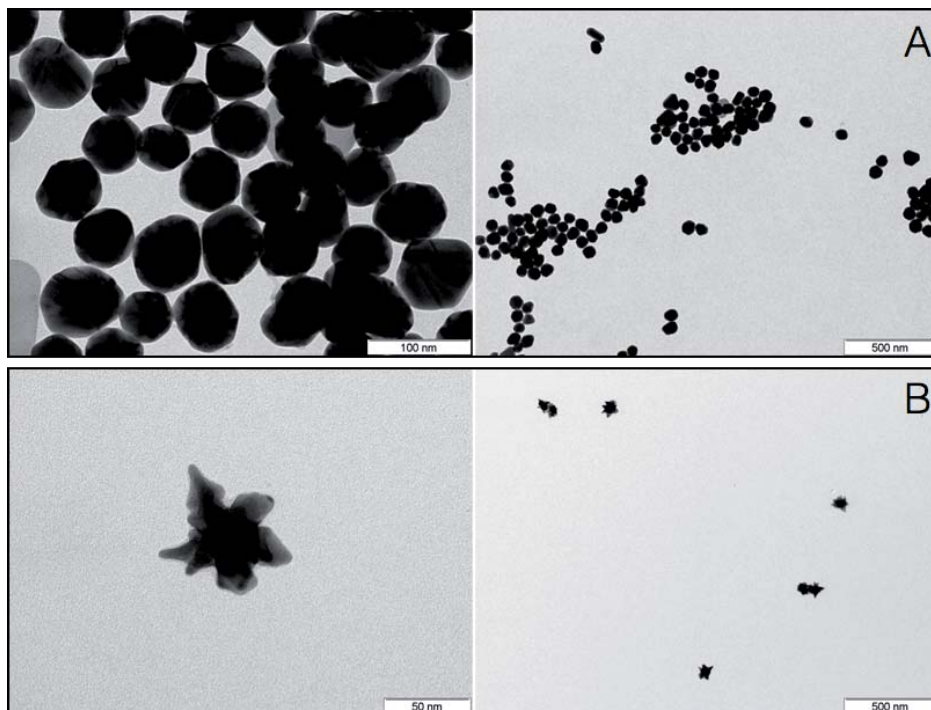

Figure 18. (A) TEM micrographs of citrate capped silver nanospheres and (B) PVP capped gold nanostars.

4.2.12   Characterization

UV-VIS spectroscopy (PerkinElmer, Lambda 19) and transmission electron microscopy (TEM, LEO 922 EFTEM operating at 80 kV) were applied to characterize the optical response, structure and size of the nanoparticles during the encoding process. SERS spectra were collected in backscattering geometry with a Renishaw Invia Reflex system equipped with a 2D-CCD detector and a Leica confocal microscope. The spectrograph used a high resolution grating (1200 grooves cm^{-1}) with

additional band pass filter optics. A 785 nm diode laser was focused onto the colloidal solution ([Au] = 1 mM) by a long-working distance objective (0.17 NA, working distance 30 mm). The spectra were acquired with an exposure time of 1 s (depending on Raman Intensity saturation) and a laser power at the sample of ca. 300 mW, using the Renishaw's StreamLine accessory.

4.3 Results and discussion

Due to the low stability of colloidal solutions upon functionalization with many Raman codes, a stabilization step is required prior to codification in order to develop a universal protocol. A schematic outline of this universal protocol for the fabrication of encoded particles is illustrated in Figure 19. The process can be divided in four different steps; 1) synthesis of citrate-capped plasmonic nanoparticles of ca. 50 nm diameter (Figure 20); 2) MUA stabilization; 3) SERS codification; and, 4) silica coating (if needed).
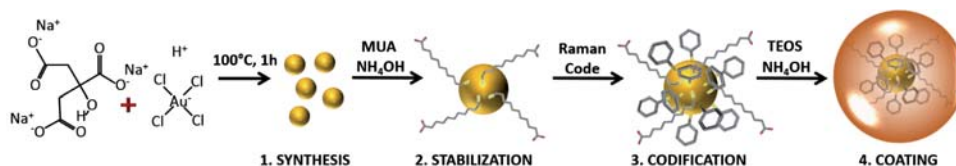

Figure 19. Schematic representation of the synthetic procedure for the production of SERS-encoded nanoparticles showing the different steps involved in the synthesis. First, citrate capped plasmonic nanoparticles are produced. Second, MUA is used to stabilize the particles in basic media. Third, SERS code is added to encode the particles. Fourth, a silica shell can be grown on the particles, as an optional step, to ensure stability for long periods of time and avoid plasmon coupling.

MUA was chosen as the stabilizing agent because it binds covalently to the gold surface through the thiol group while providing particle stability with both the long aliphatic chain (steric repulsion) and the final carboxylic group (electrostatic repulsion). On the other hand, its SERS cross section is almost negligible as compared with those of aromatic compounds due to its aliphatic nature (Figure 21).[186,187]
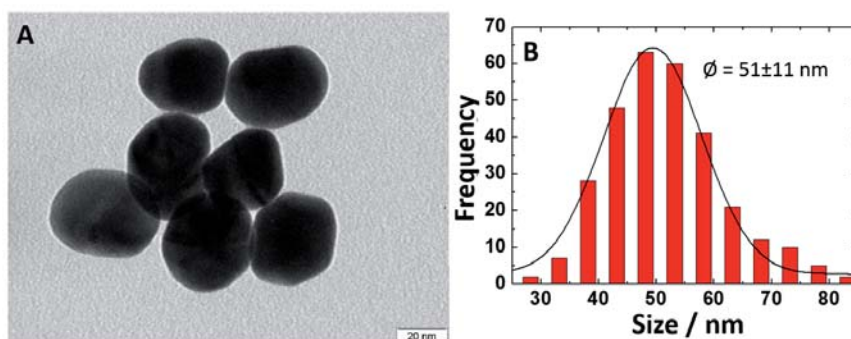
Figure 20. Representative TEM image (A) and size distribution histograms (B) of the synthesized citrate-capped gold nanoparticles.

Notwithstanding the presence of a thiol group implies that MUA should be added in the adequate proportion to avoid the formation of a compact monolayer that may passivate the metallic surface preventing the retention of the SERS codes and with extreme care to avoid heterogeneous adsorption of the molecule by some of the colloids of the solution. Thus, in a second step, MUA was rapidly added under vigorous stirring at basic pH to yield MUA functionalized gold nanoparticles (Au@MUA).
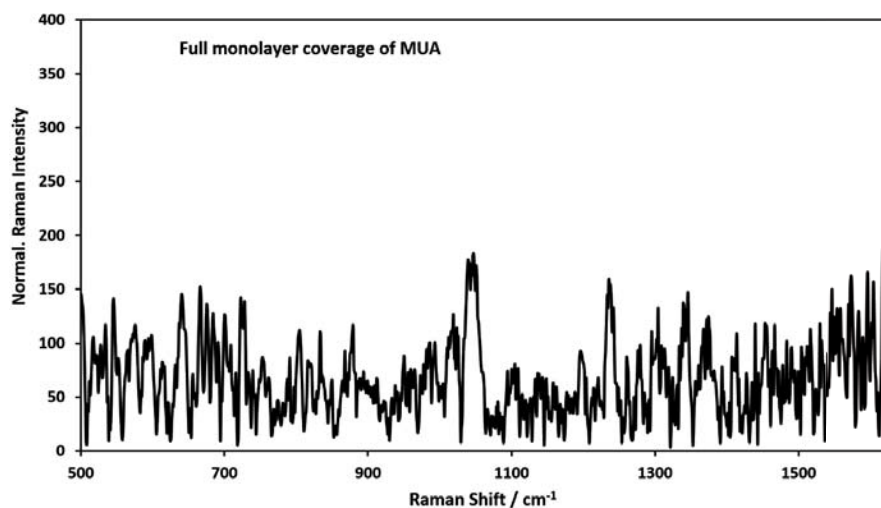

