# Estudio de tensioactivos aniónicos y catiónicos derivados de lisina: interacción con membranas celulares, desarrollo de sistemas nanoestructurados para la liberación intracelular y evaluación toxicológica *in vitro*

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**Memoria presentada por Daniele Rubert Nogueira para optar al título de Doctor por la Universitat de Barcelona**

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Que la memoria titulada **Estudio de tensioactivos aniónicos y catiónicos derivados de lisina: interacción con membranas celulares, desarrollo de sistemas nanoestructurados para la liberación intracelular y evaluación toxicológica in vitro** presentada por DANIELE RUBERT NOGUEIRA para optar al Grado de Doctor por la Universitat de Barcelona, ha sido realizada bajo nuestra dirección en el Departament de Fisiologia, y considerándola acabada, autorizamos su presentación para ser juzgada por el tribunal correspondiente.

Y para que así conste a efectos oportunos, firmamos la presente en Barcelona a 25 de enero de 2013.

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***con todo mi cariño.***

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***"Quien no haya experimentado la***

***irresistible atracción de la ciencia,***

***no podrá comprender su tiranía."***

***Mary Shelley (Frankenstein)***

# RESUMEN DE LA TESIS

*Presentación*:

Los tensioactivos son compuestos altamente versátiles con amplia utilización en la industria farmacéutica y cosmética. Aquellos derivados de aminoácidos presentan biocompatibilidad y elevada biodegradabilidad y, por lo tanto, tienen un abanico más amplio de aplicaciones y constituyen una alternativa a los tensioactivos sintéticos convencionales. Entre los compuestos derivados de aminoácidos, este trabajo se ha centrado en los tensioactivos derivados de lisina con carácter tanto aniónico como catiónico. Dentro del contexto de la búsqueda de nuevas formulaciones farmacéuticas con especificidad y eficacia para la liberación de biomoléculas en órganos o células dianas, en los últimos años existe un interés creciente en la utilización de excipientes bioactivos con actividad lítica pH-sensible en sistemas nanoestructurados. Entre estos compuestos, se destacan los péptidos, polímeros y tensioactivos, por lo que se supone que los tensioactivos biocompatibles derivados de lisina pueden ser interesantes para estas aplicaciones. Sin embargo, la potencial utilización de estos tensioactivos en sistemas nanoestructurados implica una valoración previa de su interacción con membranas celulares en función del pH, con el fin de entender los mecanismos involucrados en su actividad lítica. Del mismo modo, es necesario el estudio de su potencial toxicidad y para ello, se necesitan bioensayos rápidos, sensibles y fiables.

*Objetivos*:

Este trabajo está enmarcado dentro de una línea de investigación centrada en ampliar el rango de aplicaciones farmacéuticas de los tensioactivos derivados de aminoácidos. Para ello, se ha planteado estudiar dos grupos de tensioactivos derivados de lisina: tensioactivos aniónicos de doble cadena derivados de la $N^{\alpha},N^{\varepsilon}$-dioctanoil lisina con diferentes contraiones en su estructura y tensioactivos catiónicos de cadena simples derivados de la $N^{\varepsilon}$-acil lisina o $N^{\alpha}$-acil lisina. Dado sus características físico-químicas y sus propiedades biocompatibles y biodegradables, los objetivos de este trabajo se han postulado en base a la hipótesis de que estos compuestos también pueden presentar actividad lítica de membrana variable con el pH del medio y, por lo tanto, potencial aplicación en vehículos para la liberación intracelular de biomoléculas. Dentro de este contexto, el objetivo general de la presente Tesis se ha dividido en dos bloques:

**1.** Determinar la influencia del pH en las propiedades de interacción con membranas celulares de tensioactivos aniónicos y catiónicos derivados del aminoácido lisina.

**2.** Desarrollar sistemas nanoestructurados basados en estos tensioactivos, estudiar su potencial toxicidad *in vitro* y evaluar su efectividad en promover una liberación intracelular eficiente.

*Resultados y discusión*:

En primer lugar, se ha procedido con el estudio del potencial lítico de estos tensioactivos en función del pH del medio, utilizando el eritrocito como modelo de membrana endosomal. Se ha demostrado que los tensioactivos aniónicos tienen actividad lítica pH-dependiente independientemente del contraión; sin embargo, la presencia de diferentes contraiones influye en la potencia hemolítica. Por otra parte, las propiedades líticas de los tensioactivos catíonicos dependen especialmente de la posición de la carga catiónica y, en menor medida, de la longitud de la cadena alquílica. La actividad pH-sensible se atribuye a la presencia de la carga positiva en el grupo α-amino de la lisina, mientras que el aumento de la fluidez de la membrana y la pérdida de sus proteínas estructurales se han evidenciado como los mecanismos involucrados en el aumento de la lisis celular a pH ácidos.

Teniendo en cuenta la variabilidad inherente entre las diferentes líneas celulares y su diferente sensibilidad a los efectos citotóxicos de los más diversos compuestos bioactivos, en esta Tesis también se ha estudiado un modelo de ensayo predictivo de la citotoxicidad de tensioactivos pH-sensibles con potenciales aplicaciones farmacéuticas. Se ha planteado la utilización de seis líneas celulares, dos tumorales y cuatro no tumorales, y de dos ensayos de citotoxicidad basados en diferentes mecanismos de viabilidad celular. Con esta batería de ensayos se ha corroborado la baja toxicidad de los tensioactivos aniónicos y se ha demostrado que los diferentes contraiones tienen contribución directa en la respuesta citotóxica de estos compuestos. Además, se ha propuesto este estudio comparativo como una propuesta fiable para el cribado de nuevos excipientes farmacéuticos.

Una vez corroborado el potencial lítico pH-dependiente de los tensioactivos, se ha avanzado en el desarrollo, caracterización, estudio toxicológico *in vitro* y evaluación del comportamiento intracelular de sistemas nanoestructurados conteniendo estos compuestos. Cabe destacar que actualmente no hay protocolos reglamentados para la evaluación toxicológica de los nanomateriales y, por lo tanto, el estudio *in vitro* del potencial citotóxico de los sistemas nanoestructurados y el establecimiento de los posibles mecanismos involucrados en su toxicidad es de gran importancia en el campo de la nanotoxicología.

En primer lugar, se han desarrollado nanovesículas lipídicas con los tensioactivos catiónicos y se ha procedido con el estudio de sus efectos citotóxicos en células representativas de la piel, como propuesta de su potencial aplicación en formulaciones de administración tópica. Se han

observado efectos citotóxicos moderados, respuesta inflamatoria leve y ausencia de fototoxicidad. A continuación, se han llevado a cabo estudios de los mecanismos involucrados en la toxicidad de las nanovesículas utilizando diferentes líneas celulares y métodos *in vitro.* Además, se evaluaron su biocompatibilidad con la sangre, su capacidad de internalización celular y su capacidad de romper la membrana endosomal. Se ha propuesto que el estrés oxidativo, la disfunción mitocondrial y la apoptosis son los principales mecanismos involucrados en la toxicidad. También se demostró que las nanovesículas tienen baja actividad hemolítica, no inducen aglutinación de los eritrocitos y tampoco adsorben proteínas plasmáticas de modo significativo. Por otra parte, los estudios de internalización celular y estabilidad endosomal han mostrado la capacidad de las nanovesículas que contienen los tensioactivos con carga positiva en el grupo α-amino de la lisina para romper la membrana de los endosomas, lo que corrobora su actividad pH-dependiente.

Por último, se han desarrollado nanopartículas poliméricas con el quitosano incluyendo el tensioactivo aniónico 77KL como excipiente pH-sensible. Se ha encapsulado el fármaco antitumoral metotrexato y se ha demostrado su mayor actividad en inhibir la proliferación de las células tumorales en comparación con el fármaco libre no encapsulado. Este hecho se atribuye, especialmente, a la actividad pH-dependiente de las nanopartículas. Tras la internalización y liberación específica del fármaco a nivel intracelular, la inhibición del ciclo celular, la apoptosis y, en menor medida, el daño mitocondrial y lisosomal, se han demostrado como los mecanismos involucrados en la actividad antitumoral del metotrexato encapsulado en las nanopartículas.

*Conclusiones*:

Como conclusión general de esta Tesis, es importante destacar que la incorporación de excipientes bioactivos en nanomateriales mejora las propiedades fisicoquímicas y la actividad biológica de dichos sistemas. El desarrollo de vehículos con actividad pH-dependiente es una propuesta interesante para mejorar la liberación intracelular de fármacos y para aumentar sus efectos terapéuticos. Por lo tanto, se considera que los tensioactivos derivados de lisina estudiados en esta Tesis son potenciales excipientes bioactivos en formulaciones nanoestructuradas. Del mismo modo, los métodos *in vitro* utilizados se mostraron eficaces para demostrar los mecanismos involucrados en los efectos tóxicos de los nanomateriales, lo que contribuye para la consolidación de una nueva disciplina, la Nanotoxicología, y para disminuir las incertidumbres relacionadas con la toxicidad de los nanomateriales.

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# ABREVIATURAS

| | |
|---|---|
| 3T3-NRU-PT | Ensayo de fototoxicidad mediante captación de rojo neutro en fibroblastos 3T3 |
| 77K | $N^{\alpha}$,$N^{\varepsilon}$ –dioctanoil lisina |
| 77K22 | $N^{\alpha}$,$N^{\varepsilon}$–dioctanoil lisina-N,N-bis[metilo eter dietileno glicol]amida |
| 77KK | Sal de lisina derivada de $N^{\alpha}$,$N^{\varepsilon}$ –dioctanoil lisina |
| 77KL | Sal de litio derivada de $N^{\alpha}$,$N^{\varepsilon}$ –dioctanoil lisina |
| 77KP | Sal de potasio derivada de $N^{\alpha}$,$N^{\varepsilon}$ –dioctanoil lisina |
| 77KS | Sal de sodio derivada de $N^{\alpha}$,$N^{\varepsilon}$ –dioctanoil lisina |
| 77KT | Sal de tris(hidroximetil) amino metano derivada de $N^{\alpha}$,$N^{\varepsilon}$ –dioctanoil lisina |
| ADN | Ácido desoxirribonucleico |
| ARN | Ácido ribonucleico |
| ATC | *Acute Toxic Class Method* |
| ATP | Adenosina trifosfato |
| BCOP | *Bovine Corneal Opacity and Permeability* |
| BOE | Boletín Oficial del Estado |
| BraCVAM | *The Brazilian Center for Validation of Alternative Methods* |
| CCRSERI | Comité Científico de los Riesgos Sanitarios Emergentes y Recientemente Identificados |
| CCSC | Comité Científico de Seguridad de los Consumidores |
| CDER | *Center for Drug Evaluation and Research* |
| $CH_{50}$ | Concentración que provoca el 50% de hemólisis |
| $CI_{50}$ | Concentración inhibitoria 50 |
| CMC | Concentración micelar crítica |
| DLS | Espectroscopia de correlación de fotones |
| DMPC | Dimiristoilfosfatidilcolina |
| DPH | 1,6-difenil-1,3,5-hexatrieno |
| ECVAM | *European Centre for the Validation of Alternative Methods* |
| EEC | *European Economic Community* |
| EELS | Espectroscopía por pierda de energía de electrones |
| EFSA | Autoridad Europea de Seguridad Alimentaria |
| ELISA | Enzima inmunoensaio (*Enzyme Linked ImmunoSorbent Assay*) |

| | |
|---|---|
| EMA | Agencia Europea de Medicamentos (*European Medicines Agency)* |
| EPR | Efecto incrementado de permeabilidad y retención |
| EURL ECVAM | *European Union Reference Laboratory on Alternatives to Animal Testing* |
| FDA | *Food and Drug Administration* |
| FDP | *Fixed Dose Procedure* |
| HLB | Balance hidrófilo-lipófilo |
| HTAB | Bromuro de hexadecil trimetilamonio |
| ICE | *Isolated Chicken Eye* |
| IL-1α | Interleucina 1 alpha |
| IL-6 | Interleucina 6 |
| IL-8 | Interleucina 8 |
| ICCVAM | *Interagency Coordinating Comittee on the Validation of Alternative Methods* |
| JACVAM | *Japanese Center for the Validation of Alternative Methods* |
| LDH | Lactato deshidrogenasa |
| LSM | Microscopía confocal de barrido a láser |
| MDR | Resistencia multifármaco |
| MIC | Concentración mínima inhibitoria |
| MKM | $N^{\varepsilon}$-Miristoil lisina metil éster |
| MLM | $N^{\alpha}$-Miristoil lisina metil éster |
| MTS | Ensayo de reducción de la sal de 3-(4,5-dimetiltiazol-2-il)-5-(3-carboximethoxiphenil)-2-(4-sulfofenil)-2H-tetrazolio |
| MTT | Ensayo de reducción de la sal de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio |
| MTX | Metotrexato |
| NPs | Nanopartículas |
| NRU | Ensayo de captación de rojo neutro |
| NVs | Nanovesículas |
| OECD | *Organisation for Economic Co-operation and Development* |
| PCR | Reacción en cadena de polimerasa |
| REACH | *Registration, Evaluation, Authorisation and Restriction of Chemical substances* |
| rLLNA | *Reduced Local Lymph Node Assay* |
| ROS | Especies reactivas de oxígeno |
| SDS | Dodecil sulfato sódico |

| | |
|---|---|
| SDS-PAGE | Electroforesis en gel de poliacrilamida |
| SEM | Microscopía electrónica de barrido |
| PEN | *The Project on Emerging Nanotechnologies* |
| PKM | $N^{\varepsilon}$-Palmitoil lisina metil éster |
| TBARs | Especies reactivas del ácido tiobarbitúrico |
| TEM | Microscopía electrónica de transmisión |
| TER | Resistencia eléctrica transepitelial |
| TMA-DPH | Trimetilamonio 1,6-difenil-1,3,5-hexatrieno |
| Tox-21c | *Toxicity Testing in the 21$^{st}$ century: A vision and a Strategy* |
| TPP | Tripolifosfato |
| UDP | *Up and Down Procedure* |
| UE | Unión Europea |
| UTC | *Upper Threshold Concentration* |
| UV | Ultravioleta |
| WST-1 | Ensayo de reducción de la sal de 2-(4-Iodofenil)-3-(4-nitrofenil)-5-(2,4-disulfofenil)-2H-tetrazolio |
| XTT | Ensayo de reducción de la sal de 2,3-bis-(2-metoxi-4-nitro-5-sulfofenil)-2H-tetrazolio-5-carboxanilida) |

# OBJETIVOS

Los tensioactivos son compuestos altamente versátiles con amplia utilización en la industria farmacéutica y cosmética. Por lo tanto, la búsqueda de nuevos tensioactivos con baja toxicidad y elevada biodegradabilidad es una interesante y práctica estrategia para ampliar su rango de aplicaciones. Desde hace más de 25 años, nuestro grupo de investigación colabora en el desarrollo y caracterización toxicológica de tensioactivos derivados de aminoácidos, que constituyen una alternativa a los tensioactivos sintéticos convencionales.

En los últimos años, también existe un creciente interés en la utilización de compuestos pH-sensibles como excipientes bioactivos en sistemas nanoestructurados, lo que puede aumentar la especificidad y eficacia de dichos vehículos para la liberación de fármacos en órganos o células dianas. Sin embargo, la potencial utilización de estos tensioactivos en sistemas nanoestructurados implica una valoración previa de su interacción con membranas celulares, con el fin de entender los mecanismos involucrados en su actividad lítica. Del mismo modo, es necesario el estudio de su potencial toxicidad y para ello, se necesitan bioensayos rápidos, sensibles y fiables.

La nanotecnología constituye uno de los avances tecnológicos más importantes de este siglo. Sin embargo, los posibles efectos adversos para la salud humana que comportan los nanomateriales son poco conocidos. De este modo, los estudios de toxicidad de los sistemas nanoestructurados son fundamentales para conocer su potencial efecto tóxico. En el contexto de esta línea de investigación, este tipo de estudios ha llevado a la constitución de una nueva disciplina, la Nanotoxicología.

En este trabajo se ha planteado estudiar dos grupos de tensioactivos derivados del aminoácido lisina: una familia de tensioactivos aniónicos de doble cadena derivados de la $N^{\alpha},N^{\varepsilon}$ -dioctanoil lisina (77K), constituida por 5 tipos de sales con diferentes contraiones; y un grupo de tensioactivos catiónicos de cadena simple derivados de la $N^{\varepsilon}$ -acil lisina o $N^{\alpha}$ -acil lisina. Concretamente, dado sus características físico-químicas y sus propiedades biocompatibles y biodegradables, este trabajo se ha centrado en la búsqueda de nuevas aplicaciones farmacéuticas para estos tensioactivos. La hipótesis de partida sobre la que se han erigido los objetivos de este estudio se basa en postular que también pueden presentar propiedades líticas de membrana variables con el pH del medio y, por lo tanto, pueden ser interesantes para aplicaciones en vehículos nanoestructurados destinados a la liberación intracelular de fármacos.

En base a esta hipótesis, el objetivo general del presente trabajo se ha dividido en dos bloques:

**1.** Determinar la influencia del pH en las propiedades de interacción con membranas celulares de tensioactivos aniónicos y catiónicos derivados del aminoácido lisina.

**2.** Desarrollar sistemas nanoestructurados basados en estos tensioactivos, estudiar su potencial toxicidad *in vitro* y evaluar su efectividad en promover una liberación intracelular eficiente.

Para alcanzar estos objetivos globales se han planteado como objetivos específicos:

**1.1.** Estudiar el efecto del pH del medio sobre la actividad lítica de los tensioactivos aniónicos y catiónicos utilizando el eritrocito como modelo de membrana endosomal.

**1.2.** Evaluar la influencia de las características estructurales y propiedades fisicoquímicas de los tensioactivos en su actividad biológica, como herramienta para obtener relaciones estructura-actividad que permitan racionalizar el diseño de tensioactivos con actividad pH-dependiente y potencial aplicación en vehículos para la liberación intracelular de biomoléculas.

**1.3.** Evaluar los mecanismos de interacción con las membranas celulares de los tensioactivos catiónicos y las alteraciones inducidas en la bicapa fosfolipídica tras el tratamiento con cada compuesto a diferentes pH. Además, valorar si sus características estructurales, muy especialmente la hidrofobicidad y la posición de la carga catiónica, pueden afectar a su interacción con la membrana y relacionarse con su actividad lítica pH-dependiente.

**1.4.** Estudiar los potenciales efectos citotóxicos de los tensioactivos aniónicos en líneas celulares tumorales y no tumorales, como propuesta de un modelo de ensayo predictivo de la citotoxicidad de nuevos compuestos bioactivos con potenciales aplicaciones cosméticas y/o farmacéuticas.

Una vez establecidas la actividad lítica pH-dependiente y la toxicidad de los tensioactivos, los objetivos se han dirigido a estudiar su potencial aplicación en el desarrollo de sistemas nanoestructurados, especialmente, para la liberación intracelular de biomoléculas. Los objetivos específicos que se han perseguido son:

**2.1.** Preparar nanovesículas lipídicas biocompatibles incluyendo los tensioactivos catiónicos derivados de lisina, y establecer sus características físicoquímicas.

**2.2.** Estudiar la potencial aplicación tópica de estas nanovesículas a través del estudio de su toxicidad dérmica, utilizando modelos de ensayo *in vitro* y células representativas de la piel.

**2.3.** Establecer las condiciones óptimas de los ensayos *in vitro* para una valoración toxicológica eficiente de las nanovesículas. Evaluar su actividad pH-sensible y su comportamiento intracelular tras su internalización, utilizando marcadores de endocitosis y microscopía de fluorescencia.

**2.4.** Establecer correlaciones entre las características estructurales y fisicoquímicas de las nanovesículas con su actividad citotóxica y comportamiento intracelular.

**2.5.** Preparar nanopartículas poliméricas incluyendo el tensioactivo aniónico con el contraión litio. Aplicar dichas nanopartículas para la encapsulación del fármaco antitumoral metotrexato y establecer sus características físico-químicas.

**2.6.** Estudiar la actividad antitumoral de dichas nanopartículas utilizando modelos celulares *in vitro* y establecer los posibles mecanismos involucrados. Evaluar la actividad pH-dependiente de estos nanocomplejos a través del ensayo de hemólisis y mediante microscopía de fluorescencia, utilizando un marcador fluorescente de endocitosis y estabilidad endosomal.

# INTRODUCCIÓN

# 1. Los tensioactivos

## 1.1. Características generales

Un compuesto tensioactivo es una sustancia química natural o sintética de estructura anfífila que modifica la tensión superficial en las interfases gas/líquido (aire-agua), líquido/líquido (aceite-agua) o gas/sólido (superficie de sólidos) (Attwood y Florence, 1983). El término anfífilo hace referencia a la existencia en la misma molécula de dos regiones claramente diferenciadas. Una es la porción hidrófoba (o apolar), que presenta afinidad por disolventes orgánicos o apolares y corresponde frecuentemente a una cadena hidrocarbonada, de tipo alquilo o alquil benceno, de longitud variable. La otra es la porción hidrófila (o polar), caracterizada por mostrar atracción hacia disolventes polares, sobre todo agua.

La naturaleza dual (polar-apolar) de los tensioactivos y en particular el equilibrio entre las partes hidrófoba e hidrófila de la molécula, conocido como balance hidrófilo-lipófilo (HLB), es la característica responsable de los fenómenos de actividad superficial y de agregación supramolecular (micelas, cristales líquidos, liposomas, vesículas o geles) de los tensioactivos. Estas características proporcionan a los tensioactivos sus numerosas aplicaciones en el desarrollo de productos industriales (Rosen, 2004).

El tensioactivo es capaz de disminuir la energía de los enlaces entre las moléculas de agua, es decir, de reducir la fuerza de tensión superficial del agua. Esto es posible debido a la adsorción progresiva de las moléculas de tensioactivo en la superficie de la solución, lo que provoca un cambio en las fuerzas de interacción de las moléculas de agua de la superficie. A medida que aumenta la concentración de tensioactivo, aumenta el número de moléculas de tensioactivo en la superficie, disminuyendo la tensión superficial hasta alcanzar un valor crítico, llamado concentración micelar crítica (CMC), a partir del cual, la tensión superficial se mantiene constante y se empiezan a formar agregados moleculares de tensioactivo o micelas en la solución (Berthod, 1983; Rosen, 2004), en equilibrio con los monómeros. A partir de la CMC, cualquier cantidad de tensioactivo que se añada a la solución se incorpora en forma de agregado y no de monómero.

La formación de micelas es una característica importante de los tensioactivos ya que algunos procesos que se dan en las interfases, como la interacción de los tensioactivos con membranas biológicas, la hemólisis y la solubilización dependen de las micelas o agregados presentes en la solución (Evans y Wennerströn, 1995; Adams y col., 2003; Ross y col., 2004; Dehghan-Noude y col., 2005).

## 1.2. Clasificación de los tensioactivos

Desde el punto de vista comercial, los tensioactivos se clasifican a menudo en función de su uso. Sin embargo, este tipo de clasificación no resulta muy adecuada ya que muchos tensioactivos presentan diversos usos y podría dar lugar a confusiones. La clasificación de tensioactivos más aceptada científicamente se basa en su capacidad de disociación en agua (ya que ésta es el principal medio de aplicación de los tensioactivos) y, por lo tanto, se clasifican según la naturaleza de su grupo hidrófilo o polar (Effendy y Maibach, 1996; Salager, 2002; Porter, 1994). Sobre la base de este criterio se clasifican en los siguientes grupos:

- *Aniónicos*: Son tensioactivos que en solución acuosa se disocian en un anión anfífilo (responsable de la actividad superficial) y un contraión catiónico, que suele ser un metal alcalino (sodio, potasio…) o un amonio cuaternario.

- *Catiónicos:* Se disocian dando lugar a un catión anfífilo y un contraión aniónico, que suele ser un halogenuro. La mayoría de estos agregados son compuestos nitrogenados tales como sales de amonio y sales de amonio cuaternario.

- *Anfóteros:* Son tensioactivos que, en función del pH de su grupo polar, dan lugar tanto a disociaciones aniónicas como catiónicas y pueden poseer las características de los aniónicos y/o de los catiónicos. Este es el caso de productos sintéticos como las betaínas o las sulfobetaínas y sustancias naturales como los aminoácidos y los fosfolípidos.

- *No iónicos:* El pH de la solución no afecta a esta clase de tensioactivos y no se ionizan en soluciones acuosas ya que su grupo hidrófilo (alcohol, fenol, éter, éster o amida) no se puede disociar. Un gran porcentaje de estos tensioactivos son hidrófilos debido a la presencia de una cadena de polietilenglicol (alcoholes etoxilados).

## 1.3. Tensioactivos derivados de aminoácidos

Características como la biodegradabilidad y la biocompatibilidad de los tensioactivos se han convertido en propiedades casi tan importantes como las posibles aplicaciones que puedan tener en la industria farmacéutica y cosmética. En consecuencia, actualmente existe la necesidad de desarrollar nuevas moléculas con propiedades tensioactivas y que, además, sean biodegradables, biocompatibles y con baja toxicidad. Sin embargo, para evitar

limitaciones en su amplio rango de aplicación, estos nuevos compuestos deben conservar las propiedades fisicoquímicas que presentan los tensioactivos utilizados actualmente.