Figure 21. SERS spectra of Au particles coated with a full monolayer of MUA showing its small Raman cross section.
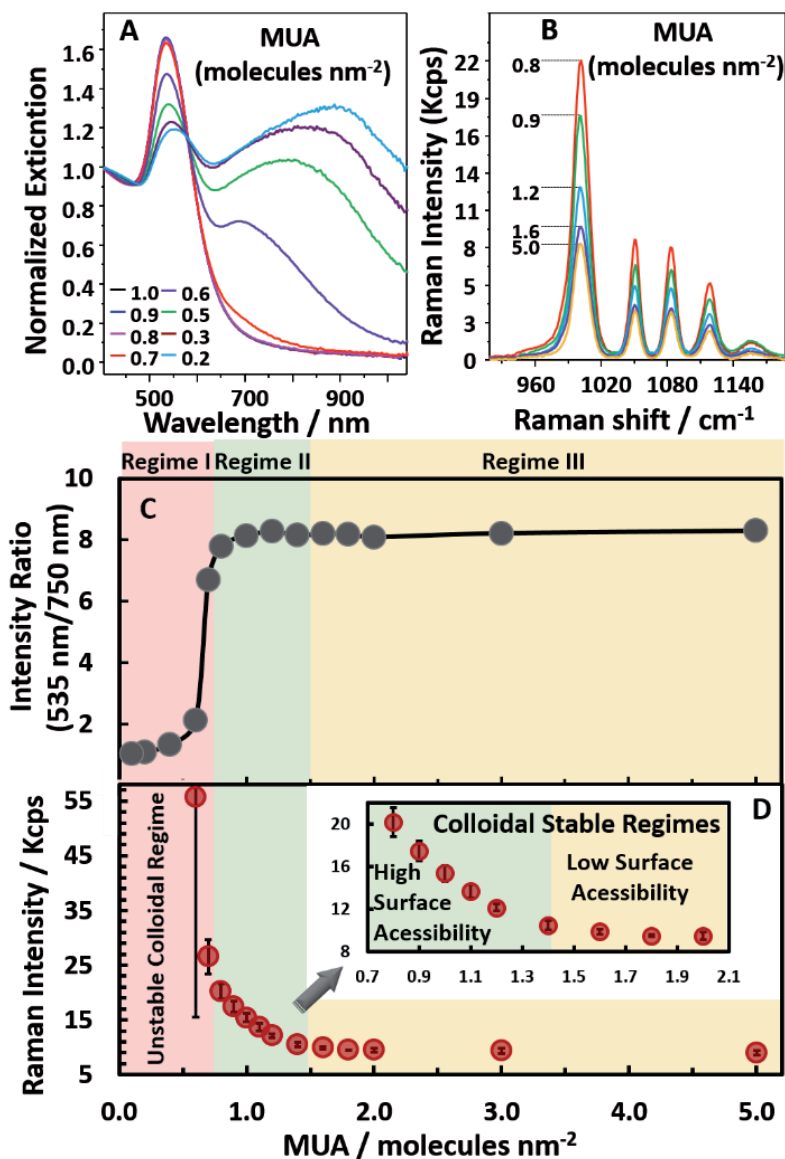
Figure 22. (A) Extinction spectra of the Au@MUA/MPy nanoparticles with different amounts of MUA (molecules nm^{-2}). MPy was always added in a large excess to guarantee the full coating of the nanoparticles. Below 0.8 MUA molecules nm^{-2}, it is possible to recognize an unstable colloidal regime (nanoparticles start aggregating). (B) SERS spectra of Au@MUA/MPy colloids ([Au] = 1 mM) with different amount of MUA (molecules nm^{-2}). (C) Intensity ratio between the Abs at 535 nm and the Abs at 750 nm for Au@MUA/MPy nanoparticles as a function of different amount of MUA. (D) SERS intensity of Au@MUA/MPy colloids ([Au] = 1 mM) as a function of different amount of MUA.

In order to maximize the final SERS efficiency of the encoded NPs, the MUA surface coverage was decreased as much as possible to provide maximum accessibility to the metal surface while preserving overall colloidal stability when exposed to an excess of the SERS code. The addition of the code is depicted as the third step in Figure 19. Among all the investigated encoding molecules, 2-mercaptopyridine (MPy) was observed to induce the fastest colloidal aggregation upon addition to the bare citrate-capped gold nanoparticles. For this reason, optimization of the protocol was performed by using this molecule (i.e. the worst colloidal stability scenario). MUA has been reported to take up an area of 0.22 nm^2, corresponding to ca. 4.5 molecules nm^{-2}. [188] Therefore, experiments decreasing the amount of MUA were designed in between 5.0 to 0.1 molecules nm^{-2}. After waiting for MUA adsorption to reach its thermodynamic equilibrium (30 min), MPy was added in a large excess (15 molecules nm^{-2}) to yield the corresponding Au@MUA/MPy nanoparticles. Aggregation of the colloidal system was monitored by UV-Vis-NIR spectroscopy (Figure 22A) by comparing the absorption of the resulting solutions at 535 nm, associated with isolated gold nanoparticles, and that at 750 nm, attributed to plasmonic contributions of interacting particles indicative of aggregation (Figure 22C).

Concurrently, SERS was also monitored in the same samples to estimate the amount of adsorbed code (Figure 22B). The SERS measurements were performed by focusing a 785 nm laser onto the colloidal suspension ([Au] = 1 mM) with a long working-distance objective. Between 5-0.8 MUA molecules nm^{-2} (stable colloidal regime), the extinction spectra of Au@MUA/MPy exhibit the characteristic LSPR of monodisperse spherical gold nanoparticles in suspension. At 0.7 molecules nm^{-2}, the appearance of a shoulder at ca. 700 nm is observed, indicating the significant formation of nanoparticle aggregates. A further decrease of the MUA surface coverage leads to a dramatic perturbation of colloidal stability upon addition of the MPy, as clearly revealed by the dominant plasmonic contribution at a longer wavelength. Therefore, the range of colloidal stability was identified as between 5 to 0.8 MUA molecules nm^{-2}.
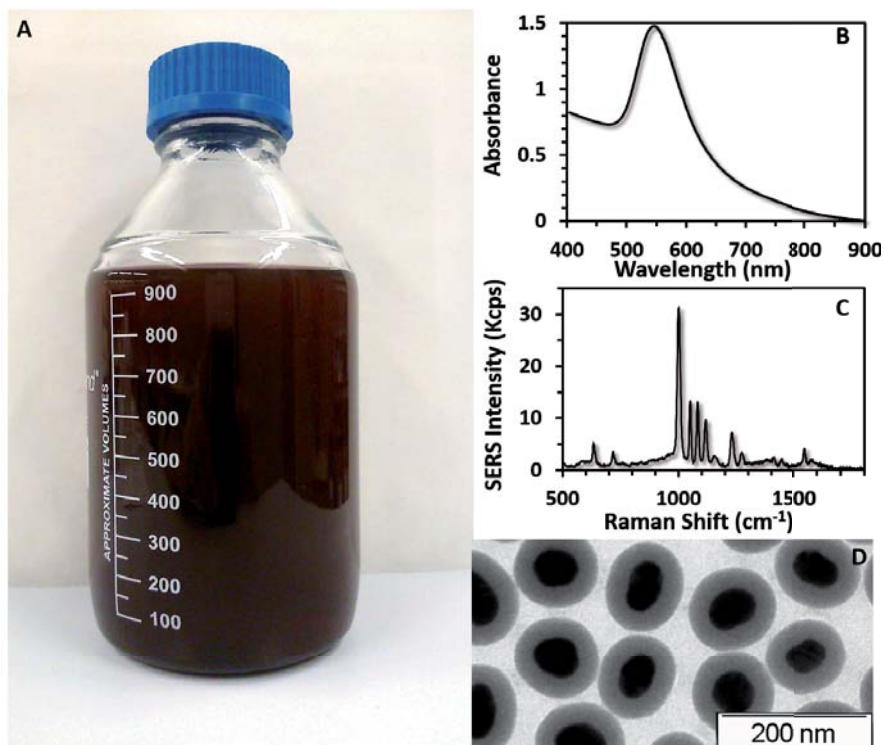
Figure 23. (A) Photography of the one-pot synthesis of a liter batch of MPy encoded nanoparticles. (B) UV-Vis, (C) SERS spectra, and (D) TEM image of the encoded nanoparticles.

Conversely, the monitoring of the SERS intensity of the MPy ring breathing mode at 1001 cm^{-1} (Figure 22D) reveals the existence of three SERS regimes. The first corresponds to the particle aggregation (below 0.8 MUA molecules nm^{-2} range) and, as expected, shows a remarkable increase in the intensity due to the uncontrolled plasmon coupling (see also the very large standard deviation). The second, between 1.4-0.8 MUA molecules nm^{-2}, reveals a progressive decrease in the SERS intensity of MPy as the MUA content increases. In this regime, an increase in MUA surface coverage is directly reflected in the decrement of code adsorption onto the metal surface. In the third regime, 1.6-5 MUA molecules nm^{-2}, the SERS intensity remains constant as MUA forms a progressively full monolayer and only a fixed amount of MPy molecules can diffuse onto the nanoparticles surfaces. Notably, for small molecules such as MPy, SERS intensity never decays to zero. This is because, differently to the crystalline arrangement of the densely packed films of alkanethiols on gold

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surfaces,[189] the coulomb repulsions between the negatively carboxylic groups of MUA limit the lateral interactions of the hydrophobic alkyl chains preventing the formation of a thick molecular packing on the surface.[190] Clearly, accessibility to the metal surface in this particular regime is highly dependent on the chemical and geometrical properties of the SERS code. For instance, for MUA concentration of 4 molecules nm^{-2} almost no SERS signals are observed for large molecular codes. Therefore 0.8 molecules nm^{-2} was identified as the optimum MUA concentration for the production of SERS-encoded gold nanoparticles, preventing colloidal aggregation and maximizing the final SERS signal.
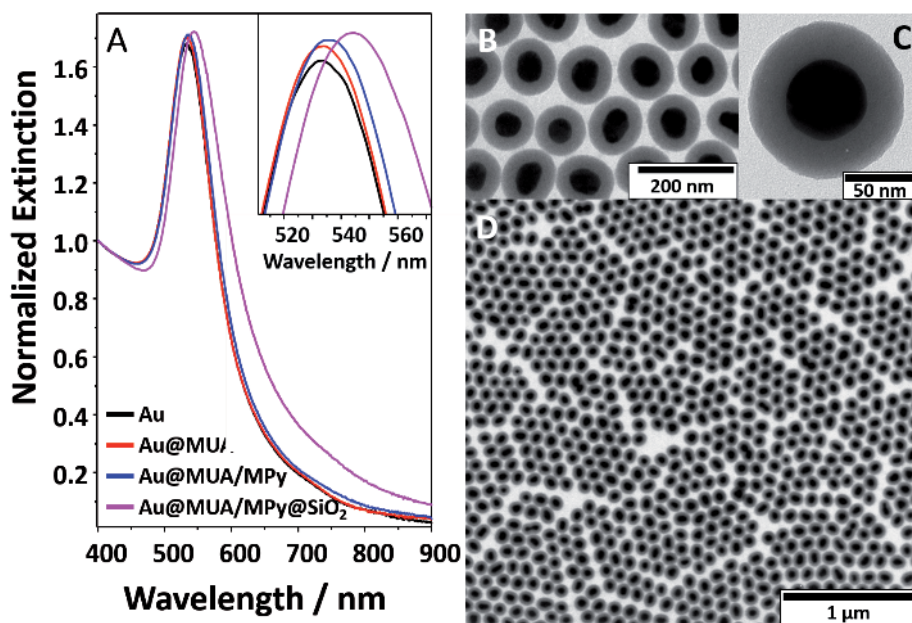

Figure 24. (A) Extinction spectra of the Au particles after each step of the MUA-based protocol (*Inset*: detail of the plasmon absorption maxima). (B, C and D) Representative TEM images at different magnifications of the MPy SERS-encoded gold nanoparticles.

Moreover, if it is necessary to further improve the colloidal stability for long periods of time and prevent plasmon coupling and therefore the uncontrolled generation of hot-spots Au@MUA/Mpy nanoparticles could be encapsulated in a silica matrix. To prove this, a silica coating was performed, as an additional step, by using a modification of the well-known Stöber method,[191] exploiting the ability of ligands with terminal carboxylic

acid like MUA to induce the silica growth.[192–195] To this end, appropriate amounts of ethanol and NH_4OH were added to the Au@MUA/MPy aqueous suspension to maintain the adequate pH of the solution and provide the correct $EtOH/H_2O$ molar ratio (1.84) for the Stöber process. Then, TEOS was added to initiate silica growth. The solution was allowed to react for 14 h at room temperature before putting it through several washing cycles (Figure 24B-D illustrates characteristic TEM images of Au@MUA/MPy@SiO$_2$ nanoparticles). A thick silica shell of ca. 30 nm was grown on top of the labelled-nanoparticles to prevent localizations of intense local fields at the interparticle junctions of possible SERS-encoded nanoparticle agglomerates. Figure 24A shows the extinction spectra of the colloidal suspension after each fabrication step. As it can be seen, the shift of the LSPRs of individual nanoparticles clearly reflects the changes in the refractive index associated with each functionalization step (Figure 24A, inset**)**. Firstly, the sub-monolayer deposition of MUA induces minimal changes in the plasmon maximum. Then, full coating with MPy results in a ca. 3 nm shift and, finally, the growth of a thick silica layer is responsible for the large displacement up to 545 nm. Importantly, the extinction spectra do not reveal any significant broadening of the LSPR. That indicates that the nanoparticles preserve their colloidal stability during the whole process with no appreciable formation of aggregates. It is worth noting that, independently of the SERS encoding process and the related colloidal stability, homogeneous silica coatings can be only achieved for a MUA concentration above 0.7 molecules nm^{-2} (Figure 25).
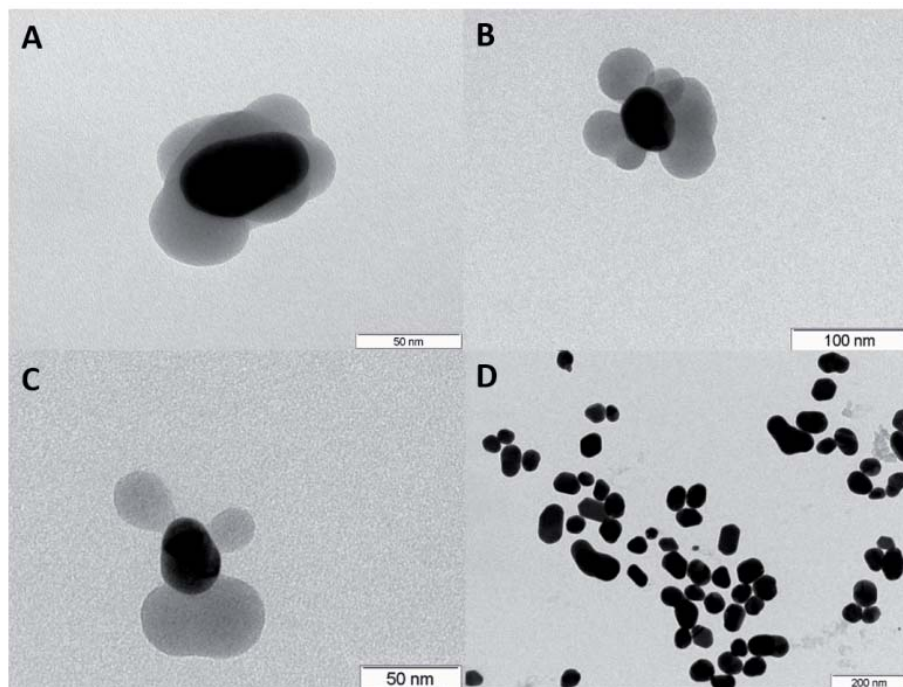
Figure 25. (A, B, and C) TEM images showing the results of encoding nanoparticles at MUA concentrations below 0.8 molecules nm^{-2} (0.7, 0.6 and 0.5 respectively). (D) TEM image showing the uncontrolled aggregation of the particles below 0.7 molecules nm^{-2}.