Para alcanzar ese objetivo, se ha propuesto el diseño de análogos estructurales de compuestos naturales como los lipoaminoácidos, los glicolípidos y los fosfolípidos. La utilización de lipoaminoácidos constituye una de las mejores opciones para obtener el mismo rendimiento de los tensioactivos sintéticos convencionales en sus aplicaciones para la industria farmacéutica y cosmética (Morán y col., 2004a). Los lipoaminoácidos son moléculas anfífilas que contienen un aminoácido como porción hidrófila y al menos una cadena larga, generalmente hidrocarbonada, como porción hidrófoba. Basado en este contexto general, nuestro grupo de investigación viene colaborando desde hace más de 25 años en el desarrollo y caracterización biológica de tensioactivos derivados de aminoácidos. Se han realizado investigaciones químicas básicas y aplicadas (productos y procesos) para obtener este tipo de compuestos como alternativa a los tensioactivos convencionales (Infante y col., 2010). Considerando los principios de la química verde, esta línea de investigación ha contemplado la adopción de estos conceptos en la preparación de tensioactivos más eficientes y seguros, utilizando materias primas procedentes de fuentes renovables en su síntesis (p. ej. aminoácidos como lisina y arginina) y empleando disolventes seguros (sistemas acuosos, procesos libres de disolventes, emulsiones concentradas). Además, con el objetivo de aumentar la eficiencia de estos compuestos, se ha realizado una caracterización completa, incluyendo ecotoxicidad, biodegradabilidad y estudios de los mecanismos de toxicidad (Infante y col., 1997, 2001; Vives y col., 1997, 1999; Piera y col., 1998; Vinardell e Infante, 1999; Vinardell y col., 2008; Pinazo y col., 2000; Morán y col., 2001a, 2002, 2004a, 2004b; Clapés y Infante, 2002; Pérez y col., 2002a, 2004, 2009; Mitjans y col., 2003; Benavides y col., 2004a, 2004b; Martínez y col., 2006; Sánchez y col., 2006a). Los numerosos estudios realizados durante estos años en la investigación de los tensioactivos derivados de aminoácidos, han demostrado que estos compuestos presentan características como multifuncionalidad, biodegradabilidad y baja ecotoxicidad. En cuanto a la toxicidad en células humanas, estos compuestos mostraron, en general, niveles bajos a moderados, estando su estructura química directamente relacionada con los efectos tóxicos. En conjunto, las propiedades de estos tensioactivos cumplen con la demanda de los fabricantes y consumidores por productos químicos más eficaces y menos peligrosos para la salud humana y el medio ambiente.

La combinación de aminoácidos polares en la región hidrófila y de cadenas hidrocarbonadas de longitud variable (de 8 a 16 átomos de carbono) para formar compuestos con estructura anfífila ha dado lugar a numerosas moléculas con elevada capacidad tensioactiva (Infante y col., 1984, 1988, 2010; Seguer y col., 1994; Clapés y col., 1999; Piera y col., 2000; Morán y

col., 2001b, 2002; Pérez y col., 2002a, 2002b, 2009). La variedad de estructuras con aminoácidos y de residuos grasos presentes en estos tensioactivos explican su amplia diversidad estructural y sus muy diversas propiedades fisicoquímicas y biológicas.

Los aminoácidos pueden combinarse con las cadenas alifáticas para formar cuatro tipos principales de tensioactivos (Figura 1) (Morán y col., 2004a), conforme a las representaciones siguientes:

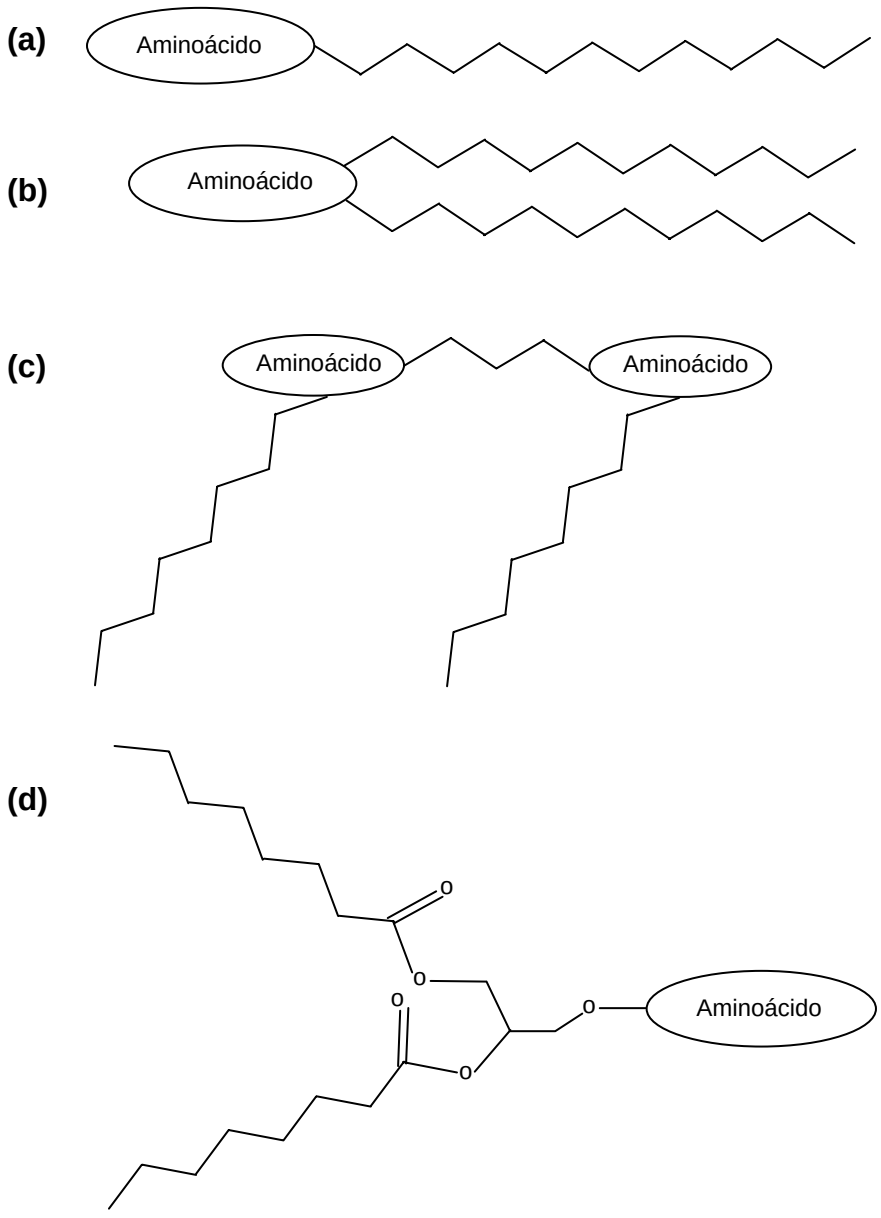


***Figura 1.*** *Estructuras de los diferentes tensioactivos derivados de aminoácidos: (a) lineales, (b) de doble cadena, (c) diméricos y (d) glicerolípidos.*

- *Lineales:* Consisten en un aminoácido (cabeza polar) unido a una cadena hidrocarbonada. A este grupo pertenecen los tensioactivos catiónicos del tipo sales de $N^{\alpha}$ y $N^{\varepsilon}$ -acil lisina (Pérez et al., 2009), objeto de estudio de esta Tesis.

- *De doble cadena:* Están formados por un aminoácido unido a dos cadenas hidrocarbonadas. Cabe destacar los tensioactivos aniónicos derivados de lisina (Vives y col., 1999; Sánchez et al., 2004, 2006a) también objeto de estudio de esta Tesis.

- *Diméricos (Gemini):* Son estructuras formadas por dos aminoácidos (cabezas polares) y dos grupos hidrófobos (cadenas hidrocarbonadas) separados por una molécula espaciadora unida covalentemente.

- *Glicerolipídicos:* Consisten en una cabeza polar (un aminoácido) y uno o dos grupos hidrófobos unidos por un esqueleto de glicerol.

En función del aminoácido incorporado/usado, cada uno de estos tipos pueden clasificarse a su vez se puede clasificar en tensioactivos aniónicos, catiónicos, anfóteros y no iónicos. La región hidrófila formada por el aminoácido determina las diferencias fundamentales en cuanto a la adsorción, la agregación y la actividad biológica del tensioactivo.

### 1.3.1. Tensioactivos derivados del aminoácido lisina

La lisina tiene gran importancia y aplicabilidad en la síntesis de compuestos tensioactivos con propiedades biocompatibles y biodegradables (Morán y col., 2010a). Por ser un aminoácido esencial que puede ser producido por fermentación bacteriana (Parekh y col., 2000), desde hace casi 50 años se están realizando esfuerzos constantes para mejorar su eficacia de producción con el objetivo de alcanzar una disponibilidad de la materia prima a larga escala, con bajo coste y con elevada estabilidad (Eggeling y Sahm, 1999).

La presencia de dos grupos aminos (catiónicos), con diferentes p*K*a/basicidad (los grupos α-amino y ε-amino tienen p*K*a = 8,9 y 10,5, respectivamente) (Pérez y col., 2009), y de un grupo carboxilo (aniónico, p*K*a = 2.2 ) (Maugras y col., 2001) en la molécula del aminoácido lisina, permite el desarrollo de una amplia variedad de tensioactivos con diferente carácter iónico (aniónico, catiónico, no iónico y derivados anfóteros), dependiendo de que grupo funcional se queda libre y en el que se introduce un grupo hidrófobo (ácido graso, amina grasa o alcohol graso). La naturaleza iónica del tensioactivo con la lisina como grupo polar depende del pH y de la modificación estructural específica en la molécula del tensioactivo, lo que permite un ajuste

fino de sus propiedades para alcanzar un amplio rango de aplicaciones (Morán y col., 2010a).

Dependiendo de su estructura química, estos compuestos pueden tener diferentes propiedades. Por lo tanto, los tensioactivos biocompatibles derivados de lisina tienen un amplio rango de potenciales aplicaciones industriales y biomédicas. Además, basándose en el conjunto de sus características fisicoquímicas y biológicas, la proyección de mercado de estos tensioactivos es muy alentadora (Morán y col., 2010a).

#### 1.3.1.1. Aniónicos

Se sintetizaron y estudiaron diversos tensioactivos no iónicos polioxietilenados, simétricos (dos cadenas iguales de ácidos grasos) y de doble cadena, del tipo *$N^{\alpha}$,$N^{\varepsilon}$ –diacil lisina* y con semejanza estructural a las lectinas naturales, con el fin de desarrollar tensioactivos no tóxicos para su aplicación en el campo farmacéutico y biológico (Seguer y col., 1994, 1996; Macián y col., 1996). Se obtuvieron homólogos de diferentes longitudes de cadena alquílica y diferente número y longitud de cadena oxietilenada. El homólogo *$N^{\alpha}$,$N^{\varepsilon}$ –dioctanoil lisina-N,N-bis[metilo eter dietileno glicol]amida* (77K22), con cadena alquílica formada por ocho átomos de carbono (C8), se ha mostrado el más eficaz como potencial tensioactivo para aplicaciones prácticas debido a sus mejores características fisicoquímicas (solubilidad en agua y actividad superficial en concentraciones normales de utilización), baja actividad hemolítica y irritante (Macián y col., 1996).

Teniendo como base el homólogo no iónico derivado de lisina con cadena alquílica doble de ocho átomos de carbono (77K22), se sintetizaron tensioactivos aniónicos de doble cadena como sales del tipo *$N^{\alpha}$,$N^{\varepsilon}$-diacil lisinatos* (Vives y col., 1999; Sánchez y col., 2004, 2006a). Se prepararon tensioactivos con excelentes propiedades superficiales mediante un método sencillo a través de la neutralización cuantitativa del intermediario ácido *$N^{\alpha}$,$N^{\varepsilon}$ –dioctanoil lisina* (77K) con su base correspondiente (Figura 2) (Seguer y col., 1994; Vives y col., 1999).

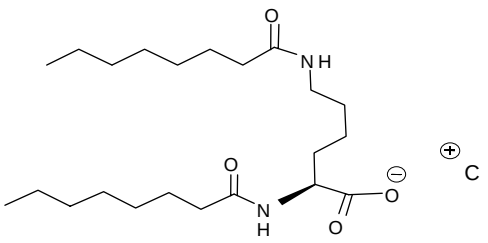


***Figura 2.*** *Esquema de las sales de $N^{\alpha}$,$N^{\varepsilon}$ -dioctanoil lisina. La letra C simboliza el contraión.*

Cabe destacar que los tensioactivos formados por dos cadenas hidrófobas resultan más eficaces cuando se comparan con los tensioactivos lineales convencionales. Este hecho se debe a que la presencia de dos cadenas hidrófobas en la misma molécula refuerza la interacción hidrofóbica inter o intracadena contribuyendo de una manera positiva a las propiedades superficiales y micelares del tensioactivo (Rosen, 1993; Zhu, 1991).

Teniendo en cuenta las consideraciones anteriores, los tensioactivos aniónicos derivados del aminoácido lisina son de gran interés debido a sus propiedades fisicoquímicas y a su biodegradabilidad, característica esta propia de los tensioactivos derivados de aminoácidos. Por lo tanto, en esta Tesis se ha estudiado una familia de lipoaminoácidos aniónicos de doble cadena: las sales derivadas de $N^{\alpha},N^{\varepsilon}$ -dioctanoil lisina (77K). Dicha familia comprende 5 tipos de sales (Figura 3): la sal de lisina (77KK), la de tris(hidroximetil) amino metano (77KT), la de sodio (77KS), la de litio (77KL) y la de potasio (77KP), representadas a continuación:

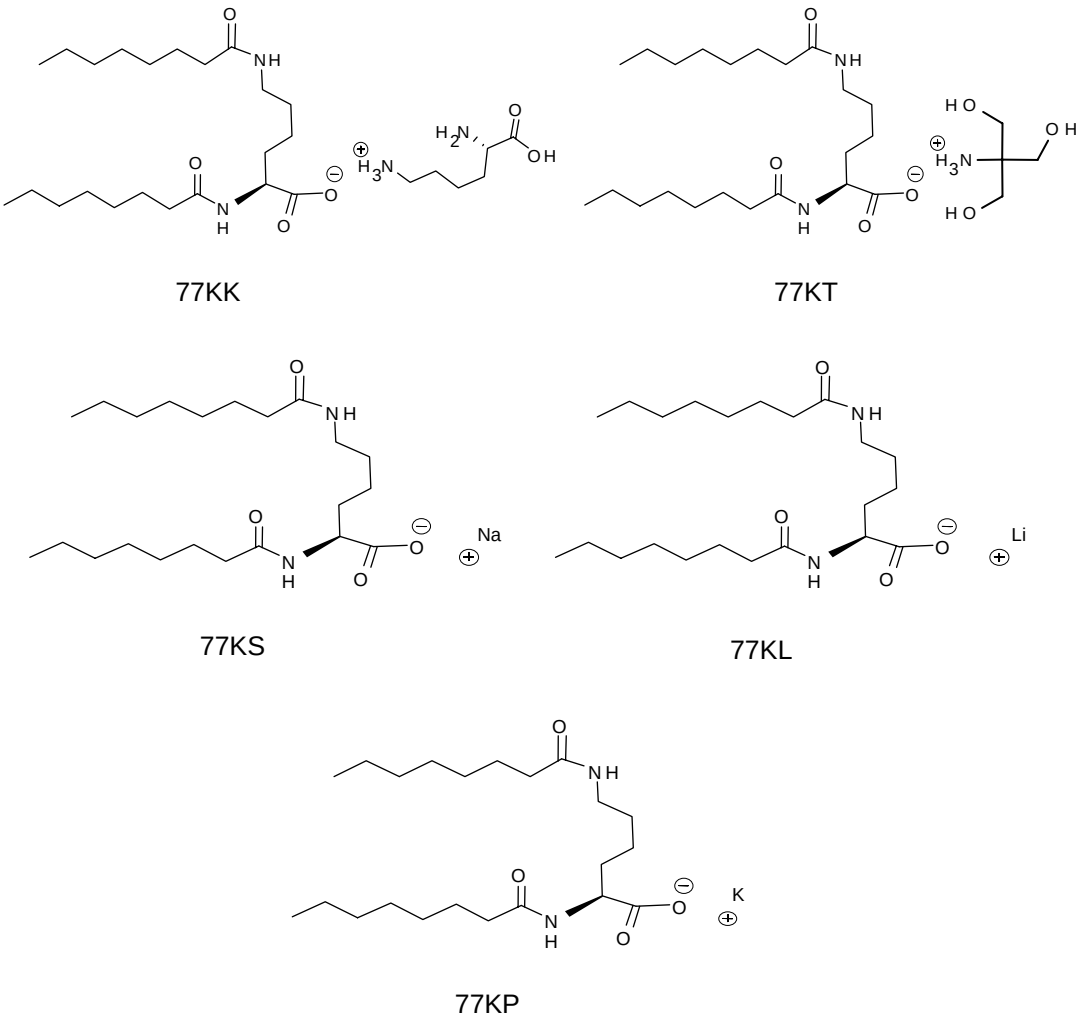


***Figura 3.*** *Estructuras químicas de los tensioactivos aniónicos derivados de la $N^{\alpha},N^{\varepsilon}$ -dioctanoil lisina.*

Estos tensioactivos tienen la siguiente estructura:

• Una parte hidrófoba: formada por la condensación de dos cadenas de ácidos grasos saturados de 8 átomos de carbono con los grupos α-amino y ε-amino de la lisina y representada en la nomenclatura por *77K*, donde "77" indica las dos cadenas alquílicas (el número 7 indica los grupos metileno de la cadena pero sin contar el grupo carbonilo del ácido graso) y la letra K, la lisina.

• Una parte hidrófila: formada por el aminoácido lisina, cuyo grupo carboxilo está ionizado en forma de sal aniónica, y el correspondiente contraión catiónico.

• Diferentes contraiones: estos pueden ser orgánicos y voluminosos, como los cationes lisina (K) y tris(hidroximetil) amino metano (T) o inorgánicos y pequeños, como los cationes sodio (S), litio (L) y potasio (P).

Este grupo de tensioactivos se ha estudiado exhaustivamente en cuanto a sus propiedades biológicas. Los ensayos de hemólisis, ampliamente utilizados para valorar la toxicidad de nuevos tensioactivos (Vinardell e Infante, 1999; Mitjans y col., 2003; Dufour y col., 2005; Martínez y col., 2006), han demostrado que estos tensioactivos aniónicos derivados de lisina presentan menor actividad hemolítica que los tensioactivos sintéticos convencionales (Vives y col., 1997, 1999; Sánchez et al., 2006a). Además, el mecanismo de interacción de estos compuestos con las membranas celulares se ha estudiado a través de la evaluación de la protección frente a la hemólisis hipotónica (antihemólisis) (Vives y col., 1999; Sánchez y col., 2007a), de los cambios en la forma de los eritrocitos en medio hipotónico (Vives y col., 1999) y también de las alteraciones en la fluidez de la membrana mediante anisotropía de fluorescencia (Martínez y col., 2007). Se observó un comportamiento bifásico de la actividad antihemolítica, con protección frente a la hemólisis hipotónica a concentraciones bajas e inducción de la hemólisis a concentraciones altas. Los experimentos de anisotropía de fluorescencia con las sondas 1,6-difenil-1,3,5-hexatrieno (DPH) y trimetilamonio 1,6-difenil-1,3,5-hexatrieno (TMA-DPH) mostraron una tendencia de los tensioactivos a perturbar la región externa de la bicapa lipídica, sin afectar la interna. Respecto a la morfología, se observaron cambios de discocitos a estomatocitos dependientes del tiempo de contacto.

El potencial efecto irritante dérmico de este grupo de tensioactivos también se ha estudiado mediante técnicas de cultivo celular, utilizando líneas celulares de fibroblastos (3T6) y queratinocitos (NCTC 2544). Los resultados de concentración inhibitoria 50 ($CI_{50}$) obtenidos sugieren que esos tensioactivos presentan un potencial efecto irritante menor que el dodecil sulfato sódico (SDS, en sus siglas en inglés), utilizado como control positivo. Además, se

observó que los tensioactivos con contraiones orgánicos presentan una tendencia a ser menos irritantes que los que tienen contraiones inorgánicos (Sánchez y col., 2004). En estudios sucesivos se determinaron sus efectos hemolíticos, fotohemolíticos y citotóxicos en células HaCaT. Los resultados mostraron baja actividad hemolítica, ningún efecto fototóxico significativo y baja citotoxicidad en comparación con los tensioactivos comerciales, siendo los de contraión orgánico significativamente menos citotóxicos (Sánchez y col., 2006a). Con el objetivo de completar el perfil toxicológico de estos tensioactivos, se valoró en queratinocitos NCTC 2544 la producción de interleucina 1 alpha (IL-1α) intracelular y la liberación al medio de cultivo. Se demostró menor potencia de los tensioactivos derivados de lisina en estimular la síntesis y la liberación de la citocina en comparación al SDS, confirmando su bajo potencial efecto irritante dérmico (Sánchez y col., 2006b).

La influencia de los diferentes contraiones asociados a los tensioactivos derivados de la $N^{\alpha},N^{\varepsilon}$ -dioctanoil lisina sobre la actividad biológica también se estudió en células U937, donde se evaluó la capacidad de producir necrosis o inducir una señal que conduce a la muerte celular por apoptosis. Se observó que la agresividad de los compuestos depende de la naturaleza de los contraiones, siendo mayor cuando los tensioactivos están asociados a contraiones pequeños (Maugras y col., 2001).

En estudios posteriores de este grupo de tensioactivos se determinaron la toxicidad acuática mediante el ensayo de inmovilización del crustáceo *Daphnia magna* y las propiedades antimicrobianas en una serie de bacterias y levaduras calculando la concentración mínima inhibitoria (MIC, en sus siglas en inglés). Los resultados obtenidos mostraron que el 77KT y 77KL presentan una menor toxicidad acuática. Por otro lado, los valores de MIC de los ensayos de susceptibilidad antimicrobiana mostraron que los tensioactivos sólo son activos frente a las levaduras y la bacteria gram-negativa *Bordetella bronchiseptica.* Además, no se encontró ninguna relación entre el tipo de contraión asociado a los tensioactivos y las propiedades estudiadas (Sánchez y col., 2007b).

Los tensioactivos aniónicos derivados del aminoácido lisina son de gran interés y, como se ha descrito anteriormente, han sido ampliamente estudiados en relación a sus propiedades fisicoquímicas y biológicas. Teniendo en cuenta sus características estructurales y su baja toxicidad en comparación con tensioactivos comerciales, y también basándose en la búsqueda de nuevas aplicaciones, en esta Tesis se ha estudiado su actividad hemolítica dependiente del pH y se ha complementado el estudio de su citotoxicidad, utilizando un modelo de ensayo predictivo basado en una combinación de diferentes líneas celulares y ensayos de viabilidad celular. A continuación, se ha valorado su efecto como excipiente bioactivo en vehículos nanoestructurados para liberación intracelular de fármacos.

#### 1.3.1.2. Catiónicos

Los tensioactivos catiónicos tienen dos propiedades importantes: son fácilmente absorbidos en la interfase sólido/líquido y pueden interaccionar con las membranas celulares, que poseen una carga neta negativa. Consecuentemente, la potencial toxicidad de estos tensioactivos catiónicos, atribuida a su fuerte interacción (por atracción electrostática) con las membranas de las células, es todavía un obstáculo para su utilización en aplicaciones biológicas. La búsqueda de compuestos menos tóxicos y más biocompatibles, pero con las mismas propiedades tensioactivas de los productos sintéticos convencionales, ha propiciado el desarrollo de tensioactivos catiónicos derivados del aminoácido lisina.

Los tensioactivos catiónicos del tipo $N^{\alpha}$- o $N^{\varepsilon}$-acil lisina se han sintetizado recientemente siguiendo las metodologías para formación de funciones amidas en fase líquida (Pérez y col., 2009; Colomer y col., 2011). El procedimiento utilizado para la síntesis de los tensioactivos $N^{\alpha}$-acil lisina metil éster o $N^{\varepsilon}$-acil lisina metil éster ha sido simple y muy efectivo, y se ha realizado a través de la reacción de los compuestos $N^{\varepsilon}$-Cbz-L-lisina metil éster o $N^{\alpha}$- Cbz-L-lisina metil éster, respectivamente, con el correspondiente ácido graso saturado.

Los tensioactivos catiónicos están generalmente compuestos por tres dominios básicos: un grupo polar positivo, una fracción hidrofóbica y un enlazador entre estas dos regiones (Colomer y col., 2012). La longitud de cadena del grupo hidrófobo es una variable de gran importancia en la determinación de las propiedades de agregación y de los efectos biológicos de un tensioactivo. Sin embargo, existen estudios demostrando que estas características de los tensioactivos no dependen únicamente del grupo hidrófobo, si no también del grupo hidrófilo, incluyendo propiedades como su tamaño, y el tipo, número y posición espacial de su carga catiónica (Colomer y col., 2011).