The described protocol was successfully extended to a large set of different codes, including thiolated and non-thiolated aromatic small molecules and dyes (phenothiazines, rhodamines, oxazines, triarylmethanes, tri- and tetrazoles, etc.), proving the universal applicability of this synthetic strategy. Figure 26-28 shows the SERS signatures and TEM images of 31 representative SERS-encoded nanoparticles. Notably, the one-pot synthetic method has been successfully employed in the fabrication of larger volumes of SERS-encoded nanoparticles (at the liter regime, Figure 23) without impacting the final characteristics of the substrate, which clearly demonstrates the scalability of the process.
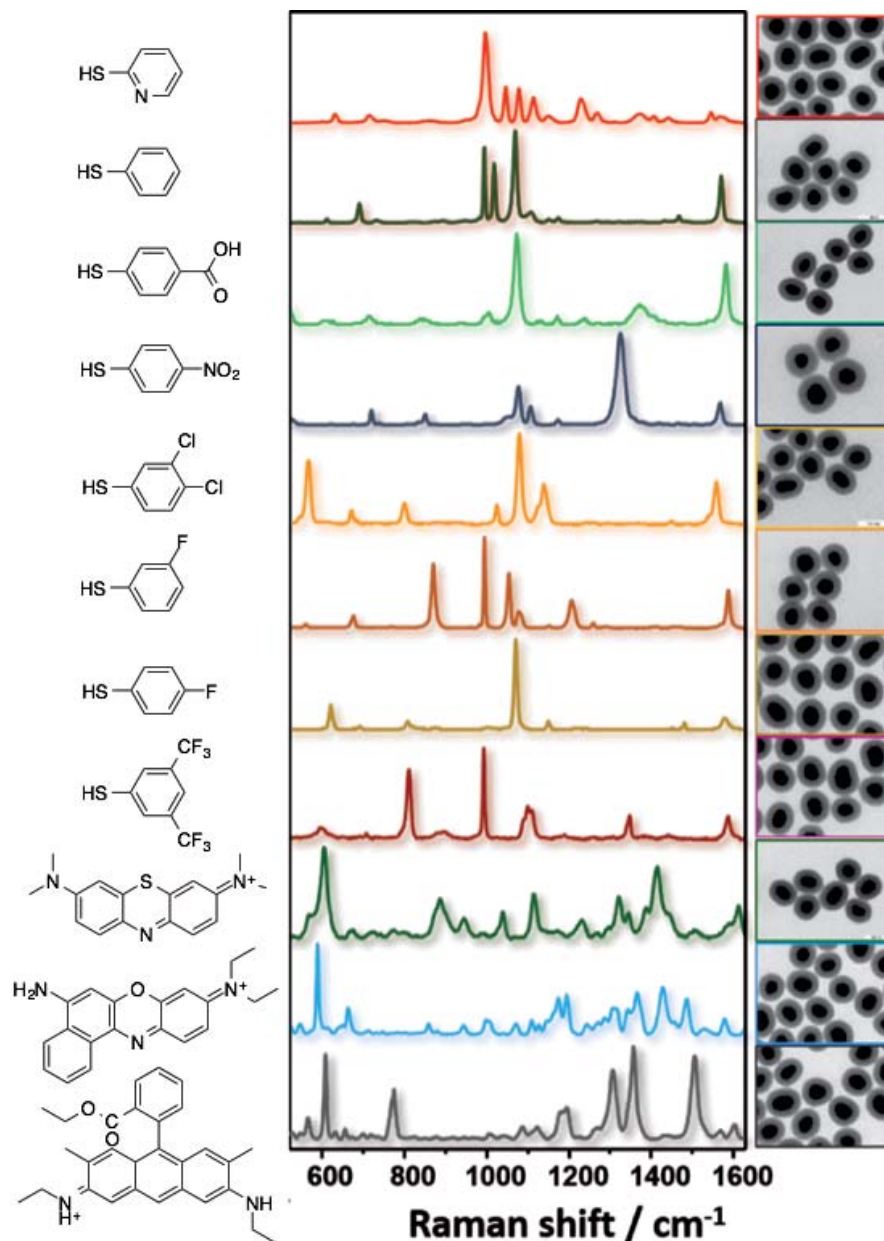
Figure 26. SERS spectra of eleven representative SERS-encoded nanoparticle colloids ([Au] = 1 mM). From the top to the bottom: 2-mercaptopyridine (MPy); benzenethiol (BT); 4-mercaptobenzoic acid (4MBA); 4-nitrobenzenethiol (4NBT); 3,4-dicholorobenzenethiol (DBT), 3-fluorothiophenol (3FTP); 4-fluorothiophenol (4FTP); 3-5-bis (trifluoromethyl) benzenethiol (3FMBT); methylene blue (MB); nile blue A (NBA); and rhodamine 6G (R6G). Right column: representative TEM images of the corresponding SERS-encoded gold nanoparticles.
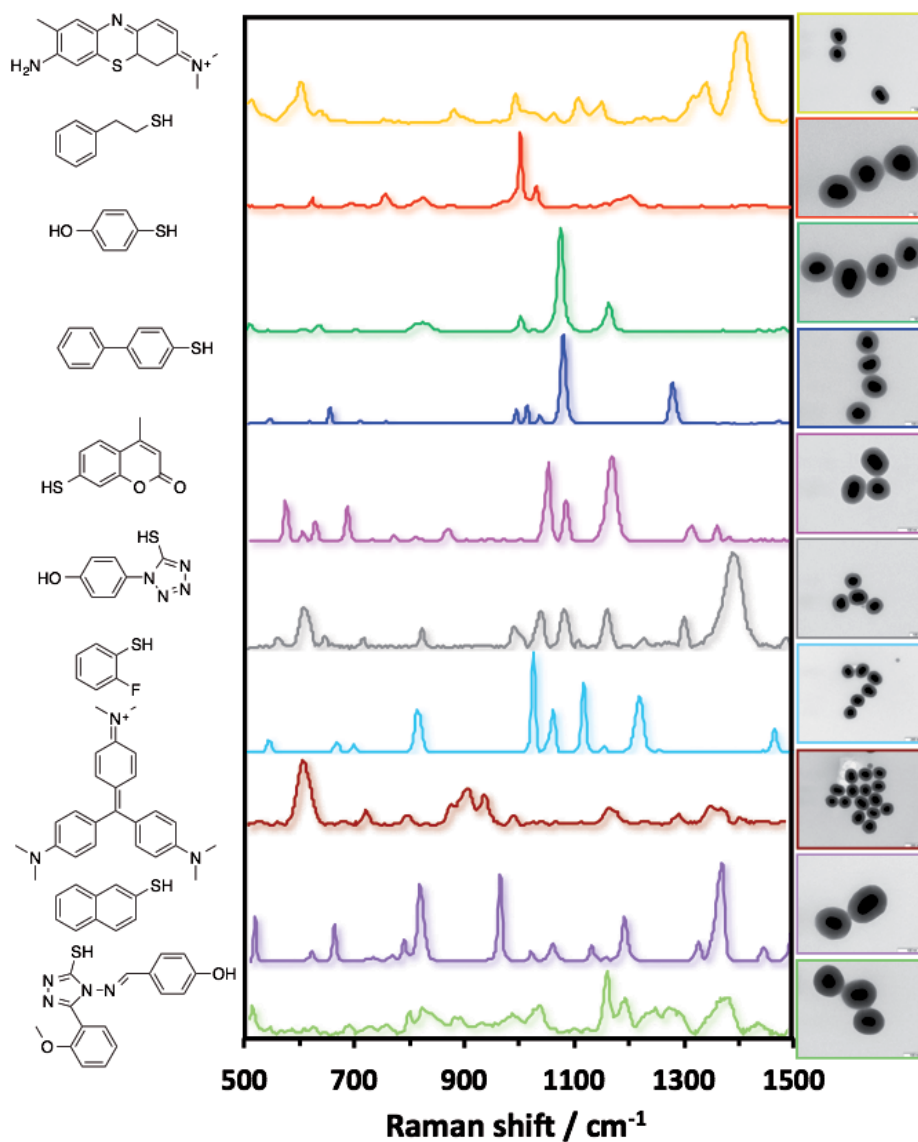
Figure 27. SERS spectra of ten representative SERS-encoded nanoparticle colloids ([Au] = 1 mM). From the top to the bottom: Toluidine Blue O (TB), 2-Phenylethanethiol (2PET), 4-Mercaptophenol (4MP), Biphenyl-4-thiol (BPT), 7-Mercapto-4-methylcoumarin (MMC), 1-(4-Hydroxyphenyl)-1H-tetrazole-5-thiol (HPHTT), 2-fluorothiophenol (2FTP), crystal violet (CV), 2-naphtalenethiol (2NT), 4-(((3-mercapto-5-(2-methoxyphenyl)-4H-1,2,4-triazol-4-yl) imino) methyl) phenol (MMPHTYIMP). Right column: representative TEM images of the corresponding SERS-encoded gold nanoparticles.
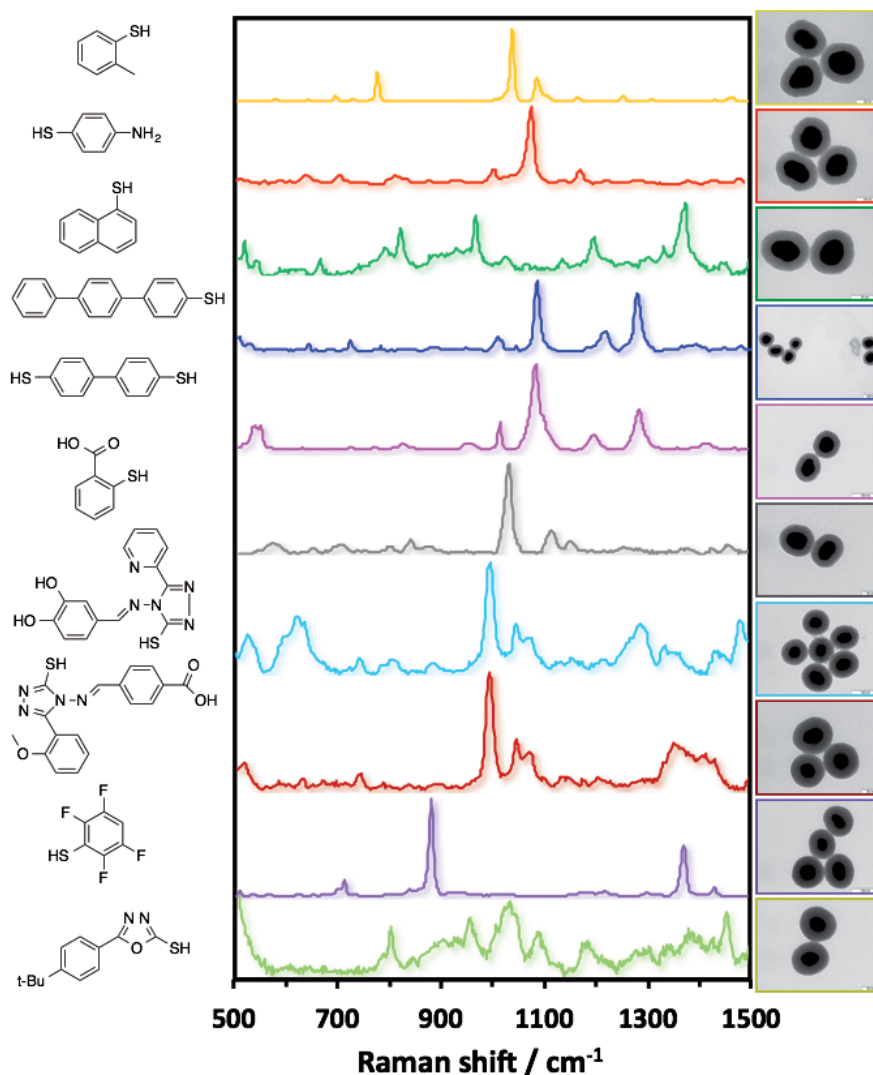
Figure 28. SERS spectra of ten representative SERS-encoded nanoparticle colloids ([Au] = 1 mM). From the top to the bottom: (2-trifluoromethyl) benzenethiol (2TFMBT), 4-aminothiophenol (4ATP), 1-naphthalenethiol (1NT), 1,1',4,,1''-terphenyl-4-thiol (TPT), biphenyl-4,4'-dithiol (BPDT), thiosalicylic acid (TSA), 4-(((3-mercapto-5-(2-pyridinyl)-4H-1,2,4-triazol-4-yl) imino) methyl)-1,2-benzenethiol (MPHTYMBDO), 4-(((3-mercapto-5-(2-pyridinyl)-4H-1,2,4-triazol-4-yl) imino) methyl)-1,2-benzoic acid (MPHTYIMBA), 2,3,5,6-tetrafluorobenzenethiol (2356TFBT), (5-(4-methoxyphenyl)-1,3,4-oxidazole-2-thiol) (MPOT). Right column: representative TEM images of the corresponding SERS-encoded gold nanoparticles.