En la búsqueda de relaciones estructura-actividad que permitan establecer los efectos de diferentes grupos hidrófobos e hidrófilos en la estructura final del tensioactivo, en esta Tesis, se ha estudiado un grupo de tensioactivos catiónicos derivados de lisina del tipo sales de hidrocloruro de $N^{\alpha}$ o $N^{\varepsilon}$-acil lisina metil éster que difieren en cuanto a la longitud de la cadena hidrocarbonada y a la posición de la carga catiónica. Estos tensioactivos tienen una estructura química caracterizada por un radical acilo (grupo hidrófobo) unido al grupo α- o ε- amino de la lisina (grupo polar) a través de un enlace amida, lo que les confiere propiedades biodegradables. Por lo tanto, con el objetivo de establecer el efecto que los cambios estructurales provocan en sus propiedades biológicas, se han estudiado los siguientes compuestos (Figura 4): MKM, $N^{\varepsilon}$-Miristoil lisina metil éster, constituido por una cadena alquílica hidrocarbonada de 14 átomos y una carga positiva en el grupo α-amino de la lisina; PKM, $N^{\varepsilon}$-Palmitoil lisina metil éster, constituido por una cadena alquílica

hidrocarbonada de 16 átomos y una carga positiva en el grupo α-amino de la lisina; y MLM, $N^{\alpha}$-Miristoil lisina metil éster, constituido por una cadena alquílica hidrocarbonada de 14 átomos y una carga positiva en el grupo ε-amino de la lisina. El MKM y el PKM tienen la cadena alquílica hidrocarbonada unida al grupo ε-amino de la lisina, mientras que el MLM tiene la cadena alquílica hidrocarbonada unida al grupo α-amino de la lisina. Sus estructuras químicas se representan a continuación:

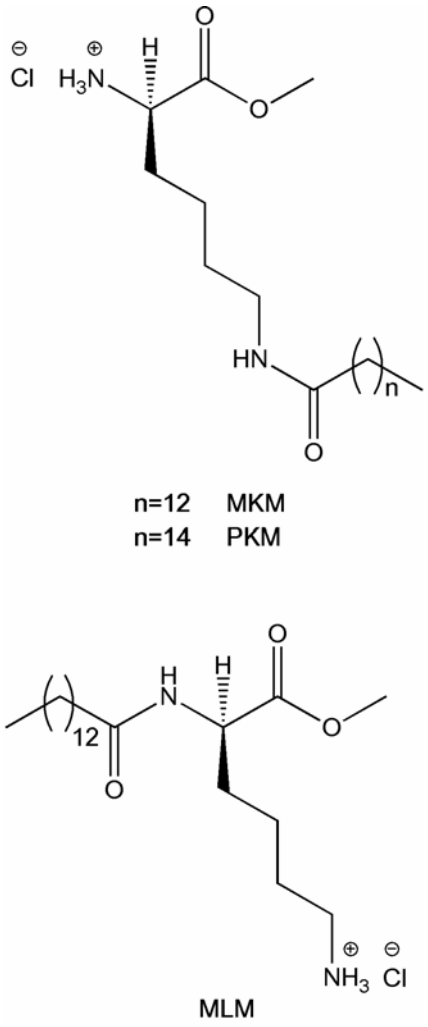


***Figura 4.*** *Estructuras químicas de los tensioactivos catiónicos derivados de la $N^{\varepsilon}$-acil lisina (MKM y PKM) o $N^{\alpha}$-acil lisina (MLM).*

Este nuevo grupo de tensioactivos catiónicos derivados de la *N-acil lisina* se estudió previamente en cuanto a su capacidad de formar micelas, actividad antimicrobiana y hemolítica, ecotoxicidad y biodegradabilidad (Pérez y col., 2009). Se ha demostrado que estos compuestos tienen actividad antimicrobiana moderada contra las bacterias gram-positivas, además de presentar una actividad hemolítica considerablemente inferior en comparación a los tensioactivos catiónicos convencionales, derivados del amonio cuaternario (Funasaki y col., 2000; Shalel y col., 2002; Vieira y Carmona-Ribeiro, 2006) y

a otros análogos catiónicos del tipo $N^{\alpha}$–acil arginina (Pérez y col., 2005). Además, se ha observado moderada toxicidad acuática y elevado nivel de biodegradación. Como conclusión, se ha observado que la posición de la carga catiónica tiene efecto sobre la actividad antimicrobiana y el potencial hemolítico, pero no afecta los parámetros biodegradabilidad y capacidad de formación de agregados micelares.

El estudio de los tensioactivos catiónicos derivados de lisina se ha extendido a la síntesis y valoración comparativa de tres grupos diferentes: monocatenarios con un aminoácido, monocatenarios con dos aminoácidos y geminales con dos lisinas en el grupo polar (Colomer y col., 2011, 2012). La estructura, número y posición de la carga catiónica han sido sistemáticamente variados en estos compuestos. Se ha observado que la concentración micelar crítica (CMC) de estos tensioactivos sólo depende del carácter hidrófobo de la molécula. Sin embargo, la actividad antimicrobiana, la citotoxicidad y el potencial hemolítico están relacionados con la hidrofobicidad de los compuestos, así como con el número y posición de la carga catiónica. Además, estos tensioactivos también han presentado un índice de biodegradación elevado y toxicidad acuática inferior a los compuestos basados en el amonio cuaternario. Por último, estos compuestos han demostrado una actividad hemolítica dependiente del pH, lo que también ha motivado el estudio del efecto del pH en la actividad de los tensioactivos catiónicos estudiados en esta Tesis. Cabe destacar que esta actividad pH-dependiente sugiere la potencial aplicación de estos compuestos en vehículos para la liberación intracelular de fármacos (Wang y col., 2007; Chen y col., 2009).

## 2. Nanomateriales

Los nanomateriales se definen clásicamente como sustancias que tienen una o más dimensiones externas en una escala inferior a 100 nm (Horie y col., 2012). De acuerdo con la Comisión Europea (Recomendación UE/696/2011) (UE, 2011), un nanomaterial se define como "un material natural, secundario o fabricado que contenga partículas, sueltas o formando un agregado o aglomerado y en el que el 50% o más de las partículas en la granulometría numérica presente una o más dimensiones externas en el intervalo de tamaños comprendido entre 1 nm y 100 nm. En casos específicos y cuando se justifique por preocupaciones de medio ambiente, salud, seguridad o competitividad, el umbral de la granulometría numérica del 50% puede substituirse por un umbral comprendido entre el 1% y el 50%". Por otro lado, los nanomateriales también se clasifican como partículas nanométricas con tamaño máximo de 200 nm (Cho y col., 2008; Singh y Lillard Jr, 2009; Paillard y col., 2010), mientras que muchos autores definen los nanomateriales como estructuras con tamaño inferior a 1000 nm (Kreuter, 1994; Sahoo y Labhasetwar, 2003; Sahoo

y col., 2007; Parveen y Sahoo, 2008), así como también lo hace la Agencia Europea de Medicamentos (EMA, en sus siglas en inglés) (EMA, 2012).

Se calcula que la cantidad total anual de nanomateriales en el mercado mundial es de unos 11 millones de toneladas, con un valor de mercado de aproximadamente 20000 millones de euros. El negro de carbón y la sílice amorfa representan, con mucha diferencia, el mayor volumen de los nanomateriales que existen actualmente en el mercado. En la actualidad, el grupo de materiales que más reclama la atención está constituido por el nanodióxido de titanio, el nanóxido de cinc, los fullerenos, los nanotubos de carbono y la nanoplata. Al mismo tiempo, se están desarrollando otros nuevos nanomateriales y aparecen nuevos usos. Muchos se utilizan en aplicaciones innovadoras como los catalizadores, la electrónica, los paneles solares, las baterías y las aplicaciones biomédicas, incluyendo el diagnóstico y las terapias oncológicas (COM, 2012). Las bondades de los nanomateriales abarcan desde salvar vidas hasta los avances que permiten la puesta a punto de nuevas aplicaciones o la reducción del impacto medioambiental, pasando por la mejora de las funciones de los productos básicos.

La nanotecnología es uno de los desarrollos tecnológicos más importantes de este siglo. Se ha visto que los materiales a estas dimensiones tienen unas propiedades que permiten múltiples aplicaciones, entre ellas hay que resaltar sus usos en el diagnóstico médico, cosméticos, preparados alimentarios y vehículos para encapsulación y liberación específica de fármacos. Además, esta nueva tecnología viene siendo investigada como una manera de mejorar las propiedades de los medicamentos, como su solubilidad o estabilidad, y para desarrollar medicamentos que puedan proporcionar nuevas maneras de liberar los medicamentos en su sitio de acción, dirigir los medicamentos hacia su sitio de acción específico, diagnosticar y tratar enfermedades y promover la regeneración de células y tejidos. Actualmente, el EMA, conjuntamente con la Comisión Europea, ya ha aprobado 20 medicamentos basados en la nanotecnología (COM, 2012; EMA, 2012), incluyendo formulaciones de liposomas y nanopartículas de sustancias activas. Además, el PEN (*The Project on Emerging Nanotechnologies*) (PEN, 2012) informa y facilita una base de datos con los productos médico/farmacéuticos basados en la nanotecnología que ya están disponibles actualmente (Tabla 1).

***Tabla 1.*** *Principales productos médicos/farmacéuticos basados en la nanotecnología actualmente aprobados por el FDA y/o EMA* (Kelly y Bogaert, 2008; EMA, 2012; PEN, 2012).*

| Producto | Categoría | Nanotecnología | Fecha aprobación | Empresa Responsable |
|---|---|---|---|---|
| ***Megace®ES*** | Control del apetito (Acetato de Megestrol) | Nanocristales | FDA 07/2004 | Par Pharmaceutical Companies, Inc (USA) |
| ***Abraxane ™*** | Cáncer (Paclitaxel) | Nanopartículas unidas a albúmina | FDA 01/2005 EMA 01/2008 | American Pharmaceutical Partners, Inc. (USA) Celgene Europe Ltd. (Reino Unido) |
| ***Doxil®*** | Cáncer (Doxorubicina) | Nanopartículas lipídicas con revestimiento de polietilenoglicol | FDA 01/2005 | ALZA Coporation (USA) |
| ***Caelyx®*** | Cáncer (Doxorubicina) | Liposomas | EMA 06/1996 | Janssen-Cilag Internacional N.V. |
| ***DaunoXome®*** | Cáncer (Daunorubicina) | Liposomas PEGilados | FDA 04/1996 | Galen Pharmaceutical Company (Irlanda) |
| ***Myocet®*** | Cáncer (Doxorubicina) | Liposomas | EMA 07/2000 | Cephalon Europe |
| ***Emend®*** | Cáncer (Aprepitant) | Nanocristales | FDA 03/2003 EMA 11/2003 | Merck & Co., Inc.(USA) Merck Sharp & Dohme Ltd (Reino Unido) |
| ***Mepact®*** | Cáncer (Mifamurtide) | Liposomas | EMA 03/2009 | IDM Pharma SAS |
| ***TriCor®*** | Colesterol (Fenofibrato) | Nanocristales | 12/2004 | Abbott Laboratorios (USA) |
| ***Rapamune®*** | Inmunosupresor (Sirolimus) | Nanocristales | FDA 08/2000 EMA 03/2001 | Wyeth (USA) Pfizer Limited (Reino Unido) |
| ***Estrasorb ™*** | Terapia hormonal (estrógeno) | Nanopartículas micelares | FDA 10/2003 | Novavax, Inc. (USA) |
| ***PegIntron*** | Hepatitis C (Peginterferon alfa-2b) | Conjugado polímero-proteína | EMA 05/2000 | Merck Sharp & Dohme Limited (Reino Unido) |
| ***Copaxone®*** | Esclerosis múltipla (acetato de glatiramer) | Sustáncia polimérica en formato "nano" | EMA 1996 | Teva Pharmaceutical Industries Ltd. |
| ***Somavert*** | Acromegalia (Pegvisomant) | Conjugado polímero-proteína | EMA 11/2002 | Pfizer Limited (Reino Unido) |
| ***Vitoss*** | Reemplazo óseo | "Scaffolds" compuesto por nanopartículas | 02/2004 | Orthovita (USA) |
| ***Óxido de zirconio*** | Reemplazo óseo | Óxido de zirconio en tamaño nanométrico | 09/2003 | Altair Nanotechnologies, Inc. (USA) |
| ***CellTracks®*** | Pruebas diagnósticas | Nanopartículas magnéticas | Entre 2004-2005 | Immunicon Corporation (USA) |

| | | | | |
|---|---|---|---|---|
| ***NanoChip® Technology*** | Pruebas diagnósticas | Tecnología "Nanogen's" | 2005 | CambiMatrix Corporation (USA) |
| ***Qdot*** | Imágenes y diagnóstico | Nanocristales | No informado | Invitrogen Corporation (USA) |
| ***TriLite™ Technology*** | Imágenes y diagnóstico | Nanocristales y nanoclusteres | No informado | Crystalplex Corportaion (USA) |

* Muchos de estos productos ya están disponibles comercialmente pero no están disponibles directamente al consumidor. Son utilizados por investigadores involucrados en el descubrimiento de nuevos fármacos, por médicos que necesitan mejores técnicas de imágenes, o como prescripciones para tratar enfermedades muy particulares.

## 2.1. Clasificación de los nanomateriales

Los nanomateriales pueden estar constituidos por una amplia variedad de materiales, incluyendo polímeros, lípidos, virus y compuestos organometálicos. De acuerdo con su composición, los namomateriales se pueden clasificar en diversos tipos (Figura 5) (Kim, 2007; Cho y col., 2008; Jabir y col., 2012; Parveen y col., 2012), entre los cuales podemos destacar:

- *Nanopartículas poliméricas*: son generalmente formadas por polímeros biodegradables y la molécula de interés terapéutico puede estar disuelta, encapsulada o dispersa en la matriz polimérica. Además, su estructura se puede formar por la conjugación del fármaco a la cadena lateral de un polímero lineal. También presentan ventajas como solubilidad acuosa, biodegradabilidad y posibilidad de modificación de la superficie.

- *Nanopartículas lipídicas sólidas*: son sistemas coloidales formados por lípidos sólidos y estabilizados por tensioactivos. Pueden estar formados por triglicéridos altamente purificados, mezclas complejas de glicéridos o por ceras. Presentan ventajas como buena tolerabilidad, biodegradabilidad, estabilidad física, protección de fármacos lábiles, fácil preparación y baja toxicidad, además de tener potencial aplicación por las vías pulmonar, intravenosa y dérmica.

- *Nanopartículas magnéticas*: son partículas de óxido de hierro, ampliamente utilizadas como agente de contraste y más recientemente como nueva tecnología en el tratamiento del cáncer. Pueden ser activadas usando campos magnéticos, lo que permite su direccionamiento a sitios específicos de acción.

- *Nanopartículas metálicas*: son estructuras compuestas especialmente por metales como el oro, el aluminio y la plata. Presentan propiedades fisicoquímicas únicas, como tamaño muy pequeño, gran área superficial y alta reactividad, además de biocompatibilidad y baja toxicidad.

- *Nanopartículas virales*: son nanopartículas revestidas por subunidades proteicas derivadas de virus, que presentan estructuras multivalentes y autoensambladas.

- *Liposomas/nanovesículas lipídicas*: son sistemas coloidales que se forman espontáneamente cuando ciertos lípidos son hidratados en medio acuoso. Están constituidos por un volumen acuoso encapsulado por una o más bicapas de lípidos sintéticos o naturales.

- *Polimersomas*: son sistemas coloidales de tipo vesicular, análogos a los liposomas, en los cuales el fármaco está confinado en una cavidad hidrofílica revestida por una membrana polimérica.

- *Micelas*: están formadas por bloques de copolímeros anfífilos que se ensamblan y forman estructuras con arquitectura similar a nanocápsulas. Estos sistemas tienen el núcleo interno compuesto por las regiones hidrófobas de los compuestos anfífilos, creando así un espacio para encapsulación y solubilización de fármacos lipófilos.

- *Dendrímeros*: estructura formada por polímero sintético radialmente hiperramificado con patrón regular y unidades repetidas. Presentan varios grupos terminales en la superficie, adecuados para la bioconjugación de fármacos, grupos de señalización, moléculas de direccionamiento específico y grupos que confieren características biocompatibles al sistema.

- *Nanotubos de carbono*: son cilindros carbónicos compuesto por un anillo de benceno. Debido a una modificación química (funcionalización orgánica) tiene características como biocompatibilidad y solubilidad en agua.

- *Nanodiamantes*: presentan mejor biocompatibilidad que otros nanomateriales derivados del carbono. Además, son estables, fotoluminescentes, presentan mínima citotoxicidad, son fáciles de purificar y están disponibles comercialmente.

- *Quantum dots*: son estructuras generalmente referidas como nanocristales. Están formados por materiales semiconductores, compuestos por 10 a 50 átomos y confinan electrones a un nivel discreto de energía cuantizada. Deben estar revestidos por moléculas especiales o anticuerpos para facilitar su captación por las células. Constituyen una clase prometedora de sondas fluorescentes para el cribado del cáncer en fluidos biológicos, la clasificación de tumores provenientes de biopsias y como artefactos para técnicas de imagen celular y biomolecular de alta resolución.

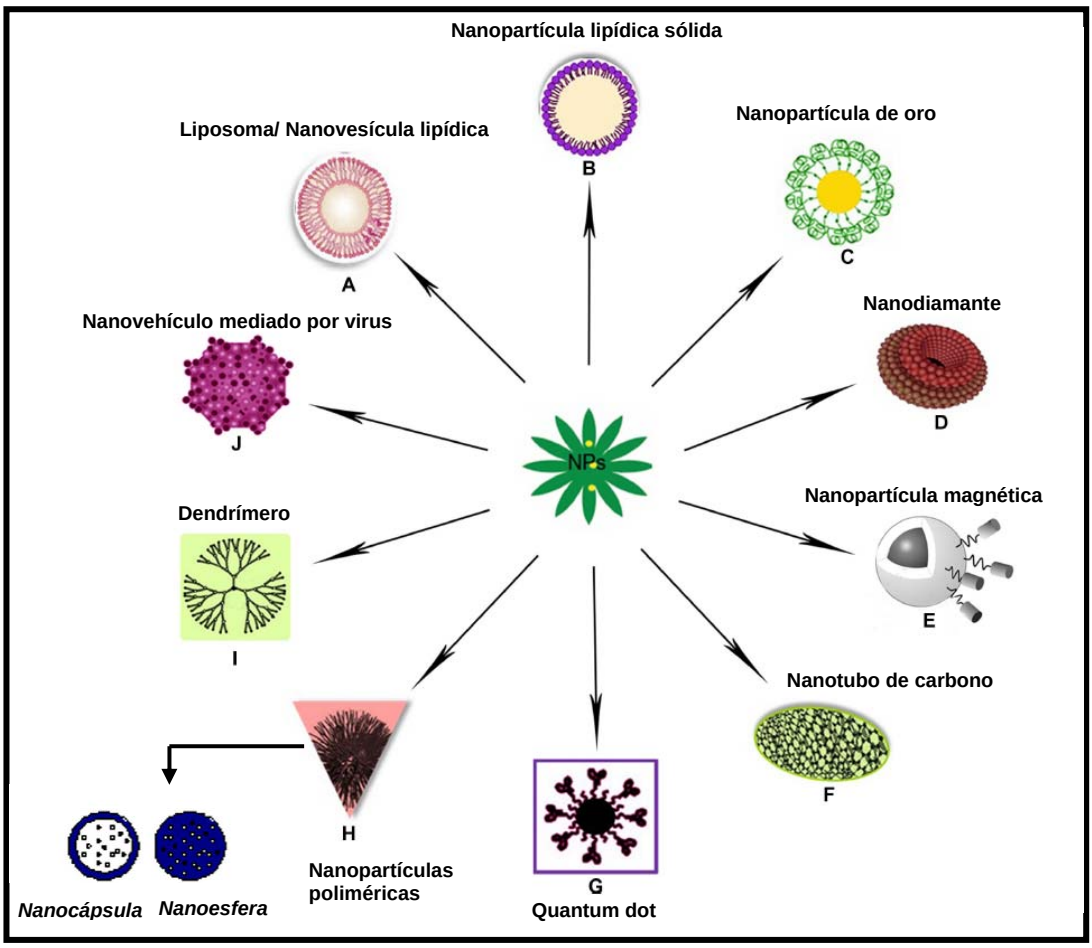


***Figura 5.*** *Representación esquemática de los diferentes nanomateriales utilizados como vehículos para la encapsulación y liberación controlada de fármacos (Adaptada de Jabir y col., 2012; Parveen y col., 2012).*

Entre los diversos tipos de nanomateriales, se destacan las nanovesículas lipídicas y las nanopartículas poliméricas, objetos de estudio en esta Tesis.

### 2.1.1. Nanovesículas lipídicas

Las nanovesículas lipídicas son estructuras coloidales que tienen forma esférica y están compuestas por una bicapa lipídica que rodea un espacio central acuoso (Cho y col., 2012). Se han demostrado útiles para la liberación de diversos agentes terapéuticos, como fármacos antitumorales y antimicrobianos, agentes quelantes, esteroides, vacunas y material genético (Sharma y Sharma, 1997; Dragicevic-Curic y col., 2010; He y col., 2010; Pollock y col., 2010). Su aplicación en el tratamiento del cáncer está bastante avanzada, con fármacos ya aprobados y disponibles comercialmente, tal y como se ha mostrado anteriormente en la Tabla 1. Además, son vehículos

eficientes para la liberación específica de fármacos hidrófobos sin provocar una respuesta inmunitaria (Ranganathan y col., 2012). Estos vehículos son estables, biocompatibles, biodegradables y están formados por membranas de fosfolípidos autoensambladas con un núcleo interno hidrófilo (Jabir y col., 2012). Además, son "sistemas de liberación de fármacos facilitado por contacto", que se basa en su unión o interacción con la membrana celular (Singh y Lillard Jr., 2009). Estas vesículas pueden transportar fármacos en su interior, solubilizados en su bicapa lipídica o adsorbidos en su interface lípido-agua (Liang y Chou, 2009; Cevc 2012). Actualmente, los sistemas basados en lípidos con aplicación terapéutica están generalmente formados por fosfolípidos (p. ej. fosfatidilcolina), suplementados o no con colesterol, debido a la amplia, pero no siempre justificada, creencia que este compuesto es un prerrequisito para la estabilidad de la bicapa lipídica de las nanovesículas (Cevc, 2012). La formación básica de las nanovesículas se basa en constituyentes lipídicos neutros. Sin embargo, su modificación estructural por compuestos bioactivos, con propiedades catiónicas o aniónicas, puede conferir mejores propiedades fisicoquímicas a dichas vesículas que, a su vez, pueden mejorar y/o ampliar su rango de aplicaciones terapéuticas (Chen y col., 2004; Zhang y col., 2004; Liang y Chou 2009).

### 2.1.2. Nanopartículas poliméricas

Las nanopartículas poliméricas preparadas con polímeros sintéticos o naturales son interesantes debido a su estabilidad y facilidad de modificación de la superficie (Herrero-Vanrell y col., 2005; Singh y Lillard Jr, 2009). Además, pueden lograr tanto una liberación controlada de fármacos, como localización enfermedad-específica a través de la regulación de las características del polímero y de la superficie química del nanomaterial resultante (Kreuter, 1994; Moghimi y col., 2001; Panyam y Labhasetwar, 2003). Dependiendo del proceso utilizado en su preparación, las nanopartículas poliméricas pueden ser nanoesferas o nanocápsulas. Las nanoesferas tienen una estructura con matriz sólida, en que los compuestos activos pueden estar adsorbidos en la superficie o encapsulados en el interior de la matriz. Por otro lado, las nanocápsulas tienen una envoltura polimérica y una matriz interior, en la que se disuelve la sustancia activa (Jabir y col., 2012; Parveen y col., 2012).

Para el desarrollo de nanopartículas poliméricas se utilizan polímeros orgánicos debido a su elevada biocompatibilidad celular, biodegradabilidad y aprobación reglamentaria. Estos polímeros pueden ser naturales, como el colágeno, la queratina, la celulosa y el quitosano; naturales modificados, a los cuales se añaden grupos polares a las cadenas para intentar disminuir el tiempo de degradación; y los sintéticos, que generalmente poseen como características la hidrofobicidad y un peso molecular menor (Jain, 2000).

Entre los polímeros naturales, el quitosano, un polímero catiónico obtenido de la desacetilación de la quitina, que a su vez, proviene del caparazón de crustáceos e insectos, se ha utilizado ampliamente en la producción de nanopartículas debido a sus propiedades fisicoquímicas (Loh y col., 2010; Mehrotra y col., 2011; Trapani y col., 2011). Además, presenta características esenciales en vehículos destinados a aplicaciones biomédicas, como biocompatibilidad, biodegradabilidad, baja toxicidad y bioadhesión (Mei y col., 2008). Para la producción de nanopartículas poliméricas y la encapsulación de fármacos existen varias técnicas, como los métodos de coacervación, reticulación de suspensión, nebulización y fluidización. Sin embargo, la mayoría de estos procesos utilizan concentraciones altas de disolventes orgánicos que pueden degradar el fármaco, otros pueden ofrecer rendimiento bajo de encapsulación, mientras que otros requieren temperaturas elevadas y pH extremos que pueden también inactivar el fármaco. En este sentido, se han buscado métodos para la producción de nanopartículas que además de garantizar la estabilidad de la molécula y el mantenimiento de su actividad biológica, puedan formar estructuras estables, solubles, con tamaño y carga favorable a la aplicación deseada. Los métodos basados en la gelificación iónica, que consiste en la formación espontánea de complejos de quitosano y polianiones, han sido muy estudiados por su simplicidad y eficiencia en la encapsulación de diversas biomoléculas (Calvo y col., 1997; Bao y col., 2008; Fan y col., 2012). Este proceso se basa en el entrecruzamiento reversible a través de interacciones electrostáticas (p. ej. entre los grupos aminos protonados $-NH_3^+$ y un anión como el tripolifosfato (TPP)) (Figura 6), en lugar de un entrecruzamiento químico, lo que, por lo tanto, previene la potencial toxicidad de los reactivos y el daño a las biomoléculas (Berger y col., 2004; Fan y col., 2012).