In order to evaluate the optical efficiency of the protocol, the SERS intensities provided by seven of the SERS-encoded nanoparticles were

compared with those yielded by their analogous counterparts fabricated (when possible) with the most common polymer-based procedures used in the literature: PVP and thiolated-PEG approaches.[179,192]

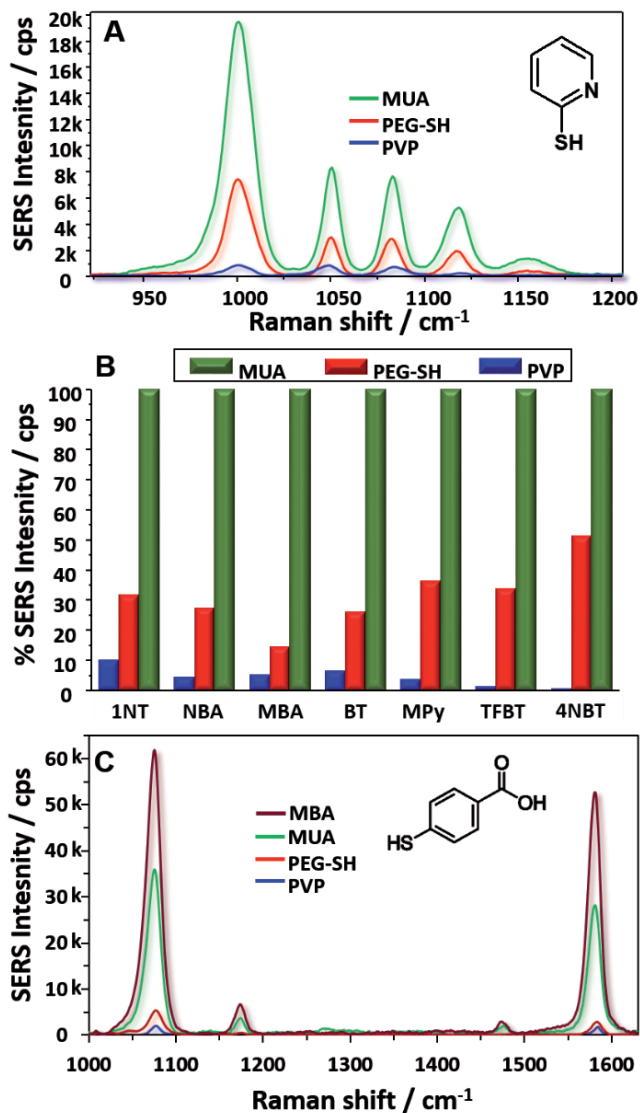

Figure 29. Comparison of the SERS efficiency of encoded nanoparticle suspensions synthesized via the three different PVP, HS-PEG$_{5000}$ and MUA approaches ([Au] = 1 mM). (A) SERS spectra in the 900-1210 cm^{-1} region for MPy encoded nanoparticles. (B) SERS intensity (%) for encoded nanoparticles using 1NT, NBA, 4MBA, BT, MPy, TFBT, and 4NBT as Raman labels (the corresponding SERS spectra are shown in Figure 30). (C) SERS spectra in the 1000-1630 cm^{-1} region for 4MBA encoded nanoparticles prepared using a full monolayer of 4MBA (purple) and with the three different approaches.

Both polymer-based strategies rely on providing the required stability to the Au particles during the codification step. To this end, the reported recipes[179,192] were followed and optimized in order to achieve the highest possible SERS signal. When PVP was used, citrate-stabilized Au nanoparticles were first produced and the subsequently transferred to EtOH using PVP. Prior to the code addition, the particles were extensively washed to remove as much as possible the PVP from the surface of the particles while maintaining colloidal stability. These washing cycles are critical to maximizing the adsorption of the code onto the metallic surface.
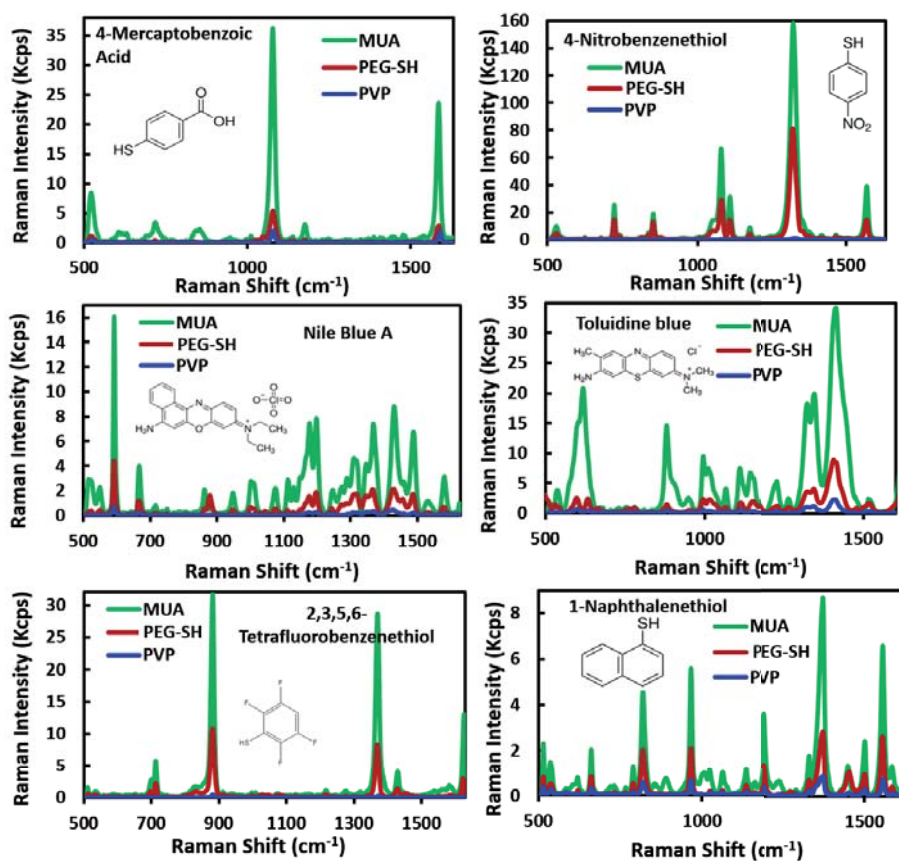

Figure 30. SERS spectra comparison of encoded gold nanoparticles prepared using the PVP, HS-PEG$_{5000}$ and MUA approaches (blue, red, and green respectively) of 4MBA, 4NBT, NBA, TB, TFBT, and 1NT.

In the case of HS-PEG$_{5000}$, it has been previously shown that the polymeric layer can significantly hinder the diffusion of molecules onto the

metal surface.[196] Consequently, due to the very limited number of Raman molecules capable to bind HS-PEG$_{5000}$-coated nanoparticles, the use of the corresponding Au@HS-PEG$_{5000}$@code particles is largely limited to surface-enhanced *resonant* Raman scattering conditions (SERRS), when the electronic excitation of the dye is in resonance with the laser beam.[179,197] A identical limitation appears if the codification step is performed prior to the HS-PEG$_{5000}$ coating (Au@code@HS-PEG$_{5000}$)[169] because, in this case, except for few fortunate cases of "nanoparticle stabilizing" Raman codes, the number of molecules per nm^2 that can be adsorbed onto the metal without perturbing the colloidal stability is very small. Therefore, as a first step, the Raman label accessibility to the metal surface was improved by progressively lowering the amount of added HS-PEG$_{5000}$ from 4 molecules nm^{-2}, as reported in the literature,[179] to 0.2. However, nanoparticle aggregation is already observed at a polymer concentration less than 3.5 molecules nm^{-2} even prior to the addition of the excess of MPy (Figure 31). Moreover, no distinguishable SERS signals were recorded upon functionalization of the HS-PEG$_{5000}$-coated gold nanoparticles with the excess of MPy unless in the presence of colloidal aggregation (Figure 31). Discarded the possibility to increase the MPy surface coverage by decreasing the HS-PEG$_{5000}$ concentration, a different strategy were pursued.[196,197] In this case, CTAB-stabilized Au nanoparticles of a similar size were prepared instead of citrate-capped colloids. The surfactant double layer offers an effective stabilizing shell preventing nanoparticle aggregation when the colloids are exposed to less HS-PEG$_{5000}$ amount (corresponding to 1.8 molecules nm^{-2} in optimized experimental conditions). The resultant HS-PEG$_{5000}$/CTAB-stabilized nanoparticles can then be put through, firstly, several wash cycles to remove excess surfactant and, secondly, to the excess MPy in the codification step without impacting the colloidal stability. This optimized protocol allows us to synthesize SERS-encoded nanoparticles with much higher SERS efficiency, generally larger than that observed for the PVP approach, but still significantly lower than that provided by the MUA-based method (Figure 29A and Figure 30). Furthermore, it is important to stress that even when optimized, the combined CTAB/ HS-PEG$_{5000}$ approach retains intrinsic