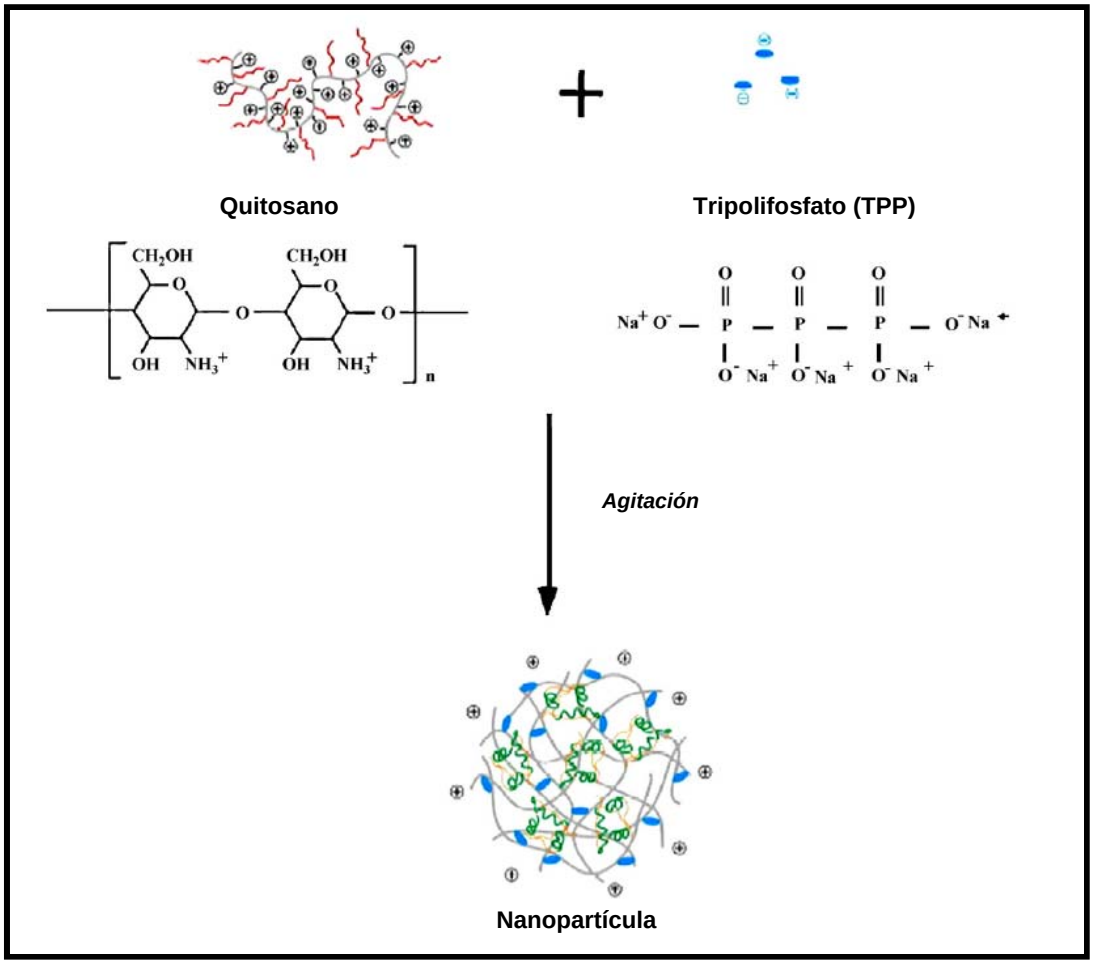


***Figura 6.*** *Representación esquemática de la formación de nanopartículas de quitosano a través del método de gelificación iónica (Adaptada de Trapani y col. 2009)*

### 2.1.2.1. Nanopartículas poliméricas en la terapia anticancerosa

El cáncer es una enfermedad altamente compleja, ya que implica sistemas celulares múltiples. Los tratamientos más comunes para esta enfermedad están limitados a quimioterapia, radiación y cirugía. Sin embargo, la eficacia clínica de dichos tratamientos sigue siendo un reto importante para el siglo XXI (Jabir y col., 2012). Dentro de este contexto, existe una gran necesidad de desarrollar tecnologías nuevas e innovadoras que puedan ayudar a lograr un tratamiento más eficiente contra las células cancerígenas. La nanotecnología se considera actualmente como un campo del conocimiento farmacéutico con varias aplicaciones potenciales en la biología del cáncer, como la detección temprana del tumor, el descubrimiento de biomarcadores de células tumorales y el desarrollo de nuevos tratamientos (p. ej. utilizando fármacos anticancerosos encapsulados en nanopartículas poliméricas) (Cai y col., 2008).

La nanotecnología como herramienta para el tratamiento de los más diversos tipos de cáncer es un campo que se encuentra en rápido crecimiento y ha demostrado contribuciones notables en las estrategias de tratamiento (Sahoo y col., 2004; Sahoo y Labhasetwar, 2005; Haley y Frenkel, 2008; Trapani y col., 2011). Estas contribuciones se pueden atribuir a las características fisicoquímicas y propiedades biológicas particulares de los nanomateriales, lo que permite una liberación específica de los agentes quimioterápicos en el tumor (Cho y col., 2008; Ranganathan y col., 2012). Como ventajas de los sistemas nanoestructurados para el tratamiento del cáncer, se pueden destacar: (1) superan la falta de selectividad de los fármacos antitumorales, (2) superan la resistencia multifármaco (MDR, en sus siglas en inglés), y (3) superan la baja solubilidad en medio acuoso de los fármacos anticancerosa (Jabir y col., 2012).

Los vehículos nanoestructurados basados en polímeros tienen gran aplicación para el tratamiento del cáncer. Se ha establecido que los nanomateriales pueden permanecer concentrados preferentemente en sitios como los tumores en virtud del efecto incrementado de permeabilidad y retención (EPR, en sus siglas en inglés) debido a la distribución de los vasos sanguíneos. Una vez acumuladas en el sitio de acción, las nanopartículas poliméricas biodegradables pueden actuar como depósito local del fármaco antitumoral, proporcionando una fuente de suministro continuo del componente terapéutico encapsulado, p. ej., en los tumores sólidos (Singh y Lillard Jr, 2009). Además, la adición de un revestimiento protector a las nanopartículas poliméricas limita su interacción con células no tumorales (Brewer y col., 2011), disminuyendo así los efectos adversos de los fármacos antitumorales. Otra tendencia para mejorar la actividad antitumoral, es acoplar ligandos y aptámeros a las nanopartículas, como un modo de activar las células cancerígenas, induciendo así la internalización celular por endocitosis mediada por receptor, que a su vez promueve una liberación intracelular eficiente del fármaco encapsulado (Woo y col., 2012).

## 2.2. Incorporación de excipientes bioactivos (tensioactivos, polímeros, péptidos, etc) en sistemas nanoestructurados

Los compuestos con propiedades bioactivas, como p. ej., que presenten actividad lítica pH-dependiente, se consideran muy importantes y son una fuente de constante estudio en el campo del desarrollo tecnológico de vehículos con especificidad y eficiencia para la liberación intracelular de genes y fármacos. Muchos compuestos terapéuticos, como proteínas, péptidos, ácido desoxirribonucleico (ADN) y una gran variedad de fármacos, tienen su sitio de acción en el interior de las células y, por lo tanto, su eficacia terapéutica depende de un eficiente mecanismo de liberación intracelular a partir de su vehículo de encapsulación (Plank y col., 1998). Muchos grupos de investigación

han dedicado esfuerzos considerables para mejorar la eficiencia de los vehículos desarrollados para liberación intracelular de fármacos. Para ello, han investigado nanocomplejos conteniendo materiales bioactivos como lípidos, polímeros, péptidos y tensioactivos. El enfoque en el diseño de estos nanomateriales combina componentes esenciales para obtener vehículos biocompatibles y niveles altos de liberación específica en el sitio de acción.

La liberación eficiente de fármacos captados por endocitosis en el citoplasma representa un reto para la liberación intracelular de fármacos. Después de entrar en las células por endocitosis pasiva o activa, los sistemas de liberación macromoleculares son comúnmente dirigidos a los lisosomas, donde los fármacos frágiles pueden ser degradados por las enzimas lisosomales (Mellman, 1996). Incluso, si la carga de la partícula no se degrada, la compartimentación en los endosomas o lisosomas pueden impedir que los fármacos lleguen a estructuras subcelulares específicas donde actúan (Sheff, 2004). Por lo tanto, teniendo en cuenta la labilidad de las biomoléculas y el hecho que una vez captadas por endocitosis son transportadas de los endosomas primarios a los lisosomas en pocas horas (Pack y col., 2005), es necesario desarrollar sistemas efectivos para su liberación intracelular. Como los endosomas presentan un pH ligeramente ácido (5,0 – 6,5), una estrategia para alcanzar ese objetivo, consiste en utilizar como transportadores, vehículos cuya estructura incluya compuestos pH-sensibles con capacidad de romper la membrana de los endosomas en condiciones levemente ácidas, evitando así el tráfico de los sistemas de liberación macromoleculares hasta el lisosoma (Figura 7) (Christie y Grainger, 2003; Yessine y col., 2003).

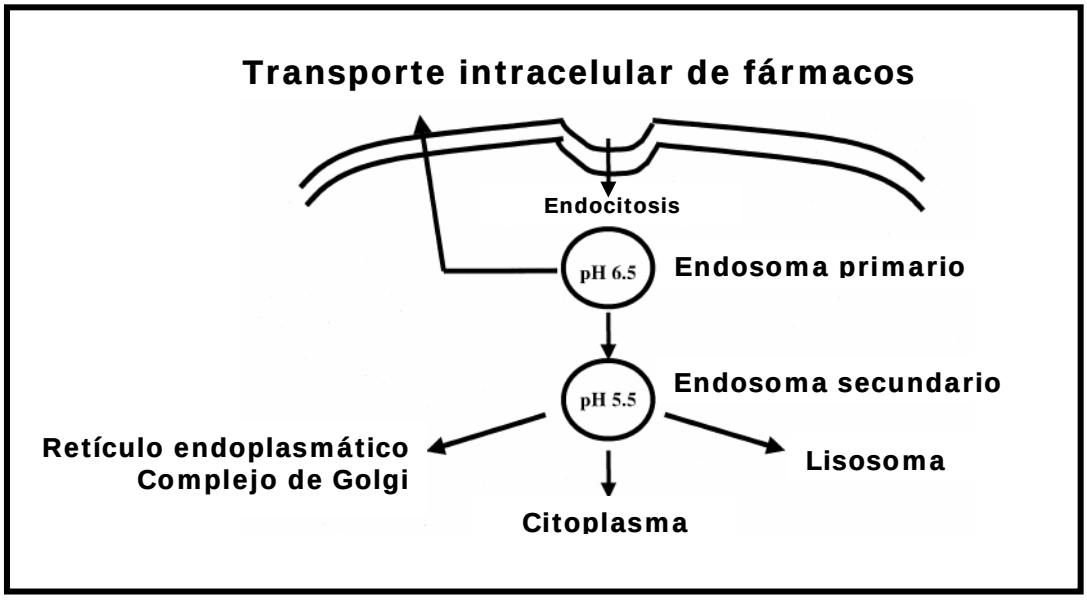


***Figura 7.*** *Ilustración esquemática de las rutas de transporte disponibles para los fármacos que actúan en compartimentos intracelulares (Adaptada de Stayton y col., 2000).*

Los vehículos basados en virus atenuados que presentan actividad lítica pH-dependiente se han estudiado exhaustivamente como transportadores para la terapia génica (Temin, 1990; Gordon y Anderson, 1994; McTaggart y Al-Rubeai, 2002). A pH ácido (4,8 – 6,5), las glicoproteínas virales adoptan una conformación hélice-alfa o lámina-beta, que tienen la capacidad de interaccionar con las membranas celulares e inducir su desestabilización (Hernandez y col., 1996). Sin embargo, a pesar de que estos compuestos tienen la capacidad de promover una elevada eficiencia de transfección, pueden provocar reacciones inmunogénicas (Temin, 1990). En este sentido, por cuestiones de seguridad se ha llevado a cabo el desarrollo de péptidos fusogénicos sintéticos que imitan el proceso por el cual los virus desestabilizan la membrana endosomal (Plank y col., 1994, 1998); sin embargo, también se han mostrado inmunogénicos *in vivo* (Kusonwiriyawong y col., 2003). Basándose en la capacidad lítica dependiente del pH demostrada con derivados virales y teniendo en cuenta la búsqueda de vehículos seguros para transportar moléculas biológicamente activas, en los últimos años se ha trabajado intensamente en el desarrollo de vehículos no virales, así como los derivados virales, con la capacidad de romper la membrana de los endosomas (Christie y Grainger, 2003). Entre los compuestos propuestos como excipientes bioactivos en este tipo de vehículos, se pueden destacar los polímeros y los tensioactivos. Se han preparado diferentes vesículas lipídicas (Kyriakides y col., 2002; Jones y col., 2003; Chen y col., 2004; Yuba y col., 2010) y nanopartículas (Akagi y col., 2010) incluyendo polímeros pH-dependientes con una eficiente capacidad de romper la membrana endosomal. Por otro lado, también se ha estudiado la potencial aplicación de los tensioactivos con este fin. Se han preparado nanopartículas modificadas con tensioactivos polimerizables con actividad pH-dependiente. Se ha demostrado una actividad lítica de membrana específica a pH ácido (nivel endosomal), lo que sugiere que estos vehículos son muy adecuados para la liberación intracelular de biomoléculas (Wang y col., 2007, 2008, 2009). También se han propuesto liposomas conteniendo tensioactivos pH-sensibles biodegradables (Liang y col., 2000) y diméricos (Asokan y Cho, 2004) como vehículos eficientes para la liberación citosólica de macromoléculas.

La incorporación de compuestos bioactivos con actividad pH-dependiente en vehículos destinados al tratamiento del cáncer también podría ser una tecnología clave en cuanto a la liberación específica del fármaco en el espacio extracelular del tejido tumoral, que tiene un pH levemente ácido (pH 6,5 – 7,2) (Lee y col., 2007). Estudios previos en este campo han demostrado que vehículos con propiedades pH-sensibles tienen su actividad antitumoral aumentada en el pH característico de los tumores (Na y col., 2003; Lee y col., 2003, 2007).

Como parte del amplio rango de aplicaciones de los tensioactivos como vehículos transportadores de sustancias biológicamente activas, también cabe destacar la utilización de tensioactivos catiónicos convencionales, derivados del

amonio cuaternario, en la preparación de vehículos para la encapsulación y liberación controlada de ADN (Morán y col. 2007a, 2007b, 2009; 2012; Clamme y col., 2000; Ruozi y col., 2007; Dass y col., 2002; Cardoso y col., 2011), como potencial aplicación en la terapia génica. Estos tensioactivos no están descritos como bioactivos (p. ej. pH-dependientes), pero tienen una elevada capacidad de complejación iónica con el ADN, que tiene carga neta negativa, o bien tienen la habilidad de formar vesículas catiónicas con gran capacidad de encapsulación de material genético. Sin embargo, estos compuestos muchas veces presentan actividades tóxicas (Morán y col., 2012; Pinnaduwage y col., 1989; Shalel y col., 2002; Funasaki y col., 2000) y, por lo tanto, no son adecuados para aplicaciones biomédicas. Además, se han utilizado en pequeñas cantidades tensioactivos catiónicos convencionales, con conocida toxicidad (Lasic, 1997), para modificar la carga de liposomas neutros (Pinnaduwage y col., 1989). Se ha justificado su uso por el bajo coste en comparación a otros lípidos sintéticos y por mejorar de forma significativa la eficiencia de transfección de estos liposomas. Recientemente, con el objetivo de mantener las propiedades fisicoquímicas de estos vehículos destinados a terapia génica, alcanzando un alto índice de transfección, pero con baja toxicidad, se han utilizado tensioactivos derivados de aminoácidos para el desarrollo de vehículos biocompatibles destinados al transporte de material genético (Morán y col., 2010b). Se ha demostrado que la interacción de tensioactivos derivados del aminoácido arginina con el ADN forma vehículos con propiedades biocompatibles y potencial aplicación en la terapia génica. Además, estos tensioactivos también han sido incorporados en formulaciones de liposomas (Lundberg y col., 2011), mientras que lípidos catiónicos con diferentes características estructurales, basados en diferentes aminoácidos dibásicos, también se han utilizado en formulaciones de vesículas catiónicas lipídicas conteniendo ADN (Heyes y col., 2002). Por último, se sintetizaron tensioactivos geminales derivados de aminoácidos para la preparación de nanopartículas para encapsulación de ADN (Singh y col., 2011). Se demostró que todas estas formulaciones modificadas con estos tensioactivos derivados de aminiácidos presentaban una mejora considerable en su capacidad de transfección. Todavía no hay datos en la literatura del empleo de tensioactivos derivados de aminoácidos que presenten actividad pH-dependiente en vehículos destinados a la terapia génica. Los tensioactivos biocompatibles con esta característica adicional podrían representar una gran ventaja en cuanto a una mayor liberación intracelular y consecuente mejora en la eficiencia de transfección.

Otra gran tendencia en la aplicación de los tensioactivos, es en el desarrollo de vesículas cataniónicas (Marques y col., 1998; Tondre y Caillet, 2001). Estas vesículas se forman por la interacción de tensioactivos con cargas opuestas, y presentan ventajas respecto a las vesículas lipídicas convencionales, como formación espontánea, bajo coste y larga estabilidad (Lasic y col., 2001), además de su potencial aplicación como vehículo para transporte y liberación de fármacos y material génico (Bramer y col., 2007;

Danoff y col., 2007; Rosa y col., 2007). Estas vesículas cataniónicas son generalmente formuladas con tensioactivos iónicos convencionales, y por lo tanto, presentan problemas en cuanto a sus propiedades toxicológicas. Con el objetivo de superar las desventajas de los sistemas convencionales, se han investigado recientemente vesículas cataniónicas desarrolladas con tensioactivos derivados de los aminoácidos lisina (Brito y col., 2006, 2009) y arginina (Lozano y col., 2011), y se ha observado un aumento considerable en su biocompatibilidad, con reducción significativa en la toxicidad de estas formulaciones (Brito y col., 2009; Lozano y col., 2011). Además, se ha demostrado que los tensioactivos incorporados en las vesículas presentaban efectos tóxicos inferiores cuando se comparaban a sus monómeros correspondientes libres en solución.

En base a todas las potenciales aplicaciones de los tensioactivos descritas anteriormente, y debido a la necesidad del desarrollo de vehículos biocompatibles, con baja toxicidad y con elevada capacidad de liberar de modo controlado y específico la biomolécula encapsulada, esta Tesis se ha centrado en el estudio de la actividad pH-dependiente de tensioactivos catiónicos y aniónicos derivados de lisina y en el desarrollo de sistemas nanoestructurados basados en estos compuestos como propuesta de potenciales vehículos no virales para la liberación intracelular de fármacos. Como se ha descrito anteriormente, existen pocos estudios que consideren la actividad pH-dependiente de los tensioactivos derivados de aminoácidos, y todavía está poco explorada su potencial utilización en vehículos propuestos para la liberación intracelular de moléculas biológicamente activas.

## 2.3. Internalización celular

El proceso de internalización celular de materiales coloidales, incluyendo las nanovesículas y las nanopartículas, se divide en diferentes rutas comunes de captación por las células, como fagocitosis, endocitosis mediada por la clatrina, endocitosis mediada por el caveolae, endocitosis por adsorción, pinocitosis y macropinocitosis (Silverstien y col., 1997; Rejman y col., 2004; Park y col., 2006; Jones y Grainger, 2009). La ruta de internalización de las macromoléculas tiene relación directa con su actividad terapéutica, ya que su destino intracelular está generalmente relacionado con el mecanismo de entrada en la célula (Khalil y col., 2006). Los mecanismos de endocitosis son los más comúnmente descritos como responsables de la internalización de los nanomateriales (Khalil y col., 2006; Harush-Frenkel y col., 2008; Banquy y col., 2009; Pollock y col., 2010), mientras que la fagocitosis (típicamente por macrófagos, monocitos y neutrófilos) es la captación de partículas mayores de 500 nm, que son generalmente atraídas por opsonización. Por otro lado, las células epiteliales y los fibroblastos generalmente captan las partículas por pinocitosis, proceso este que transporta partículas menores de 200 nm

(Mellman, 1996). La macropinocitosis (un tipo de endocitosis en fase fluida) consiste en la formación de vacuolas en la membrana celular con dimensiones de 1-5 μm, y se ha sugerido como el mecanismo de internalización responsable de la concentración de polímeros y macromoléculas en células tumorales que manifiestan mejor permeabilidad y retención (efecto EPR, en sus siglas en inglés) (Greish, 2007).

Los parámetros fisicoquímicos (p. ej. carga superficial, tamaño de partícula y elasticidad del substrato) de los nanomateriales han demostrado influir en la interacción de estos vehículos con las células o bien su destino en los compartimentos celulares después de la internalización (Khalil y col., 2006). La internalización celular de sistemas nanoparticulados puede ser monitorizada directamente en cultivo celular y proporciona informaciones relativas a la capacidad celular de captación (Jones y Grainger, 2009). Además, la caracterización *in vitro* de la captación y localización intracelular de los nanomateriales está intrínsicamente relacionada con los estudios de citotoxicidad, pues la internalización proporciona evidencias de la interacción nanomaterial-célula, lo que en consecuencia expone la maquinaria intracelular a sus efectos tóxicos (Marquis y col., 2009).

Los diferentes mecanismos de internalización celular adoptados por los nanomateriales pueden ser identificados usando diferentes inhibidores de rutas de entrada conocidas. Los experimentos realizados a 4 ºC tienen el objetivo de parar cualquier proceso celular dependiente de energía, como p. ej. los procesos de endocitosis mediados por receptores específicos (Rejman y col., 2004). La endocitosis mediada por la clatrina puede ser inhibida por la incubación previa de las células con clorpromazina, mientras que la nistatina se utiliza para inhibir la endocitosis mediada por caveolae (Rejman y col., 2004; Banquy y col., 2009). La amilorida es un inhibidor de la proteína intercambiadora de $Na^+/H^+$ y es usada para dificultar el proceso de macropinocitosis (Hewlett y col., 1994). Por último, el nocodazol se ha utilizado como inhibidor de la organización de los microtúbulos (Banquy y col., 2009), que se describen como estructuras involucradas en el transporte intracelular de vesículas (Zieve y col., 1980). La monitorización de todos estos procesos y mecanismos de internalización se realiza generalmente por microscopía de fluorescencia, utilizando nanomateriales marcados con colorantes fluorescentes (Banquy y col., 2009). Además, la captación celular de partículas ópticamente activas también puede ser valorada y cuantificada por citometría de flujo, y así permite discriminar partículas libres o agregadas, partículas asociadas a la células y células libres de partículas (Rejman y col., 2004). Asimismo, técnicas como microscopía electrónica de transmisión (TEM, de su sigla en inglés), espectroscopía de pérdida de energía de electrones (EELS, de su sigla en inglés) y microscopía confocal de barrido a láser (LSM, de su sigla en inglés) también se utilizan para estudiar la captación de partículas por orgánulos celulares específicos (p. ej. endosomas y lisosomas) o bien la penetración nuclear (Wilson y col., 2002; Lorenz y col., 2006). Por último, la co-utilización

de marcadores endosomales y lisosomales con técnicas de microscopía de fluorescencia permite la identificación de las vías intracelulares recorridas por los nanomateriales después de su internalización (Banquy y col., 2009). Además, los marcadores fluorescentes de la estabilidad de los endosomas (p. el. la calceína) son especialmente útiles en estudios de internalización celular de vehículos con actividad lítica dependiente del pH, y con cierta especificidad por las condiciones ácidas de los endosomas primarios y/o secundarios (Figura 8) (Hu y col., 2007; Chen y col., 2009).

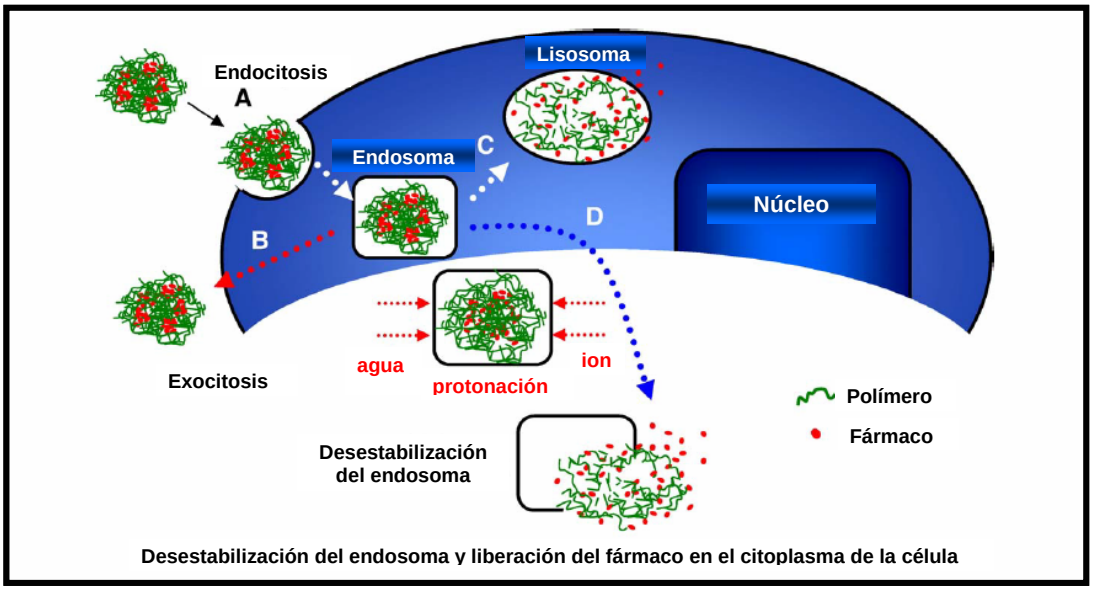


***Figura 8.*** *Representación esquemática de un modelo propuesto de internalización celular de nanomateriales y liberación del fármaco encapsulado en el citoplasma. (A) La internalización de las nanopartículas es inicializada a través de interacciones no específicas entre las nanopartículas y la membrana celular (endocitosis por adsorción). (B) Una parte de las nanopartículas son expulsadas mediante exocitosis. (C) Sin un mecanismo específico de liberación endosomal, las nanopartículas son direccionadas a los lisosomas. De este modo, fármacos sensibles a las enzimas lisosomales serán degradados y perderán su actividad. (D) En las condiciones levemente ácidas de los endosomas, nanopartículas con propiedades pH-dependientes causarán la ruptura de la membrana de los endosomas y liberación simultánea de los fármacos en el citoplasma de la célula (Adaptada de Park y col., 2006).*

## 3. Interacción de los tensioactivos con membranas celulares

Debido a las diferencias entre los compuestos bioactivos y los fosfolípidos de membrana, al intercalarse los tensioactivos en la membrana celular se inducen cambios en la organización molecular de la bicapa lipídica y aumenta la permeabilidad de la membrana, lo que puede provocar la lisis celular (Colomer y col., 2012). Alteraciones en propiedades de la membrana

como la conformación de las cadenas alquílicas, curvatura, microheterogeneidad (separación de fases, formación de dominios), grosor, potencial de superficie e hidratación de los grupos polares, son los principales mecanismos involucrados en el proceso de interacción de compuestos bioactivos con la bicapa fosfolipídica (Seydel, 2002). Por lo tanto, las potenciales aplicaciones de los tensioactivos en el campo farmacéutico y biológico hacen que el estudio de la interacción de los tensioactivos con membranas celulares y de los mecanismos involucrados en este proceso sea de gran importancia.