limitations and problems like the need for multiple cycles of centrifugation, separation and re-suspension (which are often critical for the colloidal stability as well as representing practical obstacles for large scale production) and the use of large amounts of the highly cytotoxic CTAB.[198]
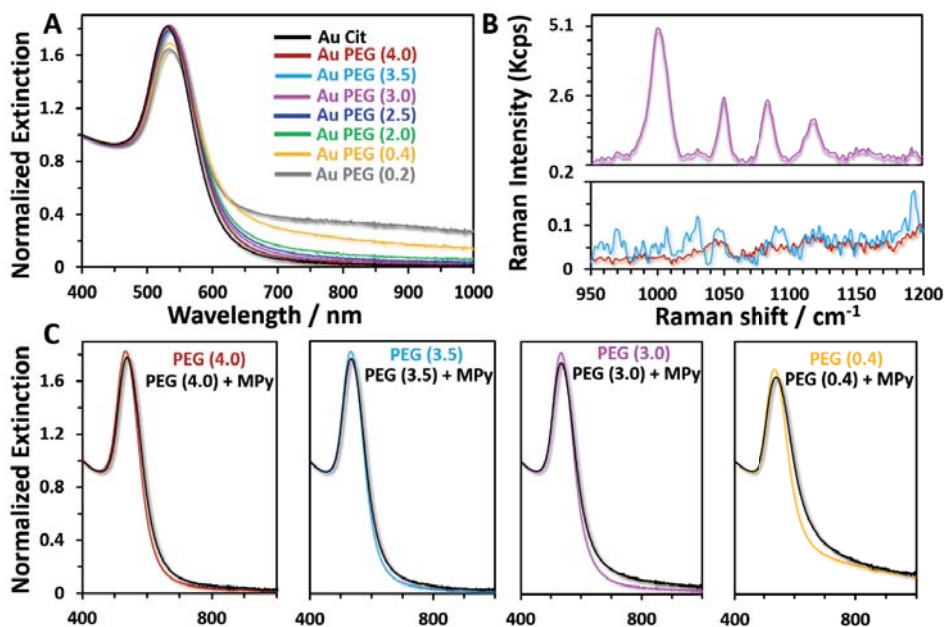

Figure 31. (A) Extinction spectra of the Au@HS-PEG$_{5000}$ nanoparticles with a different amount of HS-PEG$_{5000}$ (molecules nm^{-2}). Below 3.5 HS-PEG$_{5000}$ molecules nm^{-2}, it is clearly seen that nanoparticles start to aggregate. (B) SERS spectra of Au@HS-PEG$_{5000}$/MPy nanoparticles with a different amount of HS-PEG$_{5000}$, 4, 3.5, and 3 molecules nm^{-2} (red, blue, and magenta, respectively).The MPy SERS spectra is only obtained below 3.5 molecules nm^{-2}. (C) Comparison of the extinction spectra of Au@HS-PEG$_{5000}$ nanoparticles with different amounts of HS-PEG$_{5000}$ (molecules nm^{-2}) before and after the addition of MPy. Showing that after the addition of MPy, the particles aggregate more especially below 3.5 molecules nm^{-2} of HS-PEG$_{5000}$.

Table 1 summarizes the SERS efficiency ratios calculated by comparing the SERS intensities of seven different SERS-encoded nanoparticles obtained via a MUA-based protocol with respect to those yielded by the HS-PEG$_{5000}$ and PVP approaches (I_{MUA}/I_{PVP} and $I_{MUA}/I_{HS-PEG5000}$, respectively). For all the investigated cases, the SERS intensity achieved with the MUA-based protocol lies between 140 to 10 times higher than in the case of PVP, and from 10 to 2 times higher than in the case of thiolated- HS-

PEG$_{5000}$. Such drastic differences from one Raman label to another can be ascribed to the different chemical nature and molecular size of the codes which clearly affect their ability to diffuse through the external polymeric layer and finally be adsorbed onto the metal surface.

Code	MUA/PVP	MUA/ HS-PEG$_{5000}$
NBA	22.2	3.6
BT	14.9	3.8
2MPy	26.5	2.7
1NT	10	3.1
4MBA	19.4	7
TH60	79	2.9
4NBT	140	2

Table 1. SERS efficiency ratios calculated by dividing the SERS intensities of different SERS-encoded nanoparticles obtained via MUA-based method by those synthesized via PVP and HS-PEG$_{5000}$ approaches.

As previously indicated, modification of the nanoparticle surface chemistry by adsorption of Raman labels normally results in a decrease in colloidal stability in suspension. However, in very few cases, the specific chemical nature of code molecule can still preserve such stability by acting as a stabilizing agent. This is the case, for instance, of 4-mercaptobenzoic acid (4MBA). As for MUA, the carboxylic groups of 4MBA bound to the metal surface are oriented toward the bulk solution providing, at basic pH, the necessary electrostatic repulsion to avoid nanoparticle aggregation and, in the subsequent silica-coating step, to promote the silica growth. As a result, this SERS-encoded nanoparticle can be synthesized at full 4MBA coverage with no need for external stabilizing agents. Therefore, the SERS performance of 4MBA-encoded nanoparticles produced via the MUA synthetic method (0.8 MUA molecules nm^{-2}) was assessed with respect to the same SERS-encoded NPs obtained at full 4MBA coverage (i.e. zero MUA molecules nm^{-2}, maximum SERS efficiency). Figure 29C shows the corresponding SERS spectra as well as those of the analogous SERS-encoded NPs synthesized via the PVP and HS-PEG$_{5000}$ methods. Notably,

MUA functionalization results in a ca. 42% loss with respect to the maximum SERS efficiency, which is much less than those observed for the PVP (ca. 97% reduction) and HS-PEG$_{5000}$ (92% reduction) methods. The SERS enhancement factor for gold spherical nanoparticles fully coated with 4MBA was also calculated by direct comparison with the normal Raman spectrum of 4MBA in a 0.1 M ethanol solution (Figure 32). The estimated EF at 785 nm is 4.53×10^4 (Figure 32).
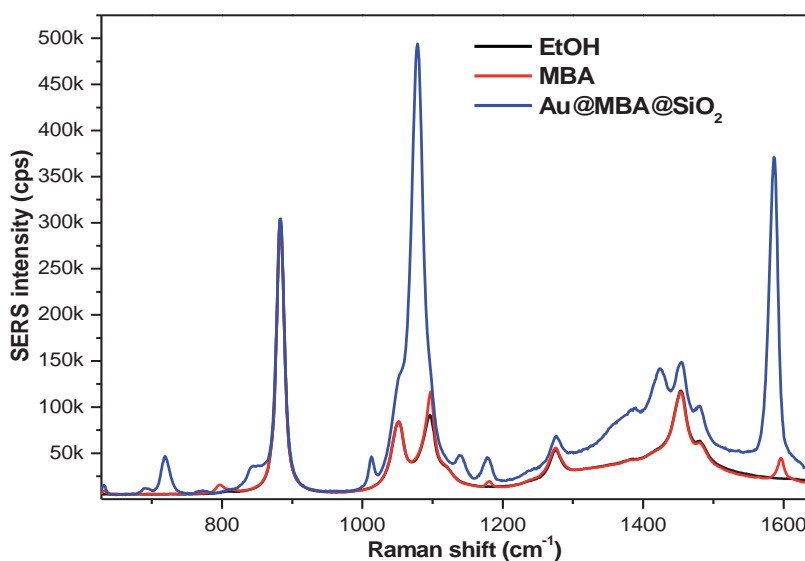