### 3.1. Eritrocito como modelo de membrana

El eritrocito representa un modelo celular muy simple (no contiene ni núcleo ni orgánulos), fácilmente disponible y manejable y, a pesar de que les falta la maquinaria de síntesis proteica y que son menos especializados que muchas otras células, sus membranas desarrollan suficientes funciones en común con ellas, como el transporte activo y pasivo y la producción de gradientes iónicos y eléctricos (Svetina y col., 2004; Kleszczynska y col., 2005; Suwaslsky y col., 2009). Además, su falta de núcleo le priva de los mecanismos de protección propios de otras células para luchar frente a las agresiones sobre la membrana (Jones, 1992). Por lo tanto, la membrana del eritrocito puede ser considerada representativa de las membranas plasmáticas celulares en general, además de constituir un buen modelo para el estudio de la interacción de sustancias bioactivas con las membranas celulares. Estas observaciones parecen ser la razón del gran número de estudios publicados que se basan en la actividad hemolítica de diferentes tensioactivos (Vives y col., 1999; Dufour y col., 2005; Rasia y col., 2007; Sánchez y col., 2007a; Suwaslsky y col., 2009).

La lisis de la membrana celular por tensioactivos es un proceso de gran importancia y por eso se han realizado muchas investigaciones al respecto, con el objetivo de entender el mecanismo subyacente a ese proceso (Shalel y col., 2002). El mecanismo involucrado en la interacción de compuestos bioactivos con las membranas celulares es un proceso muy complejo que todavía no es totalmente conocido (Dufour y col., 2005; Martínez y col., 2007; Sánchez y col., 2007a; Preté y col., 2011). Por lo tanto, son necesarios estudios específicos para alcanzar un conocimiento profundo de estos mecanismos. El uso del eritrocito como modelos de membrana permite la valoración de alteraciones en la célula y, por lo tanto, de características específicas de la bicapa lipídica, como cambios en la fluidez y deformidad, cambios en la morfología, fragmentación y degradación de proteínas que llevan a la ruptura de la membrana (Figura 9) (Srour y col., 2000; Sánchez y col., 2007a). Una definición de todos estos procesos puede, a su vez, permitir la comprensión de los mecanismos involucrados en la interacción de compuestos bioactivos con

membranas celulares. Cabe destacar que, teniendo en cuenta sus múltiples propiedades, los tensioactivos pueden constituir excipientes bioactivos eficaces cuando se incorporan en vehículos nanoestructurados destinados a la encapsulación y liberación de moléculas biológicamente activas. El conocimiento de las propiedades específicas de cómo cada sistema tensioactivo interacciona con la membrana puede tener un papel clave para ampliar su rango de aplicaciones biomédicas.

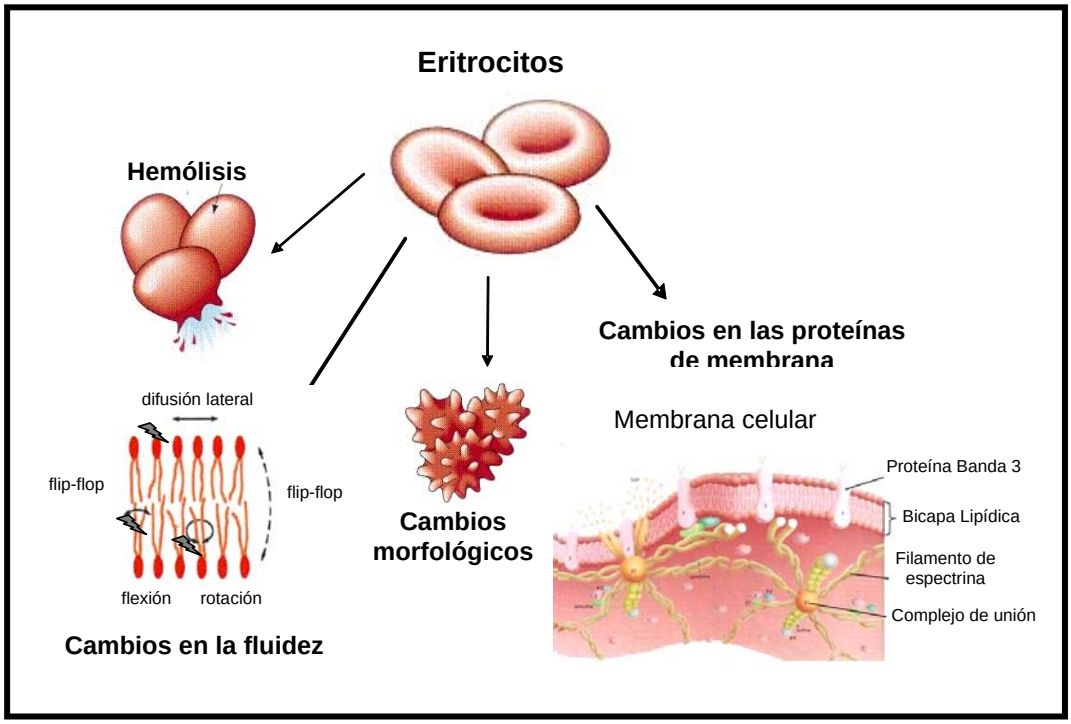


***Figura 9.*** *Principales alteraciones producidas en el eritrocito por exposición a compuestos bioactivos como los tensioactivos.*

Entre los mecanismos sugeridos como base de la lisis celular provocada por los tensioactivos, se puede destacar la solubilización de la membrana y la lisis osmótica (Bielawski, 1990; Jones, 1999). Generalmente se asume que a concentraciones suficientemente elevadas, el principal mecanismo de lisis celular es la solubilización de los lípidos y proteínas de la membrana debido a la formación de micelas mixtas (Jones, 1999). A concentraciones bajas, el principal resultado de la incorporación de los tensioactivos en la membrana, es un cambio en su permeabilidad, que provoca penetración de agua, hinchazón de la célula y lisis de la membrana (Bielawski, 1990). Teniendo en cuenta la complejidad de estos mecanismos, se han propuesto una variedad de ensayos para estudiar la interacción de compuestos químicos con las membranas celulares, utilizando el eritrocito como modelo de membrana:

- **Ensayo de hemólisis**

El ensayo de hemólisis es un método sencillo, barato, no se necesita un equipamiento sofisticado y requiere poco tiempo por producto. Además está bien caracterizado ya que los criterios principales de valoración están bien definidos. El ensayo se basa en el potencial de una sustancia química para perturbar las membranas celulares, valorando espectrofotométricamente la liberación de hemoglobina de una suspensión de eritrocitos incubada con la sustancia a ensayar y bajo condiciones estándar. Además de la utilización del ensayo de hemólisis para la evaluación del potencial efecto irritante inducido por tensioactivos (Mitjans y col., 2003; Benavides y col., 2004b; Martínez y col., 2006; Sánchez y col., 2006a), este ensayo se utiliza también para la valoración de tensioactivos y polímeros que tienen la habilidad de interaccionar con las membranas celulares de manera sensible a cambios de pH (Chen y col., 2005, 2009; Wang y col., 2007).

La hemólisis en medio hipotónico, conocida como antihemólisis, también es una técnica muy utilizada en el estudio de la interacción de compuestos bioactivos con membranas celulares (Stasiuk y col., 2004; Kleszczynska y col., 2005; Sánchez y col., 2007a; Arias y col., 2010). Este método permite valorar los cambios inducidos por los tensioactivos en la resistencia osmótica de la célula y, por lo tanto, su grado de protección frente la hemólisis hipotónica. La comprensión de estos fenómenos permite el establecimiento de algunos mecanismos involucrados en la interacción de los tensioactivos con las membranas celulares, como el nivel de incorporación en la bicapa lipídica y los cambios en el volumen y/o área superficial de la célula (Isomaa y col., 1986; Abe y col., 1991; Miseta y col., 1995; Dufour y col., 2005; Sánchez y col., 2007a).

- **Evaluación de la fluidez de membrana por anisotropía de fluorescencia**

La fluidez de la membrana es un parámetro importante relacionado con su estructura y estado funcional (Shinitzky y Barenholz, 1978). El estudio de la fluidez permite determinar la localización de los compuestos en la bicapa lipídica y sus efectos en la dinámica de lípidos en diferentes regiones de la membrana (Martínez y col., 2007; Marczak, 2009). Se estudia mediante anisotropía de fluorescencia que, utilizando sondas fluorescentes capaces de localizarse en regiones específicas de la bicapa lipídica, permite caracterizar la influencia de diferentes moléculas bioactivas en las propiedades biofísicas de las membranas (Arora y col., 2000; Hou y col., 2011). La incorporación de tensioactivos en la membrana altera significativamente sus características funcionales y, dependiendo de la magnitud de esta interacción, estos compuestos bioactivos pueden alterar la permeabilidad de la membrana por

inducción de cambios en el orden, orientación y fluidez de los lípidos (Xia y Onyuksel, 2000).

- **Análisis de las proteínas de membrana por electroforesis en gel de poliacrilamida**

En la membrana de los eritrocitos, la bicapa lipídica está anclada en una red de proteínas esqueléticas a través de proteínas transmembrana. La Banda 3, la principal proteína integral de membrana, interacciona con este citoesqueleto que mayoritariamente incluye espectrina, actina, anquirina y proteína 4.1 (Fairbanks y col., 1971). La organización de esta red proteica, en particular en la superficie más interna de la membrana, es la responsable de mantener la forma, estabilidad y deformidad de los eritrocitos (Vittori y col., 2002). Las alteraciones en el patrón proteico se observan mediante electroforesis en gel desnaturalizante de poliacrilamida (SDS-PAGE, en sus siglas en inglés) (Silva y col., 2000; Rossi y col., 2006; Hou y col., 2011). El patrón de bandas permite determinar si los tensioactivos inducen degradación o cualquier alteración de las proteínas de membrana. Se puede establecer si el daño en la integridad proteica de la membrana es un mecanismo involucrado en el proceso de interacción de los compuestos bioactivos con la membrana celular.

- **Observaciones de la morfología del eritrocito por microscopía electrónica de barrido**

La interacción y/o incorporación de compuestos bioactivos en la bicapa lipídica conduce a alteraciones en la rigidez y forma de la membrana celular. El estudio de la morfología celular por microscopía electrónica de barrido (SEM, en sus siglas en inglés) permite observar los cambios morfológicos inducidos por los tensioactivos (Vives y col., 1999; Dubnicková y col., 2000; Rasia y col., 2007). Las diferencias en la perturbación de la membrana, debido al intercalado o partición de los tensioactivos en la bicapa fosfolipídica, permiten comprobar el grado de interacción de estos compuestos con la membrana. De acuerdo con la hipótesis de la bicapa lipídica (Sheetz y Singer, 1974; Lim y col., 2002) los cambios de morfología inducidos en los eritrocitos por moléculas extrañas se deben a expansiones diferenciales de las dos monocapas de la membrana. Según esta hipótesis, los equinocitos se formarían cuando los compuestos se insertan en la monocapa más externa de la bicapa (por lo tanto, el área de esta capa se incrementaría respecto a la capa interna), mientras que los estomatocitos, eritrocitos que presentan una morfología un poco redondeada, con forma de copa, se formarían cuando los compuestos se insertaran en la monocapa interna (Figura 10). Por último, la formación de esferocitos seria debida al aumento del intercalado del tensioactivo tanto en la

monocapa externa como en la monocapa interna de la membrana (Vermehren y Hansen, 1998; Suwalsky y col., 2005).

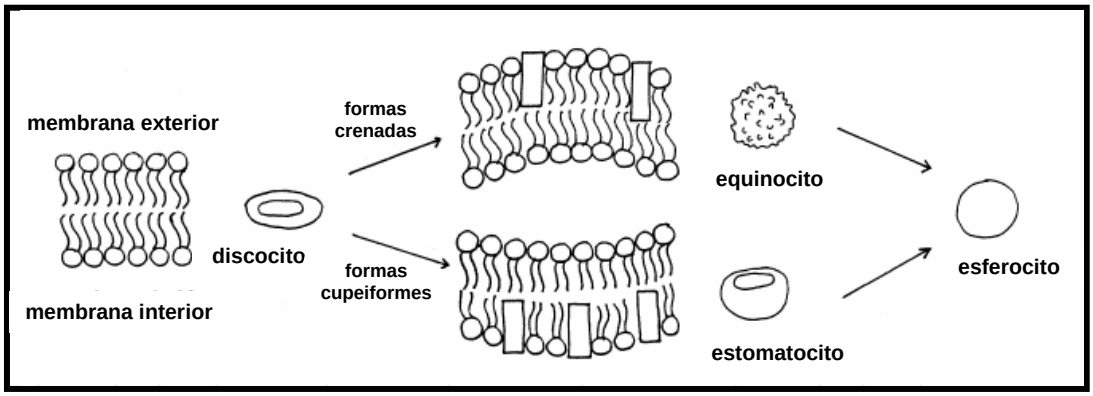


***Figura 10.*** *Representación esquemática de la hipótesis de la bicapa lipídica (Adaptada de Sheetz y Singer, 1974; Vermehren y Hansen, 1998).*

### 3.2. Eritrocito como modelo de membrana del endosoma: valoración de la actividad pH-dependiente

Además de la amplia utilización del eritrocito como modelo de membrana plasmática, como ya se ha discutido anteriormente, este tipo celular también ha sido usado como modelo de membrana endosomal en estudios de compuestos o formulaciones bioactivas desarrollados para promover una liberación intracelular específica de substancias biológicamente activas (Yessine y col., 2003; Wang y col., 2007; Chen y col., 2008, 2009; Akagi y col., 2010; Lee y col., 2010).

Se conoce que el pH en los endosomas primarios o tempranos es aproximadamente 6,5 – 6,8, mientras que en los endosomas secundarios o tardíos es aproximadamente 5,5 y en los lisosomas es 4,6 - 5,0 (Mellman, 1996; Stayton y col., 2000; Moore y col., 2008). Teniendo en cuenta la variabilidad en los valores de pH entre el espacio extracelular/fisiológico y los diferentes compartimentos intracelulares, el estudio del efecto del pH del medio en la actividad hemolítica de determinados compuestos se considera un modelo de ensayo válido y factible para determinar la potencial actividad lítica de la membrana endosomal. Una baja actividad hemolítica a pH fisiológico disminuiría la lisis no específica de la membrana celular, y por consiguiente la captación no específica por los tejidos del material liberado (Wang y col., 2007). Por otro lado, una elevada actividad lítica en el rango de pH característico de los compartimentos endosomales, demostraría la especificidad de determinado compuesto para romper la membrana de los endosomas y, por lo tanto, indicaría su potencial aplicación en vehículos para liberación intracelular de moléculas biológicamente activas. Cabe destacar que estudios

previos han demostrado que hay una fuerte correlación entre la actividad hemolítica de sustancias bioactivas y su capacidad de romper la membrana del endosoma (Plank y col., 1994).

Como ejemplos de la amplia utilización del eritrocito como modelo de membrana endosomal, podemos destacar estudios de péptidos derivados de virus (Lee y col., 2010), copolímeros derivados del ácido metacrilato (Yessine y col., 2003), polímeros derivados del ácido polipropilacrilato (Kyriakides y col., 2002), polímeros basados en el imidazol (Seo y Kim, 2010), tensioactivos polimerizables (Wang y col., 2007, 2009), derivados imidazólicos (Chen y col., 2003) y pseudo-péptidos (Chen y col., 2005, 2008, 2009). El mecanismo por el cual estos compuestos desestabilizan las membranas no está de todo dilucidado, pero se propone que el aumento en el grado de protonación de la molécula del tensioactivo o polímero a un pH reducido sería un factor clave (Wang y col., 2007). Los compuestos con mayor protonación y que alcanzan una mayor densidad de carga catiónica a pH ácido, tenderían a interaccionar electrostaticamente con las membranas de manera más fuerte, lo que llevaría a la desestabilización de la bicapa lipídica (Figura 11). En el caso de los tensioactivos, además de la atracción electrostática, el aumento del grado de protonación volvería la molécula más anfifílica y, por lo tanto, más activa para interaccionar con la membrana celular (Wang y col., 2007).

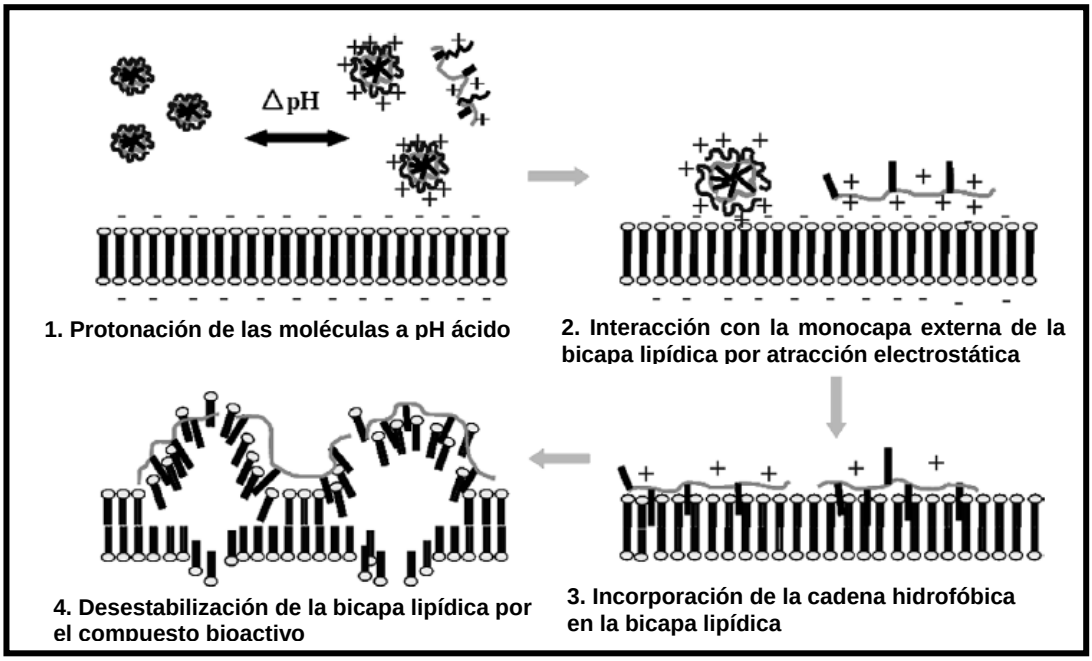


***Figura 11.*** *Posible mecanismo de interacción de compuestos bioactivos con las membranas celulares a un pH ácido (Adaptada de Seo y Kim, 2010).*

### 3.3. Liposomas como modelo de membrana

Existen métodos alternativos al uso de eritrocitos como modelo de membrana y consisten en la utilización de vesículas lipídicas compuestas por fosfolípidos. El liposoma se utiliza ampliamente como modelo de membrana por ser un modelo simple y bien caracterizado (Ahyayauch y col., 2010; Almeida y col., 2010a, 2010b; Beck y col., 2010; Ortiz y col., 2010; Fadel y col., 2011; Manrique-Moreno y col., 2011). Muchos investigadores describen innumerables ventajas para su utilización en comparación con modelos más complejos como el eritrocito. En primer lugar, este modelo puede ser obtenido a través de metodologías simples y con bajo índice de variabilidad y, por lo tanto, se considera adecuado para la valoración de un amplio rango de condiciones y productos. Además, al actuar como un modelo estándar, permite una comparación directa con otros resultados descritos en la literatura. Sin embargo, la amplia utilización del eritrocito como modelo de biomembrana, como ya se ha descrito anteriormente, tiene una ventaja y es que los eritrocitos constituyen un modelo natural que se asemejan a células más especializadas y, por lo tanto, los resultados obtenidos pueden ser indicadores más fiables de la actividad biológica del compuesto ensayado en condiciones fisiológicas (Klezczynska y col., 2005; Suwalsky y col., 2009).

Cabe destacar que los liposomas se utilizan mayoritariamente como modelo de biomembrana en estudios de mecanismos biofísicos específicos de interacción de tensioactivos con membranas, utilizando técnicas físicoquímicas como calorimetría, fluorimetría, microscopía de fuerza atómica, tensión superficial y difracción de rayos X (Xia y Onyuksel, 2000; Thorén y col., 2007; Almeida y col., 2010b; Ortiz y col., 2010; Gupta y col., 2011).

## 4. Estudios toxicológicos *in vitro*

El objetivo principal de los estudios toxicológicos es proteger al ser humano frente a posibles efectos adversos de diversas clases de compuestos químicos, incluyendo compuestos farmacéuticos y cosméticos. Actualmente, la evaluación de la seguridad preclínica final de un compuesto o formulación se basa en la experimentación en animales. La búsqueda de métodos alternativos, que podrían usarse para reemplazar totalmente los ensayos en animales, ha sido el objetivo principal de la investigación toxicológica en los últimos años, tanto por razones éticas y científicas, como económicas (Balls y col., 1995; Gettings y col., 1996; Worth y Balls, 2002; Garthoff, 2005). Debido a la creciente preocupación social respecto al uso de animales de experimentación y en vistas de su posible prohibición en el futuro, tal como ha sucedido en Europa con la evaluación toxicológica de cosméticos (Reglamento No.

1223/2009 sobre los productos cosméticos; Real Decreto 209/2005, BOE No. 49), es necesario desarrollar y fomentar métodos alternativos *in vitro*. Asimismo, la nueva ley de la Unión Europea sobre las sustancias químicas y su uso y seguridad, conocida como REACH (***R****egistration,* ***E****valuation,* ***A****uthorisation and Restriction of* ***Ch****emical substances, European Community Regulation on chemicals and their safe use*), que entró en vigor el 1 de junio de 2007, tiene como objetivos: proteger mejor la salud y el medio ambiente de los posibles riesgos derivados de las sustancias químicas, promover el uso de métodos alternativos de comprobación de la seguridad, y mejorar la manipulación y el uso de estas sustancias con total seguridad en todos los sectores de la industria.

Los métodos *in vitro* han sido desarrollados para cubrir la mayoría de áreas de toxicología, incluyendo toxicidad aguda, irritación local, mutagénesis, etc. y constituyen una herramienta muy valiosa para las industrias cosmética, química y farmacéutica. Son relativamente económicos en comparación con los estudios realizados con animales y también resultan de gran utilidad, no solo para establecer clasificaciones de compuestos químicos a través de la determinación de su toxicidad, sino también para estudiar los mecanismos de acción responsables de los efectos adversos (Pappinen y col., 2005). Otra ventaja de las pruebas *in vitro* es la rapidez con la que se pueden realizar (a menudo con varios compuestos químicos a la vez) y en muchos casos la facilidad con que se aplican los criterios de valoración. Además, permiten detectar pequeñas diferencias entre las propiedades biológicas de cada compuesto y así, el proceso de selección de compuestos químicos para el desarrollo de nuevos productos es más rápido y eficiente (Osborne y Perkins, 1994; Martínez y col., 2006).

Las bases de los métodos *in vitro*, alternativos al uso de animales de laboratorio, se basan en el principio de las 3Rs –reducción, refinamiento y reemplazo- de Russell y Burch (Russel y Burch, 1959). De acuerdo con este principio, se considera un método alternativo a la experimentación animal aquél que cumpla uno de los siguientes principios:

- Incorpora un **refinamiento** de los procedimientos, de modo que disminuyen el dolor o malestar de los animales, o bien aumentan su bienestar.

- Permite una **reducción** en el número de animales necesario.

- Facilita el **reemplazo** del uso de animales por sistemas que no requieren seres vivos, o emplean otras especies animales que sean inferiores en la escala filogenética animal.

En Europa, el *European Council* y el *European Economic Community* (EEC) se ocupan de la regulación de estas materias y el espíritu de las 3Rs quedó recogido en el documento oficial *Directiva Europea* 86/609/EEC *del*

*Consejo de 24 de noviembre de 1986 relativa a la protección de los animales utilizados para experimentación y otros fines científicos* (EEC, 1986; Louhimies, 2002), y ha sido ratificada recientemente con la nueva Directiva 2010/63/UE (UE, 2010). La actual Directiva constituye un importante paso hacia el objetivo final de pleno reemplazo de los procedimientos con animales vivos para fines científicos y educativos, tan pronto como sea científicamente posible hacerlo. En la actualidad, la mayor parte de los países europeos sigue, en mayor o menor medida, las normas comunitarias sobre esta materia.

Por otro lado, el desarrollo de métodos alternativos al uso de animales ha de basarse en conocimientos científicos sólidos y, por este motivo, existen organizaciones internacionales que se dedican a la validación y armonización de estos métodos. Entre estas organizaciones se encuentran el EURL ECVAM (*European Union Reference Laboratory on Alternatives to Animal Testing*) o anteriormente ECVAM (*European Centre for the Validation of Alternative Methods*), el ICCVAM (*Interagency Coordinating Comittee on the Validation of Alternative Methods*), el JACVAM (*Japanese Center for the Validation of Alternative Methods*) y la OCDE (*Organisation for Economic Co-operation and Development*). Recientemente, en septiembre de 2011, se fundó así mismo el BraCVAM (*The Brazilian Center for Validation of Alternative Methods),* con el objetivo de unir a todos los grupos brasileños que trabajan en métodos alternativos en experimentación y docencia.

### 4.1. Modelos celulares

El cultivo celular se utiliza habitualmente como modelo de ensayo en estudios toxicológicos *in vitro*. Una de las mayores ventajas de los sistemas de cultivo celular es la posibilidad de obtener células de un órgano diana determinado e incluso, en muchos casos, de origen humano. Los cultivos celulares se pueden desarrollar con líneas celulares establecidas o con células derivadas de cultivos primarios. Las muestras necesarias para los cultivos primarios son limitadas y variables y además la posibilidad de observar cambios en la respuesta tóxica aumenta con el número de pasajes. La ventaja principal de las líneas celulares respecto a los cultivos primarios es que evitan las diferencias en los resultados debidas a la variación de los donantes y proporcionan resultados reproducibles (van de Sandt y col., 1999; Lee y col., 2000).

El tipo de modelo o línea celular y de biomarcador que se escoja dependerá de los efectos, mecanismo de acción, propiedades y actividades que se quieran evaluar. El uso de modelos celulares es útil como método de cribado inicial para evaluación toxicológica de nuevos compuestos o formulaciones farmacéuticas, ya sean convencionales o basadas en la nanotecnología. Clásicamente se utilizan muchos tipos celulares en los diversos ensayos *in*

*vitro*, incluyendo células neuronales, hepáticas, epiteliales, endoteliales, cancerígenas y sanguíneas (Jones Y Grainger, 2009). La elección de la línea celular tiene gran relevancia en la planificación de un estudio toxicológico, ya que diferentes modelos celulares pueden responder a un estímulo determinado de forma muy distinta (Lestari y col., 2005; Burlando y col., 2008; Tan y col., 2008; Backorová y col., 2011).

Cuando un compuesto o formulación que se evalúa va a ser administrado por vía tópica, interesará determinar su potencial efecto tóxico en modelos representativos de la piel. Como ejemplos de estos modelos se pueden citar células aisladas de la piel (fibroblastos y queratinocitos) y modelos tridimensionales de equivalentes de piel que permiten realizar experimentos a nivel molecular, celular y tisular (Faller y col., 2002). Los queratinocitos son las células constituyentes de la epidermis y existen muchas evidencias de que este tipo celular juega un papel relevante en el inicio, modulación y regulación de la irritación dérmica y en la patogénesis de enfermedades cutáneas, siendo las células más utilizadas para valorar el potencial efecto irritante de la piel (Zhu y col., 2005). Los queratinocitos también intervienen en las reacciones de vigilancia inmunitaria que tienen lugar en la epidermis, por lo que desempeñan un papel crucial en la inflamación (Coquette y col., 2000). Además, los fibroblastos son las células mayoritarias de la dermis, y también son comúnmente utilizados como modelo celular de la piel en estudios toxicológicos *in vitro* (Benavides y col., 2004a; Sanchez y col., 2004).

Por otro lado, la administración de formulaciones por vía sistémica requiere una valoración de sus efectos tóxicos en células representativas de los más diversos órganos y sistemas. Como un modelo bastante genérico, también se utilizan los fibroblastos, que son células representativas de los tejidos conectivos y, por lo tanto, se utilizan ampliamente en estudios toxicológicos de sustancias o formulaciones destinados a las más diversas aplicaciones (Li y col., 2013; Browne y col., 2012; Colomer y col., 2012; Pramod y col., 2012). Un modelo celular también muy utilizado en estudios de citotoxicidad son las células tumorales (Chen y col., 2005, 2008, 2009). Estas células se suelen usar sin ninguna razón aparente más que por su fácil cultivo, rápido crecimiento y disponibilidad comercial (Jones Y Grainger, 2009). Sin embargo, su utilización se justifica por su alta habilidad fagocítica para la captación de partículas (Takeuchi y col., 1999), además de su gran relevancia en estudios que predicen el potencial farmacológico de compuestos o formulaciones con actividad antitumoral (Jin y col., 2009; Mei y col., 2009; Backorová y col., 2011).

### 4.2. Métodos *in vitro* para valoración de la toxicidad

La mayoría de métodos alternativos *in vitro* se basan en el empleo de cultivos de células de mamífero y en la aplicación de ensayos de citotoxicidad, ya que son sencillos y reproducibles y permiten obtener criterios de valoración definidos. También cabe destacar que los modelos *in vitro* no presentan los síntomas "visibles" observados *in vivo* y, por lo tanto, se utilizan otros tipos de biomarcadores para su evaluación. De forma más específica, la citotoxicidad se puede evaluar basándose en diferentes propiedades de las células (Mosmann, 1983; Borenfreund y Puerner, 1985; Cook y Mitchell, 1989; Müller-Decker y col., 1994; Anderson y Russell, 1995; Eun y Suh, 2000; Park y col., 2000):

- Cambios en la morfología celular (observación microscópica para evaluar el tamaño y la forma, contactos célula-célula, número, tamaño y forma del núcleo, inclusiones intracelulares).
- Viabilidad celular (número de células).
- Metabolismo celular (concentración de adenosina trifosfato (ATP, en sus siglas en inglés), reducción de la sal de tetrazolio o MTT).
- Integridad de membrana (exclusión de azul de tripán, captación de rojo neutro (NRU, en sus siglas en inglés), pérdida de enzimas, p. ej. la lactato deshidrogenasa (LDH), e iones).
- Proliferación celular (incremento del número de células, del número de colonias por área o del contenido total de ADN, ácido ribonucleico (ARN) y proteína).
- Adhesión celular (adherencia a la superficie del cultivo, desprendimiento de la superficie del cultivo, adhesión célula-célula).
- Captación o incorporación de precursores radioactivos (síntesis de aminoácidos y proteínas, timidina y síntesis de ADN, uridina y síntesis de RNA).

A pesar de la frecuente falta de consistencia o predicción entre los modelos *in vitro* y las observaciones *in vivo*, hay poca justificación racional o ética para proceder directamente de la síntesis de un nuevo compuesto o del desarrollo de una nueva formulación (sea esta convencional o basada en la nanotecnología) a ensayos con modelos animales. Muchos de los ensayos *in vitro* basados en modelos celulares aún necesitan validación, sin embargo resultan muy útiles en los procesos de cribado y valoración inicial de nuevos compuestos o formulaciones. Por otro lado, es importante destacar que la citotoxicidad general no es considerada un modelo predictivo adecuado por si solo (Fentem y col., 2001) y por eso es necesario acompañar los ensayos de citotoxicidad de otros métodos, como p. ej., ensayos específicos para determinar los mecanismos involucrados en el proceso de toxicidad. Entre los ensayos más comunes para estudiar de manera más definida los procesos implicados en los efectos tóxicos, se pueden destacar:

- *Liberación de mediadores inflamatorios*: Citocinas como la IL-1α y la IL-8 son mediadores inflamatorios muy importantes, p. ej., como biomarcadores de irritación/inflamación y sensibilización en la piel, respectivamente (Coquette y col., 2000; Martínez y col., 2006; Mitjans y col., 2008). Son evaluadas comúnmente por técnicas enzimáticas de inmunoensayo (ELISA, en sus siglas en inglés).

- *Análisis de ciclo celular*: Se determina el número de células, el número de colónias por área o el contenido total de ADN. El ciclo celular comprende una secuencia compleja de fenómenos que conducen al crecimiento de la célula y a la división celular. Los estudios de ciclo celular se realizan por citometría de flujo utilizando un marcador fluorescente (Jin y col., 2009).

- *Determinación de apoptosis*: Se realiza por microscopía óptica, fluorescente o electrónica, para observar cambios morfológicos como la condensación de la cromatina y la deformación de la membrana. También se hacen análisis por citometría de flujo, que mide las características físicas y químicas de las células o sus componentes celulares (Vermes y col., 2000).

- *Análisis del daño del ADN y genotoxicidad*: La técnica más utilizada para determinar la extensión del daño en el ADN celular es el ensayo del cometa ('Comet assay' o 'Single cell gel-electrophoresis'). El grado de daño al ADN se determina mediante clasificación visual del tamaño de la cola (rotura del ADN) que aparece al migrar las células en la electroforesis (Singh y col., 1988).

- *Determinación del stress oxidativo*: Se puede determinar mediante la cuantificación de especies reactivas de oxígeno (ROS, en su sigla en inglés) producidas por las células en respuesta a la exposición a determinado tratamiento (Long y col., 2007), o bien por la cuantificación de la formación de especies reactivas del ácido tiobarbitúrico (TBARs, en sus siglas en inglés) en el medio de cultivo como marcador de la peroxidación lipídica (Smith y col., 1982; Choi y col., 2007).

- *Estudio de vías de transducción de señales y de modulación de actividades enzimáticas*: La expresión de proteínas relacionadas con procesos inflamatorios y de carcinogénesis se puede valorar mediante técnicas de *Western blotting* (Cui y col., 2004).

- *Estudio de fototoxicidad*: La evaluación de la fototoxicidad de nuevos compuestos o formulaciones, desarrollados para aplicación tópica, es esencial para valorar los posibles daños o lesiones que puede sufrir la piel tras la exposición solar. La fototoxicidad o fotoirritación es definida como una reacción aguda no inmunológica de la piel inducida por la luz

visible o la ultravioleta como consecuencia del contacto con una sustancia o compuesto fotoactivo. En Europa, no está permitido evaluar el potencial fototóxico agudo en animales, ya que se ha aceptado un método alternativo con propósitos reguladores. Debido a su elevada especificidad y sensibilidad, el ensayo validado de fototoxicidad *in vitro* mediante captación de rojo neutro en fibroblastos 3T3 (3T3-NRU-PT, en sus siglas en inglés) es el ensayo esencial de fototoxicidad que se requiere según la legislación vigente (OECD, 2004).

## 5. Nanotoxicología

Existe un interés creciente en el estudio de los posibles efectos adversos para la salud humana de los nanomateriales ya sea por aplicación directa (fármacos, cosméticos, etc) o por la exposición accidental a las mismas (Dechsakulthron y col. 2007). Se considera que, debido a sus propiedades fisicoquímicas particulares y al poco conocimiento de sus efectos adversos para la salud humana y el medio ambiente, los nanomateriales deben ser tratados como nuevos productos químicos desde el punto de vista de su evaluación toxicológica. En los últimos años, el número de artículos en toxicología de los nanomateriales está creciendo de forma exponencial. Sin embargo, no existen aún resultados claros de sus mecanismos de acción en los sistemas biológicos. Se han propuesto mecanismos para explicar su efecto tóxico, como es el incremento del estrés oxidativo (Gurr y col., 2005), la producción de citocinas inflamatorias (Yamakawi y Iwai, 2006) o la muerte celular. También hay que destacar que la disminución en el tamaño de partícula suele provocar un aumento en la superficie total resultando en una mayor actividad biológica (Nel y col., 2006; Gornati y col., 2009). A pesar de existir una correlación entre el incremento de la superficie total y los efectos biológicos, no siempre es posible predecir estos efectos en base a la superficie exclusivamente y existen otros factores que se tienen que estudiar (Sayes y col., 2005). Todavía existe un gran desconocimiento de la manera de actuar de los nanomateriales y se han de incrementar los esfuerzos para investigarlos. Además, a pesar de que los riesgos que comportan estos nanomateriales se reconocen a nivel científico, no hay una reglamentación específica que defina este nuevo campo de la toxicología. De una forma general, estas variaciones en los efectos tóxicos de los nanomateriales han llevado a un rápido desarrollo de una nueva disciplina, la nanotoxicología (Donaldson y col., 2004; Linkov y col., 2008). La nanotoxicología ha sido caracterizada como una ciencia pre-normal, en la cual los investigadores no tienen paradigmas ampliamente aceptados para dirigir sus investigaciones (Elliott, 2007).

La nanotoxicología comprende los determinantes fisicoquímicos, las rutas de exposición, biodistribución, los determinantes moleculares,

genotoxicidad y por último, los aspectos regulatórios (Figura 12). Además, la nanotoxicología está implicada en proponer protocolos fiables, robustos y basados en datos concretos para la evaluación de los riesgos de los nanomateriales para el medio ambiente y la salud humana (Donaldson y col., 2004; Lewinski y col., 2008).

Cuando se compara un compuesto en su forma convencional con su forma nanoestructurada, hay que considerar que los efectos adversos probablemente no serán los mismos debido a, entre otros factores, su diferente cinética. La absorción, distribución, el metabolismo y la excreción de un nanomaterial pueden diferir significativamente de partículas mayores o sustancias solubles. Por lo tanto, simples cambios en la biodisponibilidad son suficientes para crear efectos adversos adicionales (Holl, 2009). Además, las propiedades fisicoquímicas no usuales de los nanomateriales, atribuidas a su pequeño tamaño, composición química, estructura de superficie, solubilidad, morfología y agregación, pueden llevar al desarrollo de nuevas interacciones biológicas y, por lo tanto, de nuevos efectos tóxicos (Walker y Bucher, 2009; Arora y col., 2012).

También debe tenerse en cuenta que mientras que el número de tipos y aplicaciones de nanomateriales continúa en aumento, los estudios para caracterizar sus efectos tras una exposición intencional o accidental, y para abordar su potencial toxicidad, son comparativamente pocos (Lewinski y col., 2008). Se puede decir que se han desarrollado relativamente pocos ensayos para valorar el potencial citotóxico de la amplia gama de nanomateriales desarrollados actualmente (Arora y col., 2012).

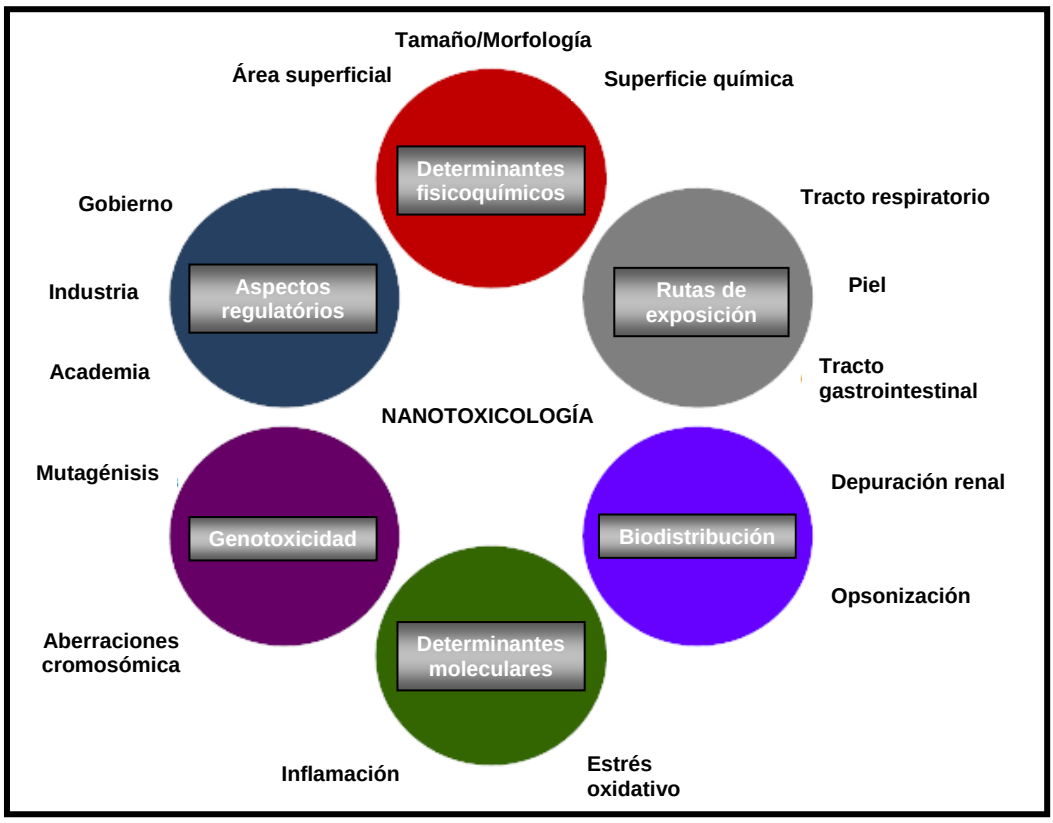


***Figura 12.*** *Representación esquemática del complejo conjunto de cuestiones relacionadas con la toxicidad de los nanomateriales (Adaptada de Arora y col., 2012).*

### 5.1. Normativas para estudios de nanotoxicidad

Dentro de los términos de la Unión Europea (UE), la Comisión Europea determina que la legislación REACH establece el mejor marco posible para la gestión del riesgo de los nanomateriales en caso de que se permitan como sustancias o mezclas, aunque se ha demostrado que son necesarios más requisitos específicos para los nanomateriales en este marco. REACH es la legislación europea responsable del registro, la autorización y la restricción de las sustancias y preparados químicos. Los nanomateriales son regulados por REACH, a pesar que no son citados explícitamente en esta legislación, pues son considerados como "sustancias" químicas de acuerdo con las definiciones establecidas y, por lo tanto, su reglamentación sigue el reglamento REACH (REACH, 2006). Sin embargo, los proyectos RIPoN sobre requisitos de información (RIPoN2) y sobre la evaluación de la seguridad química (RIPoN3) abordan, entre otras cosas, la cuestión de si los actuales requisitos de REACH y las directrices correspondientes son adecuados para evaluar los nanomateriales. El RIPoN2 concluyó que, con algunas salvedades, las directrices en el momento del proyecto y los requisitos de información se consideraban aplicables para la evaluación de los nanomateriales. Por otro

lado, el RIPoN3 concluía que los métodos conocidos de evaluación de la exposición eran por lo general aplicables, pero aún podían plantear problemas metodológicos (REACH, 2012). Por lo tanto, se puede concluir que, con su flexibilidad intrínseca, el enfoque de determinación del peligro y caracterización del riesgo, REACH resulta globalmente adecuado para los nanomateriales.

En la búsqueda de aspectos reglamentarios específicos que definan la seguridad biológica de los nanomateriales, el Comité Científico de los Riesgos Sanitarios Emergentes y Recientemente Identificados (CCRSERI), el Comité Científico de Seguridad de los Consumidores (CCSC), la Autoridad Europea de Seguridad Alimentaria (EFSA, en sus siglas en inglés) y la Agencia Europea de Medicamentos (EMA, en sus siglas en inglés) han estado trabajando desde 2004 en la determinación de los riesgos de los nanomateriales. Como parte de este mismo ámbito de trabajo, la Comisión Europea viene desde el año 2004 publicando comunicaciones sobre las características tecnológicas y reglamentarias de los nanomateriales. En 2004, a través de la Comunicación "Hacia una estrategia europea para las nanotecnologías" (COM, 2004), la Comisión declaró que el progreso tecnológico necesita ser acompañado por investigaciones científicas y la valoración de los posibles riesgos ambientales y para la salud humana asociados con la nanotecnología. A continuación, en 2005, por medio de la Comunicación "Nanociencias y nanotecnologías: un plan de acción para Europa 2005 - 2009" (COM, 2005), la Comisión especificó que todas las aplicaciones y usos de nanociencias y nanotecnologías deben cumplir con el alto nivel de salud pública, seguridad, protección a los consumidores y trabajadores, y protección ambiental. Por último, en 2008, la Comisión Europea publicó su primera Comunicación relacionada con los aspectos reglamentarios sobre los nanomateriales (COM, 2008). En esta comunicación se definieron los aspectos reglamentarios relacionados con un amplio grupo de posibles riesgos atribuidos a los nanomateriales. Dando continuidad a la legislación europea de control de los riesgos asociados a la exposición humana y ambiental a los nanomateriales, en 2012 se ha publicado una nueva Comunicación (COM, 2012) que se enmarca dentro del seguimiento de la Comunicación de la Comisión de 2008 sobre los aspectos reglamentarios de los nanomateriales. En ella se evalúan la adecuación y la aplicación de la legislación de la UE en materia de nanomateriales, se indican las acciones de seguimiento, y se responde a las cuestiones planteadas por el Parlamento Europeo, el Consejo y el Comité Económico y Social Europeo. Además, esta revisión va acompañada de un documento de trabajo de los servicios de la comisión sobre tipos de nanomateriales y su utilización, incluidos los aspectos relativos a su seguridad (SWD, 2012). Dicho documento responde a la preocupación de que el enfoque de los nanomateriales por parte de la Comisión Europea corre peligro por la falta de información sobre el uso y la seguridad de los nanomateriales que ya se encuentran en el mercado.

La OECD (*Organisation for Economic Co-operation and Development)* es una organización formada actualmente por 34 países de todo el mundo, más

otros países que actúan como observadores. La misión de la OECD es promover políticas que favorezcan el desarrollo económico y bienestar de la población de todo el mundo. Entre sus muchas actividades la OECD tiene un papel importante en la seguridad de productos químicos y en la protección ambiental y de la salud humana. Como parte de su responsabilidad por cuestiones emergentes, la OECD identificó la necesidad de analizar los potenciales problemas de seguridad causado por los nanomateriales. Como estos materiales empezaron a ser utilizados comercialmente, la OECD lanzó en 2006 un programa de trabajo para asegurar que las propuestas para valoración de los riesgos relacionados con los nanomateriales sean de gran calidad, con bases científicas y armonizadas internacionalmente. Después de 6 años de trabajo, la OECD y sus países miembros han llegado a la conclusión de que los métodos para valoración de productos químicos tradicionales son, en general, apropiados para evaluar la seguridad de los nanomateriales. Sin embargo, estos métodos pueden tener que ser adaptados a las especificidades de los nanomateriales (OECD, 2011). La OECD también destaca que no habrá necesidad de desarrollo de metodologías completamente nuevas específicas para los nanomateriales. Además, esta entidad se compromete a continuar revisando todos los métodos existentes con el objetivo de identificar e implementar los cambios necesarios para su aplicación a la valoración de los nanomateriales. Además, el sector de la OECD responsable de las cuestiones relacionadas con la seguridad biológica de los nanomateriales, mantiene en su página web una serie de publicaciones con el objetivo de proporcionar informaciones actualizadas respecto a las más diversas características de los nanomateriales, desde su seguridad ambiental hasta sus efectos sobre la salud humana (OECD, 2012).

La EMA, entidad europea encargada de controlar los medicamentos, sigue los últimos avances nanotecnológicos que son relevantes para el desarrollo de nuevas formulaciones farmacéuticas y reconoce que estas nuevas tecnologías representan nuevos desafíos para la agencia. Esto incluye las discusiones sobre si el marco reglamentario actual es apropiado para estos medicamentos y si las directrices y requisitos existentes sobre la forma en que se evalúan los medicamentos son adecuados. Sin embargo, hasta que se defina una legislación específica para la valoración toxicológica de los nanomateriales, la EMA, sigue las determinaciones de otras entidades, como la OECD, que de forma general, acepta que los modelos de determinación del riesgo utilizados para la valoración de compuestos y formulaciones convencionales, también son apropiados para la valoración de los nanomateriales (OECD, 2011; EMA, 2012). Además, la Comisión Europea considera que la legislación actual sobre los medicamentos permite efectuar adecuadamente un análisis riesgo/benefício y una gestión de los riesgos de los nanomateriales (COM, 2012).

Fuera del espacio europeo, la FDA (*US Food and Drug Administration*) en conjunto con el CDER (*Center for Drug Evaluation and Research*) también

han trabajado durante los últimos años para comprender las propiedades de los nanomateriales utilizados, especialmente, en los productos farmacéuticos, con el objetivo de informar y garantizar el desarrollo de un marco regulador que evalúe adecuadamente el impacto de sus propiedades fisicoquímicas en la seguridad y eficacia de los medicamentos (FDA, 2012a). Los proyectos actuales llevados a cabo por estas entidades buscan identificar las limitaciones de los métodos disponibles actualmente para valorar la calidad y seguridad biológica de medicamentos basados en la nanotecnología, además de evaluar el efecto de la nanotecnología en las características del producto final, incluyendo estabilidad y uniformidad de contenido. En el campo de los cosméticos, recientemente la FDA ha publicado un guión para la industria contemplando la seguridad de los nanomateriales en productos cosméticos (FDA, 2012b).

En resumen, aunque está ampliamente extendida la aplicación de metodologías de determinación del riesgo de materiales convencionales para los nanomateriales, todavía es preciso ahondar más en determinados aspectos relacionados con estos. Es algo que continuará planteándose hasta que se disponga de suficiente información científica para caracterizar los efectos nocivos de los nanomateriales sobre los seres humanos y el medio ambiente. A la luz de los conocimientos actuales y de los dictámenes de los comités consultivos y científicos de la UE y de los evaluadores independientes de riesgos, los nanomateriales son similares a las sustancias y los productos químicos normales, en el sentido de que algunos pueden ser tóxicos y otros no. Los posibles riesgos están relacionados con nanomateriales específicos y con usos específicos. Por tanto, es necesario realizar una determinación del riesgo de los nanomateriales, que debe hacerse caso por caso, utilizando la información pertinente. Los actuales métodos de determinación del riesgo se pueden aplicar a los nanomateriales, si bien aún es necesario trabajar sobre aspectos particulares de dichas metodologías. Además, la Comisión Europea está trabajando actualmente en los métodos de detección, medición y control de los nanomateriales y en su validación para garantizar la correcta aplicación de la definición de nanomateriales (UE, 2011). Existen retos importantes fundamentalmente en cuanto al establecimiento de métodos e instrumentos validados de detección, caracterización y análisis, a completar información sobre los peligros de los nanomateriales y a elaborar métodos de evaluación de la exposición a los mismos (COM, 2012).