Figure 32. Normal Raman spectrum of EtOH and 4MBA (0.1 M in EtOH). SERS spectrum of Au@4MBA@SiO$_2$ suspension in EtOH ([Au] = 1 mM), [NP] = 0.244 nM). All the spectra were acquired under the same experimental conditions (785 nm laser line, 100% laser power, 1 s exposition time, 5 accumulations). The SERS enhancement factor was calculated using the definition $EF = \dfrac{I_{SERS}/N_{surf}}{I_{Raman}/N_{vol}}$, where N_{Vol} is the average number of molecules in the scattering volume, V, for the normal Raman measurement, and N_{Surf} is the average number of adsorbed molecules in the same scattering volume for the SERS measurement.[148] As normal Raman and SERS intensities (I_{Raman} and I_{SERS}) selected the height of the $\nu_{C=C}$ band at ca. 1600 cm^{-1}. Both Raman and SERS measurements were performed under the same experimental set-up (i.e. identical scattering volume). Nanoparticle concentration of 0.244 nM was calculated by Lambert-Beer's law using the extinction coefficient for gold nanoparticles of 4.2×10^{10} M^{-1} cm^{-1}, derived from literature.[23] The molecular footprint of 4MBA on gold, for a full monolayer, was reported to be 0.44 nm^2.[149] Thus, for gold spheres of ca. 50 nm diameter functionalized with a monolayer of 4MBA molecules, 18,570 molecules per particle were estimated, which corresponds to 4.53×10^{-6} M in the colloidal suspension. The estimated EF at 785 nm is therefore ca. 4.53×10^4.
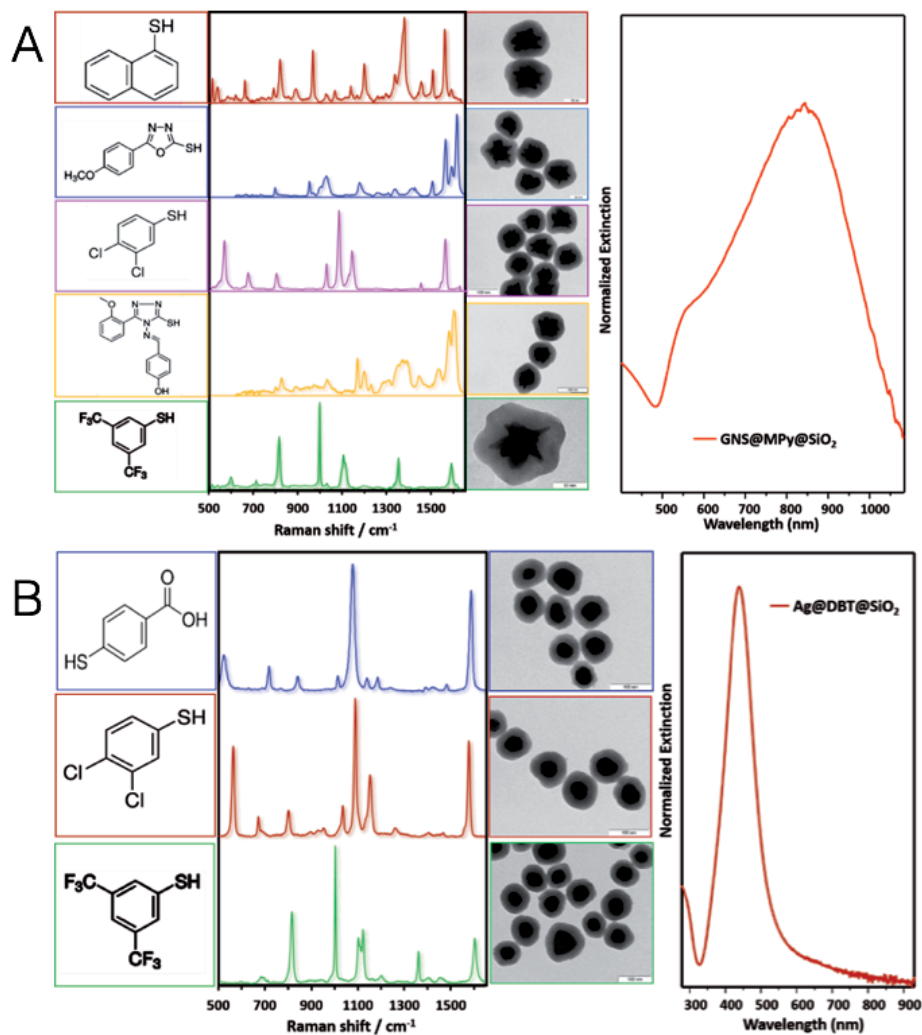
Figure 33. (A) SERS spectra and TEM images of different SERS encoded gold nanostars prepared using the MUA approach. Normalized extinction spectra of GNS@MPy@SiO$_2$ encoded nanoparticles. (B) SERS spectra and TEM images of different SERS encoded silver nanoparticles prepared using the MUA approach. Normalized extinction spectra of Ag@DBT@SiO$_2$ encoded nanoparticles.

Finally, in addition to spherical gold nanoparticles, the straightforward application of the MUA-based encoding protocol was demonstrated for other SERS enhancing platforms such as gold nanostars prepared via the standard PVP-protocol[185] (i.e. same material but a different shape) or citrate-reduced silver colloids (i.e. similar shape but a different material). Gold nanostars were selected as a representative example of highly SERS-

active spiked nanostructures. Figure 33 illustrates representative SERS spectra and TEM images of different encoded silver nanoparticles and gold nanostars. Enhancement factors associated with these structures result in 9.12×10^5 for the gold nanostars and 6.73×10^7 for the silver spherical particles.

4.4 Conclusions

In summary, herein a universal, one-pot, inexpensive and scalable synthetic protocol for the fabrication of SERS-encoded nanoparticles was described. This method relies on the functionalization of plasmonic nanoparticles with a submonolayer of mercaptoundecanoic acid providing high colloidal stability during the codification process while allowing Raman labels to easily diffuse onto the metal. Furthermore, in a subsequent step, the carboxylic groups of MUA also act as functional sites promoting silica growth on the outer shell of the nanoparticles. This synthetic strategy has proven to be successfully applicable to every Raman code tested (31 codes) and scalable up to two liters without affecting the final properties of the encoded structures. It is worth noting that the MUA-based method averts the use of high-molecular-weight polymers and their associated issues of reproducibility from batch-to-batch and for different manufacturers. The SERS efficiency of the so-fabricated encoded nanoparticles has been shown to be from 2 to 140 times higher than the corresponding particles prepared via the common polymer-based methods (PVP and HS-PEG$_{5000}$). The MUA-based protocol can be readily applied to metallic nanoparticles, such as citrate-reduced silver colloids or gold nanostars.