### 5.2. 3Rs: Una base ética para la nanotoxicología

Un gran número de investigadores considera que los métodos alternativos actualmente disponibles para la caracterización y evaluación toxicológica de los nanomateriales no presentan suficiente sensibilidad, fiabilidad, correlación y sofisticación para proporcionar algo más que informaciones limitadas, y a veces equivocadas, sobre el funcionamiento

general de los nanomateriales en modelos fisiológicos realistas (Jones y Grainger, 2009). En este sentido, hay una gran discusión en cuanto a la necesidad de métodos especiales y específicos para estudios de nanotoxicología (Hartung, 2010). Sin embargo, es extremadamente elevado el número de nanomateriales que han de ser analizados en la búsqueda de una definición de criterios y relaciones estructura-actividad (efecto de la morfología, tamaño y material de revestimiento del nanomaterial) para un estudio toxicológico más eficaz y realista. Por lo tanto, esto sugiere la importancia de los métodos alternativos, que a pesar de tener algunas limitaciones, permiten una gran velocidad de análisis, incluyendo varias replicas y ensayos adicionales en paralelo. Dentro del contexto de la razonabilidad en utilizar métodos alternativos en nanotoxicología, se puede destacar el estudio de Choi y col. (2009), en el que se demostró que el coste total para una evaluación toxicológica de las nanopartículas que ya están siendo comercializadas (en las más diversas aplicaciones) seria entre $250 millones y $1,2 billones, mientras que el tiempo requerido seria de 34 a 53 años.

Actualmente la incertidumbre y el desconocimiento general sobre los potenciales efectos tóxicos de los sistemas nanoestructurados lleva a un gran debate entre la comunidad científica sobre si lo que se necesita son métodos tradicionales o alternativos para una evaluación toxicológica segura e eficaz. Cuando la pregunta en cuestión es "¿Realmente hay suficientes razones que hagan los métodos alternativos disponibles menos aplicables a los nanomateriales?", la respuesta muchas veces es sí, ya que la biocinética de un sistema nanoparticulado afectaría los resultados *in vitro* e *in vivo* de modo muy diferente, pudiendo llevar a aumentos significativos en la biodisponilidad y toxicidad de la sustancia activa encapsulada. Sin embargo, el hecho de que es más fácil de desarrollar modelos de cultivos celulares *in vitro* para identificar los riesgos después de una sobreexposición, se argumenta otra vez a favor del uso de métodos alternativos.

En cuanto a las ventajas de los sistemas *in vitro* utilizando varias líneas celulares, también cabe destacar: (1) demostración de los efectos primarios de las células dianas en ausencia de efectos secundarios causado por inflamación; (2) identificación de los mecanismos primarios de toxicidad en ausencia de factores fisiológicos y compensatorios que confunden la interpretación de los estudios en modelos animales; (3) eficiencia, rapidez y buena relación coste-eficacia; (4) ámbito para mejorar el diseño de los ensayos subsecuentes utilizando modelos animales *in vivo* (Huang y col., 2010); (5) reducción en la variabilidad entre experimentos; (6) necesidad de menor cantidad de muestra y generación de cantidades limitadas de residuos tóxicos; y (7) posibilidad de utilización de células transgénicas que llevan genes humanos específicos (Takhar y Mahant, 2011).

Los modelos *in vitro* representan un compromiso razonable entre esfuerzo y ganancia de información, permitiendo la comparación directa entre varios nanomateriales y sus compuestos originales. Un amplio cribado basado en modelos animales no es factible con respecto a costes y capacidad de un laboratorio, además de no ser un método deseado desde el punto de vista de las políticas éticas y de bienestar animal (Hartung, 2010). Por otro lado, la meta expresada en el informe "*Toxicity Testing in the 21st century: A vision and a Strategy (Tox-21c)*" (National Research Council, 2007), para proponer la utilización de conceptos de sistemas biológicos para valorar los efectos de compuestos y materiales en las vías de respuesta en lugar de los efectos a nivel apical u órgano-específico, permanece como un abordaje tan importante para la nanotoxicología como los métodos de evaluación toxicológica más tradicionales. En conclusión, lo que se requiere es una evaluación sistemática de los métodos alternativos disponibles antes de aplicarlos a los nanomateriales y, si es necesario, proceder a una modificación en el modelo de predicción (Hartung, 2010). Los modelos *in vitro* prometen proporcionar una base de datos accesible sobre las actividades biológicas de los nanomateriales para ayudar a entender sus riesgos.

### 5.2.1. Métodos alternativos validados para productos químicos y farmacéuticos, y su posible relevancia para los nanomateriales

Existen considerables incertidumbres sobre cualquier recomendación para un conjunto de métodos alternativos o una estrategia de ensayo totalmente integrada y definida para la valoración toxicológica de los nanomateriales (Silbergeld y col., 2011). Dentro del concepto de las 3Rs (reducción, refinamiento, reemplazo), cabe destacar que actualmente existen varios métodos alternativos validados que cumplen con este principio. Estos métodos han sido validados para productos químicos, farmacéuticos y/o cosméticos, pero no en cuanto a su propuesta para la utilización en nanomateriales. Sin embargo, a pesar de la falta de validación, pueden ser útiles y tener gran relevancia para la valoración toxicológica de nanomateriales (Tabla 2) (Hartung, 2010). En un futuro próximo, lo que se necesita es la adaptación y validación de las herramientas actualmente disponibles o bien la validación de nuevas metodologías, lo que ofrecería fiabilidad, rapidez y relevancia para valorar las características claves de los nanomateriales que están relacionadas con sus potenciales efectos adversos.

***Tabla 2.*** *Métodos alternativos validados para productos químicos y farmacéuticos y su probable relevancia para los nanomateriales (Hartung, 2010).*

| Nº | Método | Fecha de aprobación por la ESAC | Impacto en el principio de las 3Rs | Adaptabilidad para los nanomateriales |
|---|---|---|---|---|
| 1 | 3T3 NRU phototoxicity test | 03/11/1997 | Reemplazo | Probable aplicación, pero poca demanda de ensayo |
| 2 | EpiSkin skin corrosivity test | 03/04/1998 | Reemplazo | Probable aplicación |
| 3 | Rat TER skin corrosivity test | 03/04/1998 | Reemplazo | |
| 4 | Application of 3T3 NRU phototoxicity test to UV filter chemicals | 20/05/1998 | Reemplazo | Probable aplicación, pero poca demanda de ensayo |
| 6 | Local lymph node assay for skin sensitization | 21/03/2000 | Reducción/ Refinamiento | Probable aplicación |
| 7 | EpiDerm skin corrosivity test | 21/03/2000 | Reemplazo | Probable aplicación |
| 8 | CORROSITEX skin corrosivity test | 06/12/2000 | Reducción | |
| 11 | Micromass embryotoxicity assay | 01/05/2002 | Reducción | Relevancia no muy clara; puede necesitar una combinación con un modelo de barrera placentaria |
| 12 | Whole rat embryotoxicity assay | 01/05/2002 | Reducción | |
| 13 | Embryonic stem cell test for embryotoxicity | 01/05/2002 | Reducción | |
| 17 | Upper Threshold Concentration (UTC) step-down approach for acute aquatic toxicity testing | 21/03/2006 | Reducción | Probable aplicación |
| 18 | CFU-GM assay for predicting acute neutropenia in humans | 21/03/2006 | Reducción | Relevancia no muy clara; posible relevancia para valoración de la toxicidad aguda |
| 19 | Human whole blood IL-1 for *in vitro* pyrogenicity testing | 21/03/2006 | Reemplazo | Probable aplicación; especialmente importante debido a la gran área superficial de los nanomateriales y su aplicación cuestionable en el ensayo Limulus (LAL) |
| 20 | Human whole blood IL-6 for *in vitro* pyrogenicity testing | 21/03/2006 | Reemplazo | |
| 21 | PBMC IL-6 for *in vitro* pyrogenicity testing | 21/03/2006 | Reemplazo | |
| 22 | MM6 IL-6 for *in vitro* pyrogenicity testing | 21/03/2006 | Reemplazo | |
| 23 | Human Cryopreserved whole blood IL-1 for *in vitro* pyrogenicity testing | 21/03/2006 | Reemplazo | |
| 24 | *In vitro* micronucleous test as an alternative to the *in vitro* chromosome aberration assay for genotoxicity testing | 17/11/2006 | Mejora de la batería de ensayos *in vitro* | Probable aplicación; alta relevancia |
| 25 | Application of the SkinEthic human skin model for skin corrosivity testing | 17/11/2006 | Reemplazo | Probable aplicación |
| 27 | Bovine Corneal Opacity and Permeability (BCOP) test method | 27/04/2007 | Reducción | Probable aplicación |
| 28 | Isolated Chicken Eye (ICE) test method | 27/04/2007 | Reducción | |
| 29 | Reduced Local Lymph Node Assay (rLLNA) | 27/04/2007 | Reducción | Probable aplicación |

| | | | | |
|---|---|---|---|---|
| 30 | EpiDerm (with MTT reduction) for skin irritation | 27/04/2007 | Reemplazo | Probable aplicación; alta relevancia |
| 31 | EpiSkin (with MTT reduction) for skin irritation | 27/04/2007 | Reemplazo | |
| 32 | Fixed Dose Procedure (FDP) | 31/10/2007 | Reducción | Probable aplicación |
| 33 | Acute Toxic Class Method (ATC) | 31/10/2007 | Reducción | |
| 34 | Up and Down Procedure (UDP) | 31/10/2007 | Reducción | |
| 35 | EpiDerm SIT (with MTT reduction) for skin irritation | 05/11/2008 | Reemplazo | Probable aplicación; alta relevancia |
| 36 | SkinEthic (with MTT reduction) for skin irritation | 05/11/2008 | Reemplazo | |
| 37 | CellSystems human skin model EST-1000 for skin corrosivity testing | 12/06/2009 | Reemplazo | Probable aplicación |
| 38 | Cellsensor Microphysiometer for eye irritation | 10/07/2009 | Reemplazo | Probable aplicación |
| 39 | Fluorescente leakage assay for severe eye irritation | 10/07/2009 | Reemplazo | |

### 5.2.2. Limitaciones de los métodos *in vitro* en la nanotoxicología

La aplicación de los métodos *in vitro*, sean validados o no, en estudios toxicológicos de los nanomateriales es un tema que genera mucha discusión entre la comunidad científica. Al mismo tiempo que existen suficientes argumentos defendiendo su aplicabilidad y fiabilidad, hay mucho razonamiento negativo que considera los métodos alternativos *in vitro* poco aplicables a los nanomateriales. Se considera que la cinética de las partículas en solución, p. ej. en medio de cultivo celular, puede diferir de un compuesto en su forma original no particulada, lo que podría alterar la exposición celular al tratamiento y, por lo tanto, la obtención de la curva dosis-respuesta. Además, la exposición al aire y a condiciones de cultivo no fisiológicas pueden afectar los experimentos. Por el contrario, también es difícil decir si los resultados de la potencial toxicidad de los nanomateriales obtenidos *in vivo,* en diferentes modelos animales, son mejores o peores cuando son extrapolados a humanos. Por lo tanto, a pesar de las particularidades de los nanomateriales y de las limitaciones de los métodos existentes para valorar sus efectos adversos, la nanotoxicología es probablemente una fuerza positiva y no un obstáculo hacia el uso de métodos modernos, *in vitro* y alternativos al uso de animales, en toxicología (Hartung, 2010).

Más específicamente, se pueden destacar algunos de los principales problemas en la nanotoxicología *in vitro* (Hartung, 2010):

- *Aglomeración*: la agregación o aglomeración de los nanomateriales es muy común y difícil de prevenir. La agregación en soluciones acuosas puede tener efectos sustanciales en su toxicidad (Holl, 2009). Soluciones ricas en iones y proteínas, como los medios de cultivo,

tienen mayor tendencia a inducir la formación de agregados (Fröhlich y col., 2012).

- *Estabilidad*: la gran superficie libre de las nanopartículas representa un problema, ya que esto no solo atrae sustancias por ofrecer sitios para unirse (p. ej. para los pirógenos), como también desencadena reacciones químicas como la oxidación.

- *Dosimetría*: el peso, el número de partículas y el área superficial son típicamente las medidas de dosis para los nanomateriales, pero la morfología, el revestimiento y las propiedades electrofísicas también pueden tener su impacto. La caracterización química y la dosimetría de los sistemas nanoestructurados claramente requieren más atención que los compuestos químicos tradicionales (Powers y col., 2007; Walker y Bucher, 2009).

- *Biocinética in vitro*: la biocinética *in vitro* de los nanomateriales comprende la adsorción (p. ej. por el material plástico utilizado en los ensayos o bien por proteínas, como la albúmina, presente en el medio de cultivo celular), solubilidad o precipitación, captación y/o metabolización por las células u oxidación por el aire. La adsorción de factores del medio (proteínas, iones, aminoácidos) en la superficie de los nanomateriales también puede cambiar la composición del medio, influyendo así en la respuesta tóxica del material ensayado (Boverhof y David, 2010). La situación *in vitro* no tiene el mismo grado de complejidad que la cinética *in vivo*, pero ciertamente la concentración efectiva que llega a la célula no será la misma añadida inicialmente (Bouvier D'Yvoire y col., 2007).

- *Contacto celular de los nanomateriales*: las células pueden no estar realmente expuestas a los nanomateriales, pues estos pueden flotar en el medio de cultivo. Además, la densidad celular en cultivos típicos es solo 1% de aquella de un tejido normal (Hartung, 2007), lo que cambia la dosimetría y la probabilidad del contacto nanomaterial-célula.

- *Artefactos especiales de los nanomateriales in vitro*: artefactos utilizados en los procedimientos *in vitro*, como los colorantes usados en los ensayos de viabilidad, pueden interaccionar negativamente con los nanomateriales (Worle-Knirsch y col., 2006; Monteiro-Riviere y col., 2009, 2010). Este tipo de interacción puede ser debida a características particulares de los sistemas nanoestructurados, como su gran área superficial, propiedades electrostáticas y/o alta reactividad.

A parte del comportamiento y alteraciones que puedan sufrir los nanomateriales en las condiciones de cultivo, el tipo celular utilizado en el ensayo puede afectar significativamente la respuesta biológica (Sohaebuddin y

col., 2010; Fröhlich y col., 2012). La función fisiológica de la línea celular parece ser el factor clave en la determinación de su sensibilidad a los efectos tóxicos de los nanomateriales. Además, un mismo nanomaterial puede inducir diferentes respuestas intracelulares y mecanismos de toxicidad en distintas líneas celulares (Sohaebuddin y col., 2010). Por lo tanto, es de gran importancia analizar los efectos tóxicos de los nanomateriales en los modelos celulares que, además de relevantes, sean el tipo de célula que normalmente estaría expuesta a estos nanomateriales en condiciones *in vivo*. También con relación a las limitaciones de la utilización de células *in vitro*, Donaldson y col. (2009) han declarado que las células en cultivo no experimentan el rango de efectos patogénicos que comúnmente son observados *in vivo*, lo que está parcialmente relacionado con temas de translocación, toxicocinética y respuesta coordinada de los tejidos.

### 5.2.3. Caracterización de los nanomateriales para estudios toxicológicos *in vitro*

Para valorar los riesgos tras la exposición a los nanomateriales, es necesaria una completa caracterización tanto de los compuestos químicos como de las propiedades fisicoquímicas de los sistemas nanoestructurados resultantes. Estas propiedades están estrechamente relacionadas con la actividad biológica y, por lo tanto, deben ser cautelosamente evaluadas antes de proceder con cualquier valoración biológica y/o toxicológica (Boberhof y David, 2010; Horie y col., 2012). Dicha caracterización es necesaria para obtener una validez científica de las respuestas obtenidas (Jones y Grainger, 2009). La valoración de la presencia de contaminantes de superficie (p. ej. endotoxinas bacterianas, hidrocarbonos poliaromáticos y reactivos de síntesis no reaccionados) (Vakharia y col., 2001; Gorbet y Sefton, 2005; Dobrovolskaia y McNeil, 2007) en los nanomateriales es de gran importancia, ya que la composición de su superficie es un requisito para entender las interacciones de los sistemas nanoestructurados con las células. Además, la caracterización de su tamaño, estado de agregación, floculación, potencial zeta e índice de polidispersidad también son aspectos críticos para comprender las respuestas biológicas.

La formación de agregados o aglomerados en medio de cultivo durante el tiempo del ensayo de toxicidad *in vitro* es uno de los parámetros más importantes a tener en cuenta en el momento de proceder con la caracterización de los nanomateriales. Esta gran tendencia a la agregación se debe a la naturaleza altamente iónica de la solución y a las interacciones electrostáticas/van de Waals entre los nanomateriales y las proteínas presentes en el medio. Todo este proceso resulta en la formación de partículas secundarias, que pueden afectar de modo significativo la internalización celular y la citotoxicidad resultante (Horie y col., 2009, 2012). Por lo tanto, no solo el

tamaño de las nanopartículas primarias, si no también el tamaño de las nanopartículas secundarias, se debe usar como un parámetro característico para determinar la toxicidad *in vitro* de los nanomateriales en medio de cultivo celular (Kato, 2011). Con el objetivo de evitar o por lo menos disminuir la agregación de las partículas, muchos investigadores proponen la realización de ensayos *in vitro* utilizando medio de cultivo libre de suero fetal bovino (Fisher y col., 1999; Murdock y col., 2008). Sin embargo, este aspecto introduce cuestiones en cuanto a la relevancia y equivalencia biológica del ensayo, ya que ninguna exposición *in vivo* ocurriría en ausencia de proteínas (Schulze y col., 2008).

Las técnicas analíticas comúnmente utilizadas para la caracterización del tamaño y estado de agregación de los nanomateriales incluyen microscopía electrónica de transmisión (TEM, en sus siglas en inglés) (Soto y col., 2005, 2007), microscopía electrónica de barrido (SEM, en sus siglas en inglés) (Albrecht y col., 2002; Magrez y col., 2006), espectroscopia óptica (UV-visible) (Burda y col., 2005; Jain y col., 2007), espectroscopia de correlación de fotones (DLS, en sus siglas en inglés) (Lin y col., 2006; Long y col., 2007) y polarización fluorescente (Bruno y col., 2006). Estos métodos muchas veces pueden proporcionar informaciones no concluyentes cuando se aplican a nanomateriales en sistemas muy complejos. Además, cada método tiene sus propias incertidumbres inherentes, lo que hace necesaria una corroboración de los resultados obtenidos con uno o más métodos adicionales (Jones y Grainger, 2009).

Horie y col. (2012) han propuesto un protocolo para la evaluación *in vitro* de los nanomateriales (Figura 13). Este protocolo está constituido por 5 pasos: (1) preadsorción de los componentes del medio para prevenir la inanición de lo mismo, (2) determinación de los elementos liberados de los nanomateriales y sus contaminantes, (3) observación del proceso de internalización celular de los nanomateriales, (4) determinación del tamaño de las partículas secundarias y (5) confirmación de la estabilidad de los nanomateriales en medio de cultivo. A través de este protocolo, es posible llegar al conocimiento de las propiedades de los nanomateriales en medio de cultivo, lo que es esencial para entender el comportamiento de estos materiales durante los ensayos de toxicidad *in vitro*.

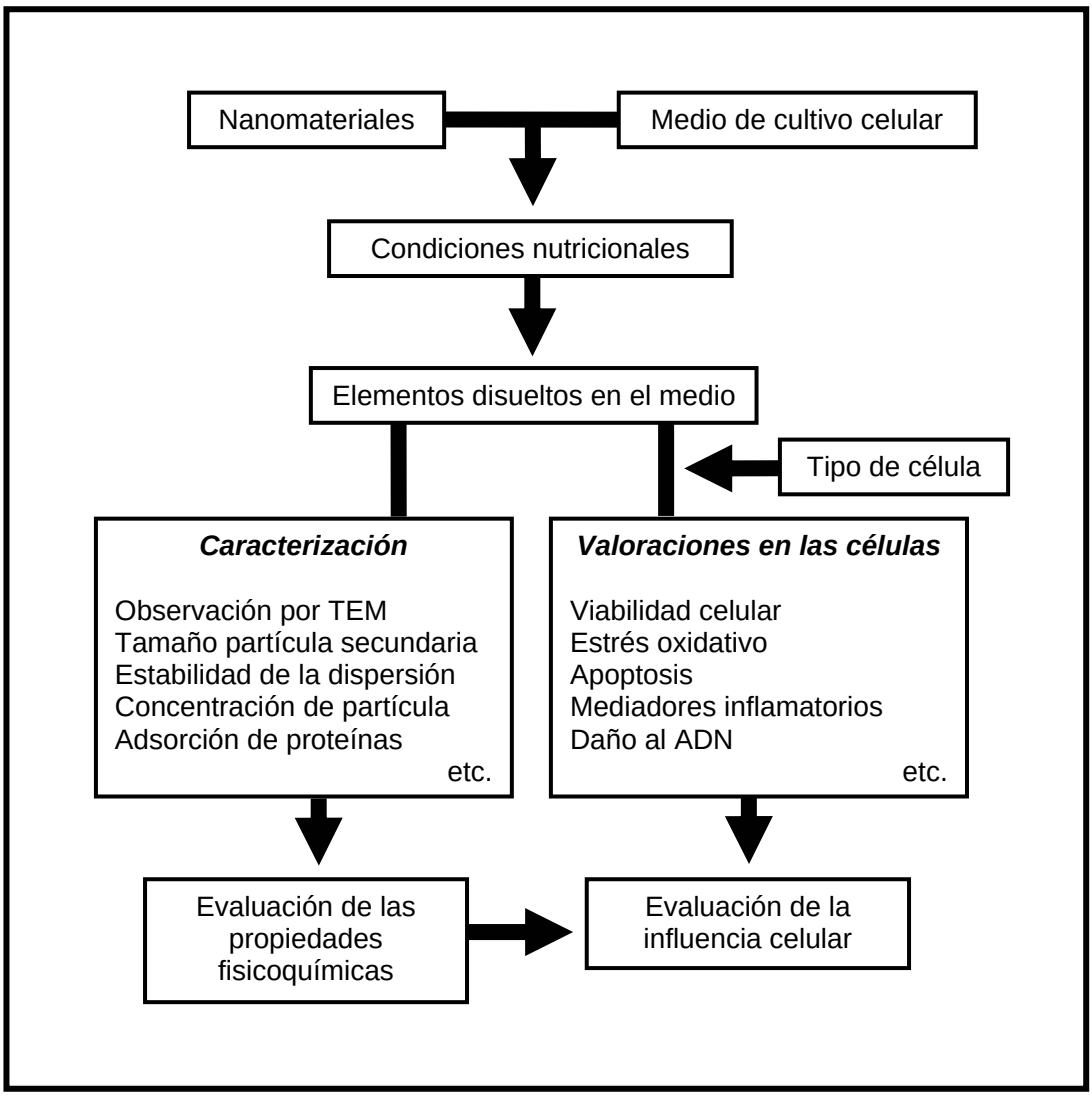


***Figura 13.*** *Protocolo propuesto para la evaluación toxicológica in vitro de los nanomateriales (Adaptada de Horie y col., 2012).*

### 5.2.4. Elucidación de los mecanismos involucrados en la toxicidad de los nanomateriales utilizando métodos *in vitro*

Los modelos *in vitro* constituyen un método rápido y efectivo para evaluar los nanomateriales en cuanto a un amplio rango de criterios de valoración toxicológicos. También permiten el desarrollo de ensayos que incluyan la valoración de los mecanismos involucrados en el proceso de interacción de los nanomateriales con los más diversos tipos celulares. Además, los estudios *in vitro* pueden ser utilizados para establecer relaciones concentración-efecto, efecto-umbrales específicos y estructura-actividad (Arora y col., 2012).

La toxicidad de los nanomateriales no es atribuida a propiedades y mecanismos definidos. Además, no hay un único método que sea satisfactorio para obtener toda la información relativa a la potencial toxicidad de los sistemas nanoestructurados. Por lo tanto, se requiere una combinación de ensayos para estudiar los mecanismos involucrados en la toxicidad de los diferentes nanomateriales (Arora y col., 2012).

La internalización celular de los nanomateriales es un fenómeno importante que puede determinar los efectos tóxicos en el compartimiento intracelular. En muchos casos, los sistemas nanoestructurados forman estructuras secundarias (agregados o aglomerados), sin embargo estas estructuras también pueden ser captadas por endocitosis al interior de las células. Una vez en el citoplasma, los nanomateriales pueden desencadenar, dependiendo de sus características fisicoquímicas, diferentes reacciones que pueden ser dañinas para las células. Estas reacciones pueden llevar a disfunción mitocondrial y del metabolismo celular, induciendo directa o indirectamente producción de especies reactivas de oxígeno (ROS, en su sigla en inglés) intracelular. Esta liberación de radicales libres puede también inducir efectos apoptóticos, que a su vez puede llevar a daños en el ADN, alteraciones en el ciclo celular y producción de mediadores inflamatorios (Figura 14).

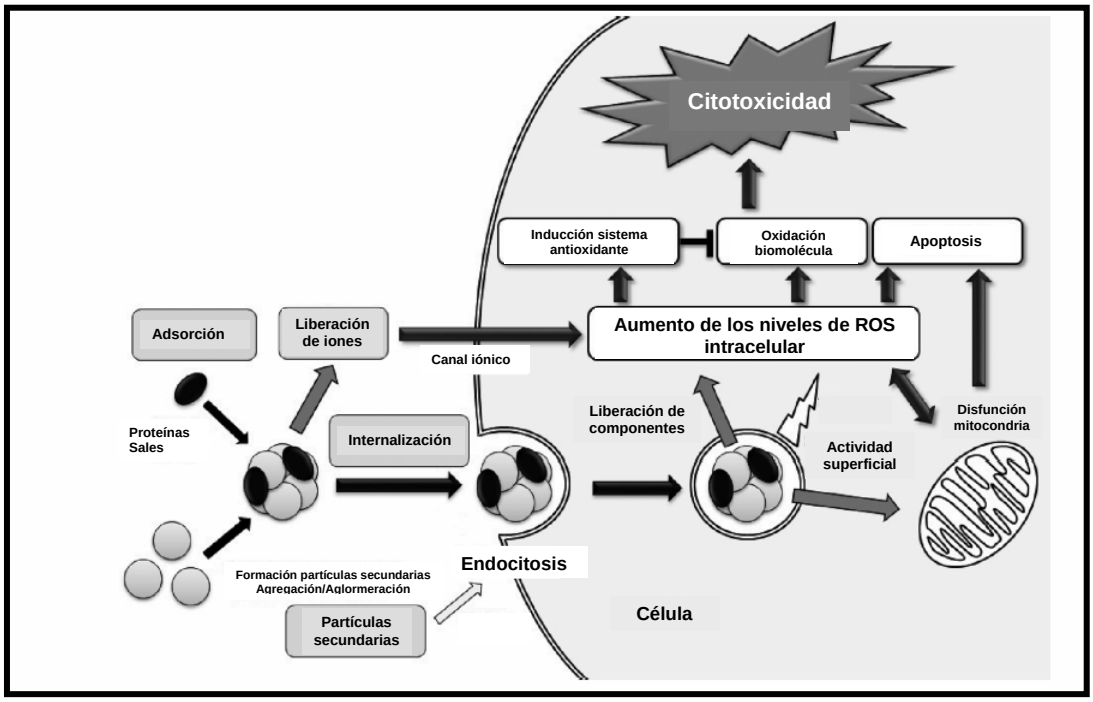


***Figura 14.*** *Representación esquemática de un posible mecanismo de citotoxicidad inducido por los nanomateriales (Adaptada de Horie y col., 2012).*

Como se puede observar, los posibles mecanismos involucrados en la nanotoxicidad son complejos y requieren métodos adecuados para un correcto entendimiento. Entre los principales métodos *in vitro* utilizados para estudiar la

toxicidad de los nanomateriales y sus mecanismos, se pueden destacar:

- Ensayos de viabilidad/proliferación celular:

- *Actividad metabólica*: MTT (Schins y col., 2002; Jia y col., 2005; Davoren y col., 2007), XTT, WST-1, MTS (Dong y col., 2008), Azul de Alamar (Davoren y col., 2007)

- *Integridad de membrana*: LDH (Lin y col., 2006; Monteiller y col., 2007), Azul de Tripano (Schins y col., 2002), NRU (Huang y col., 2004), Yoduro de propidio (Jin y col., 2007).

- Ensayos de los mecanismos de toxicidad:

- *Apoptosis y necrosis*: son procesos generalmente estudiados por citometría de flujo (AshaRani y col., 2009), utilizando el yoduro de propidio como marcador fluorescente, o microscopía óptica (Magrez y col., 2006) y de fluorescencia (Arora y col., 2009), utilizando los colorantes fluorescentes naranja de acridina y bromuro de etídio.

- *Ciclo celular*: se estudia por citometría de flujo, utilizando el yoduro de propídio como marcador fluorescente (AshaRani y col., 2009).

- *Estrés oxidativo*: se determina especialmente a través de la detección de ROS intracelular (Yang y col., 2009; AshaRani y col., 2009) y de la peroxidación lipídica (Sayes y col., 2005; Arora y col., 2009).

- *Integridad lisosomal*: se estudia por citometría de flujo (Xia y col., 2008) o microscopía de fluorescencia (Sohaebuddin y col., 2010), utilizando naranja de acridina como marcador lisosomal.

- *Genotoxicidad*: se valora el daño al ADN celular generalmente usando el Ensayo del Cometa (AshaRani y col., 2009; Yang y col., 2009).

- *Mediadores inflamatorios:* la producción de citocinas es tradicionalmente cuantificada por el enzima inmunoensayo (ELISA, en sus siglas en inglés) (Davoren y col., 2007; Monteiller y col., 2007; Singh y col., 2007).

- *Expresión de proteínas y genes:* se puede detectar por las técnicas Reacción en cadena de polimerasa (PCR, en sus siglas en inglés), *Western blotting* o Ensayo de proteína total (p. ej. Bradford) (Jones y Grainger, 2009).

#### 5.2.4.1. Toxicidad de los nanomateriales a los componentes de la sangre

El potencial empleo de los nanomateriales para la administración sistémica de fármacos requiere la evaluación de su impacto en los componentes de la sangre. El creciente número de estudios sobre la hemocompatibilidad de diferentes nanomateriales refleja la relevancia clínica de dichos estudios (Koziara y col., 2005; Dobrovolskaia y col., 2009; Mayer y col., 2009; Dash y col., 2010; Kuznetsova y col., 2012; Sasidharan y col., 2012). A pesar de que los nanomateriales están comúnmente formulados con constituyentes biocompatibles, la organización espacial de grupos químicos en la superficie del nanomaterial puede ser crucial para su hemoreactividad (Kuznetsova y col., 2012).

Todavía no hay un protocolo general establecido sobre las condiciones bajo las cuales la sangre o sus componentes deben ser sometidos a los nanomateriales, de modo que proporcionen datos relevantes *in vitro* que puedan predecir su potencial reactividad *in vivo* (Dobrovolskaia y col., 2008). Sin embargo, los métodos *in vitro* son de gran importancia en la determinación de una potencial incompatibilidad de los nanomateriales con determinados componentes de la sangre, además de sugerir los posibles mecanismos por los cuales los nanomateriales interactúan con la sangre (Kuznetsova y col., 2012). Por lo tanto, los estudios *in vitro* son necesarios y relevantes clínicamente antes de llevar a cabo estudios más a fondo *in vivo*. Entre los principales parámetros de análisis utilizados en los estudios de compatibilidad con los componentes de la sangre, se pueden destacar: hemólisis, morfología y aglutinación de los eritrocitos, integridad plaquetaria, cascada de coagulación, activación del sistema inmunológico y interacción con proteínas plasmáticas (Dutta y col., 2007; Kuznetsova y col., 2012; Sasidharan y col., 2012).

# RESULTADOS

Los resultados obtenidos en esta Tesis han dado lugar a siete publicaciones científicas, que se agrupan en dos bloques basados en los objetivos generales planteados:

**Respecto a las propiedades de interacción con membrana celulares:**

- *Artículo 1*: The role of counterions in the membrane-disruptive properties of pH-sensitive lysine-based surfactants.

- *Artículo 2*: Membrane-destabilizing activity of pH-responsive cationic lysine-based surfactants: role of charge position and alkyl chain length.

- *Artículo 3*: Phospholipid-bilayer perturbing properties underlying lysis induced by pH-sensitive cationic lysine-based surfactants in biomembranes.

- *Artículo 4*: Comparative sensitivity of tumor and non-tumor cell lines as a reliable approach for *in vitro* cytotoxicity screening of lysine-based surfactants with potential pharmaceutical applications.

**Respecto al desarrollo de sistemas nanoestructurados, su evaluación toxicológica y su comportamiento intracelular *in vitro*:**

- *Artículo 5*: New cationic nanovesicular systems containing lysine-based surfactants for topical administration: toxicity assessment using representative skin cell lines.

- *Artículo 6*: Lysine-based surfactants in nanovesicle formulations: the role of cationic charge position and hydrophobicity in *in vitro* cytotoxicity and intracellular delivery.

- *Artículo 7*: *In vitro* antitumor activity of methotrexate via pH-sensitive chitosan nanoparticles.

## Artículo 1

**THE ROLE OF COUNTERIONS IN THE MEMBRANE-DISRUPTIVE PROPERTIES OF pH-SENSITIVE LYSINE-BASED SURFACTANTS**

**(Efecto de los contraiones en las propiedades líticas de membrana de tensioactivos pH-sensibles derivados de lisina)**

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**Acta Biomaterialia 2011; 7: 2846-2856.**

**Índice de impacto (SCI 2011): 4,865**
**Categoría (posición): Engineering, Biomedical (3/72)**
**Material Science, Biomaterials (2/25)**

Los resultados de este artículo han sido presentados en formato póster en el congreso siguiente:

- XII International Congress of Toxicology (IUTOX). Barcelona, España, Julio 2010.
  Rubert D, Mitjans M, Vinardell MP. 'pH dependence on the hemolysis induced by anionic surfactants'.
  Publicado en *Toxicology Letters* 2010; 196: S131.

# Resumen

*Objetivos*

En la búsqueda de compuestos pH-sensibles con potencial aplicación en vehículos para la liberación intracelular de fármacos, se propuso estudiar la actividad hemolítica dependiente del pH de tensioactivos aniónicos derivados del aminoácido lisina, utilizando el eritrocito como un modelo de membrana endosomal.

*Material y métodos*

Se estudió una familia de tensioactivos aniónicos de doble cadena derivados de la $N^{\alpha},N^{\varepsilon}$-dioctanoil lisina (77K), comprendiendo 5 tipos de contraiones: tres inorgánicos (sodio, 77KS; litio, 77KL; y potasio, 77KP) y dos orgánicos (lisina, 77KK; y tris (hidroximetil) amino metano, 77KT). El tensioactivo comercial dodecil sulfato sódico (SDS) se utilizó con fines comparativos. Se utilizó el ensayo de hemólisis para la valoración de la interacción de estos compuestos bioactivos con membranas celulares. Los eritrocitos se aislaron de sangre de rata y se utilizaron como modelo de la membrana de los endosomas. Se prepararon disoluciones de solución amortiguadora de fosfatos (PBS) a diferentes pH (5,4–8,0), reproduciendo las condiciones fisiológicas y la de los endosomas primarios y secundarios. Además del estudio del efecto del pH en la actividad hemolítica, se estudió la hemólisis en función de la concentración y del tiempo de incubación. También se evaluaron los mecanismos de interacción con la membrana celular y su lisis resultante, a través del estudio de la morfología de los eritrocitos por microscopía electrónica de barrido (SEM), del efecto de compuestos de diferentes pesos moleculares en el proceso de lisis de la membrana y del recuento de los eritrocitos por microscopía óptica. Por fin, se estudió el potencial citotóxico *in vitro* de estos tensioactivos, utilizando la línea celular de fibroblastos 3T3 y los ensayos de viabilidad MTT y NRU.

*Resultados*

Los tensioactivos derivados de lisina presentaron, independientemente del contraión, actividad hemolítica concentración-dependiente y pH-sensible, con aumento significativo de la hemólisis especialmente a pH 5,4. Además, estos tensioactivos también mostraron cinética mejorada de su potencial lítico de membrana en los valores de pH característico de los endosomas. El tensioactivo SDS utilizado como control no demostró actividad sensible al pH. Las evaluaciones del mecanismo de lisis de la membrana celular demostraron la existencia de un mecanismo osmótico involucrado en la actividad hemolítica de los tensioactivos. Además, a través del recuento de los eritrocitos, se comprobó que la membrana celular se rompió tras el tratamiento con los tensioactivos, lo que indica que no se formaron canales o poros por los cuales la hemoglobina pudiera ser liberada al medio extracelular. Los estudios mediante SEM indicaron que los tensioactivos indujeron cambios en la morfología normal de las células, lo que corrobora la interacción de estos compuestos con la membrana celular. Los estudios de viabilidad celular mostraron, en general, una citotoxicidad leve a moderada de los tensioactivos a las concentraciones que también demostraron actividad hemolítica pH-dependiente.

*Conclusiones*

La variación del tipo de contraión influye en la intensidad de la respuesta hemolítica de los tensioactivos, pero no afecta su actividad lítica pH-sensible. Teniendo en cuenta la actividad lítica de membrana dependiente del pH, se sugiere la potencial aplicación de estos tensioactivos como excipientes bioactivos en vehículos para la liberación intracelular de fármacos.

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# The role of counterions in the membrane-disruptive properties of pH-sensitive lysine-based surfactants

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ABSTRACT

Surfactants are among the most versatile and widely used excipients in pharmaceuticals. This versatility, together with their pH-responsive membrane-disruptive activity and low toxicity, could also enable their potential application in drug delivery systems. Five anionic lysine-based surfactants which differ in the nature of their counterion were studied. Their capacity to disrupt the cell membrane was examined under a range of pH values, concentrations and incubation times, using a standard hemolysis assay as a model for endosomal membranes. The surfactants showed pH-sensitive hemolytic activity and improved kinetics at the endosomal pH range. Low concentrations resulted in negligible hemolysis at physiological pH and high membrane lytic activity at pH 5.4, which is in the range characteristic of late endosomes. With increasing concentration, the surfactants showed an enhanced capacity to lyse cell membranes, and also caused significant membrane disruption at physiological pH. This observation indicates that, at high concentrations, surfactant behavior is independent of pH. The mechanism of surfactant-mediated membrane destabilization was addressed, and scanning electron microscopy studies were also performed to evaluate the effects of the compounds on erythrocyte morphology as a function of pH. The *in vitro* cytotoxicity of the surfactants was assessed by MTT and NRU assays with the 3T3 cell line. The influence of different types of counterion on hemolytic activity and the potential applications of these surfactants in drug delivery are discussed. The possibility of using pH-sensitive surfactants for endosome disruption could hold great promise for intracellular drug delivery systems in future therapeutic applications.



## 1. Introduction

Many therapeutic agents, such as proteins, peptides, DNA and some drugs, act at intracellular sites, and thus their therapeutic efficacy depends on efficient intracellular trafficking pathways [1]. One of the challenges for the efficient intracellular delivery of therapeutic compounds is to manipulate or circumvent the non-productive trafficking from endosomes to lysosomes, where degradation may occur. This would allow delivery systems to escape endosomal compartments and consequently facilitate subsequent drug release into cytoplasm after internalization of the systems through endocytosis [2].

Carriers based on attenuated viruses have been studied extensively as pH-dependent membrane-disruptive components in gene delivery systems to enhance transport from endosomes to the cytoplasm; however, clinical use of these carriers is potentially limited by their antigenicity and toxicity, and the difficulties of large-scale production [3–5]. Safety issues have prompted the development of synthetic peptides structurally derived from viruses specifically to disrupt endosomal membranes [1]. However these peptides are also likely to be immunogenic *in vivo* [6,7]. To circumvent these problems, a variety of non-viral delivery vectors have been developed, such as synthetic polymers and surfactants. Polymerizable surfactants with tunable pH-sensitive amphiphilicity have recently been designed. These allow carriers to change their amphiphilic structure at endosomal–lysosomal pH, which results in the disruption of endosomal–lysosomal membranes [8,9]. The functionalization of one of these polymerizable pH-sensitive amphiphilic surfactants for the preparation of a peptide-directed siRNA delivery system has also been reported [10]. Moreover, cationic amino acid-based surfactants have been used to prepare novel biocompatible devices for the controlled encapsulation and release of DNA [11]. Cationic and anionic polymers with pH-sensitive activity, including non-biodegradable polymers and biodegradable poly(amino acids) and pseudo-peptides [2,7,12–18], have also been developed to promote endosomal escape. Cationic compounds have commonly been used to form stable cationic complexes; however, they show cytotoxicity and non-specifically adsorb serum proteins, thereby leading to rapid blood clearance as a result of the strong cationic surface charge [19–21]. In

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contrast, anionic pH-responsive polymers are considered of interest as drug carriers, because they mimic the structure and pH-dependent membrane–lytic behavior of endosomolytic viral peptides [1]. Moreover, recharging cationic complexes with anionic compounds has been reported as a promising method to overcome the adverse effects of cationic complexes [22].

Considerable research effort is devoted to delivery systems that specifically destabilize endosomal membranes in mildly acidic conditions following endocytic uptake [23]. In this context, amino acid-based surfactants with pH-sensitive activity and low toxicity deserve particular attention and could be a promising choice for application in non-viral drug delivery systems. Here, $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine derivatives, a class of amino acid-based surfactants synthesized as lecithin analogs, were selected, since homologs with eight carbon atom chains are the least hemolytic and show the least irritant activity, thus proving the most suitable for practical applications [24]. Moreover, earlier studies by the authors' group demonstrated the biocompatibility and low *in vitro* toxicity of this series of anionic lysine-based surfactants [25–28].

In the present study, the membrane lytic properties were studied as a function of pH of five anionic lysine-based surfactants differing in the nature of their counterion. To evaluate the potential applications in cytoplasmic delivery carriers, the pH-sensitive cell membrane disruptive activity of these compounds was examined using a standard hemolysis assay of rat erythrocytes at a range of pH values as a model for endosomal membranes. The mechanism involved in cell membrane disruption, the kinetic properties of each surfactant in the endosomal pH range, and their effects on erythrocyte morphology as a function of pH are also presented, together with *in vitro* cytotoxicity assays in the 3T3 fibroblast cell line. Furthermore, to gain insight into the structure-dependent interaction of these compounds with membrane bilayers, the influence of the surfactant structure and counterions on hemolytic activity is also discussed.

## 2. Materials and methods

### 2.1. Materials

L-Lysine monohydrochloride, L-lysine, Tris, the bases NaOH, LiOH and KOH, sodium dodecyl sulfate (SDS), glutaraldehyde, NaCl, $Na_2HPO_4$ and $KH_2PO_4$ were purchased from Merck (Darmstadt, Germany). PEG-10,000, D-glucose, dimethyl sulfoxide (DMSO), 2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT) and neutral red dye (NR) were from Sigma–Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin–EDTA solution (170,000 U $l^{-1}$ trypsin and 0.2 g $l^{-1}$ EDTA) and penicillin–streptomycin solution (10,000 U $ml^{-1}$ penicillin and 10 mg $ml^{-1}$ streptomycin) were obtained from Lonza (Verviers, Belgium). The 75 $cm^2$ flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland).

### 2.2. Surfactants tested

Five anionic amino acid-based surfactants derived from $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine and with counterions of distinct chemical nature were evaluated: two salts with organic counterions—lysine salt (77KK) and tris(hydroxymethyl) aminomethane salt (77KT); and three salts with inorganic counterions—sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP) (Fig. 1). These surfactants were synthesized in the authors' laboratory as previously described [25,29]. The commercial anionic surfactant SDS was used as reference compound.

### 2.3. Preparation of red blood cell suspensions

Rat blood was obtained from anesthetized animals by cardiac puncture and drawn into tubes containing EDTA. The procedure was approved by the institutional ethics committee on animal experimentation. Red blood cells were isolated by centrifugation at 3000 rpm at 4 °C for 10 min, and washed three times in an isotonic PBS containing 123.3 mM NaCl, 22.2 mM $Na_2HPO_4$ and 5.6 mM $KH_2PO_4$ in distilled water (pH 7.4; 300 mOsmol $l^{-1}$). The cell pellets were then suspended in PBS solution at a cell density of $8 \times 10^9$ cell $ml^{-1}$.

### 2.4. Hemolysis assay

The membrane–lytic activity of the surfactants was examined by hemolysis assay. PBS buffers in the pH range 5.4–8.0 were prepared to be isosmotic to the inside of the erythrocyte and cause negligible hemolysis. The 25-μl aliquots of erythrocyte suspension were exposed to various concentrations (from 100 to 800 μg $ml^{-1}$) of the surfactants dissolved in PBS solution in a total volume of 1 ml. Two controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). The samples were incubated at room temperature under constant shaking for various periods up to 90 min,

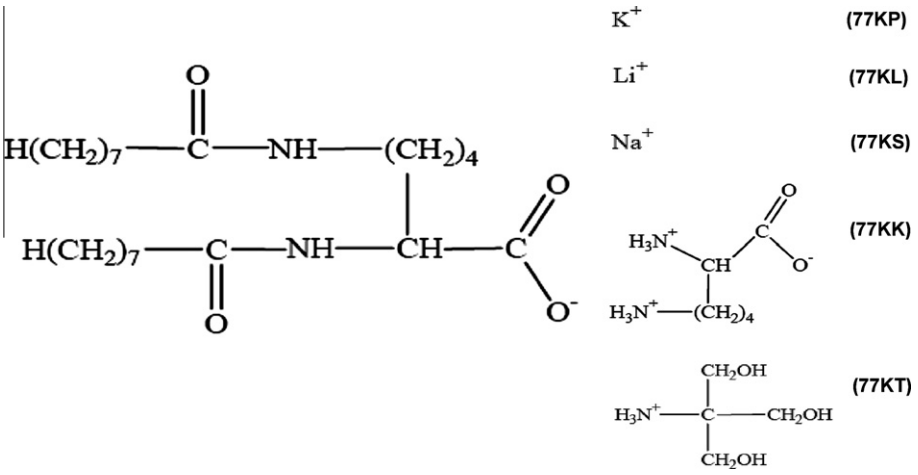


**Fig. 1.** Molecular structure of lysine-based anionic surfactants with different counterions. Codes P, L, S, K, and T represent potassium, lithium, sodium, lysine and Tris, respectively.

and then centrifuged at 10,000 rpm for 5 min. Supernatants were taken, the absorbance of the hemoglobin release was measured at 540 nm using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan), and the percentages of hemolysis were determined by comparison with the positive control samples totally hemolyzed with distilled water. Dose–response curves were obtained from hemolysis results, and the concentrations inducing 50% hemolysis ($HC_{50}$) were calculated.

### 2.5. Mechanism of cell membrane disruption

#### 2.5.1. Osmotic protection experiment

To determine the effect of osmolytes with diverse molecular weights on the hemolytic activity of the surfactants, PBS buffers at the pH range studied were prepared by adding D-glucose (180 Da) and PEG 10,000 (10 kDa) separately at a concentration of 10 mM, which is below the range at which these molecules alone induce hemolysis as a result of osmotic pressures [19]. Red blood cells were incubated with these buffers and exposed to a concentration of each surfactant which achieved ~100% hemolysis in PBS buffer alone (400–800 μg $ml^{-1}$). Hemolysis was determined after incubating the cells for 10 min at room temperature, following the procedure described above.

#### 2.5.2. Red blood cell count

Red blood cells were counted in a Zeiss Axioskop optical microscope (Zeiss, Jena, Germany) using a Bürker counting chamber (Brand, Wertheim, Germany). The percentages of lysed erythrocytes at the required concentration of each surfactant (400–800 μg $ml^{-1}$) was determined relative to the total cell number in PBS buffer alone. The hemolysis experiment was performed following the above procedure at the same pH range, and each sample was diluted four times for cell counts.

### 2.6. Scanning electron microscopy (SEM) studies of rat erythrocyte morphology

Interaction of the surfactants with the erythrocyte membrane in the pH range under study was determined by incubating intact cells with a sub-lytic concentration (100 μg $ml^{-1}$) of each surfactant. After 10-min incubation, samples were fixed by adding 1 ml of 2.5% glutaraldehyde in PBS solution and incubating at 4 °C for 2 h. The samples were then centrifuged (1500 rpm for 5 min), the supernatant was discarded, and 500 μl of 1.25% glutaraldehyde in PBS was added. Fixed samples were washed with PBS solution, postfixed with 1% osmium tetroxide, placed over a glass coverslip, dehydrated in an ascending series of ethyl alcohol (50–100%), air-dried by the critical point drying method using a CPD 7501 apparatus (Polaron, Watford, UK), and finally mounted on an aluminum stub and gold-coated in an SEM coating system SC 510 (Fisons Instruments, East Grinstead, UK). The resulting specimens were examined by SEM in a Zeiss DSM 940A instrument (Carl Zeiss SMT AG, Jena, Germany).

### 2.7. Cell culture

The murine Swiss albino 3T3 fibroblast cell line was grown in DMEM medium (4.5 g $l^{-1}$ glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U $ml^{-1}$ penicillin and 100 μg $ml^{-1}$ streptomycin at 37 °C, 5% $CO_2$. The 3T3 cells were routinely cultured in 75 $cm^2$ culture flasks and were trypsinized using trypsin–EDTA when the cells reached ~80% confluence.

### 2.8. Cytotoxicity assays

The cytotoxic effect of the surfactants was measured by tetrazolium salt MTT assay [30] and neutral red uptake (NRU) assay [31]. 3T3 cells were seeded into the central 60 wells of a 96-well plate at a density of $8.5 \times 10^4$ cells $ml^{-1}$. After incubation for 24 h under 5% $CO_2$ at 37 °C, the spent medium was replaced with 100 μl of fresh medium supplemented with 5% FBS containing 0.22-μm filter-sterilized surfactant solution at the required concentration range (150–300 μg $ml^{-1}$). After 24 h, the surfactant-containing medium was removed, and 100 μl of MTT in PBS (5 mg $ml^{-1}$) diluted 1:10 in medium without FBS and phenol red was then added to the cells. Similarly, 100 μl of 50 μg $ml^{-1}$ NR solution in DMEM without FBS and phenol red was added in each well for the NRU assay. The plates were further incubated for 3 h, after which the medium was removed, and the cells were washed once in PBS. Thereafter, 100 μl of DMSO was added to each well to dissolve the purple formazan product (MTT assay). Similarly, for the NRU assay, 100 μl of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water was added to extract the dye. After 10 min on a microtiter plate shaker at room temperature, the absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as a percentage of cell viability inhibition against the respective controls.

### 2.9. Statistical analysis

Each hemolysis experiment was performed at least three times using three replicate samples for each surfactant concentration tested. Results are expressed as mean ± standard error of mean (SE). Statistical analyzes were performed using one-way analysis of variance to determine the difference between the sets of data, followed by Bonferroni's or Dunnett's post hoc tests for multiple

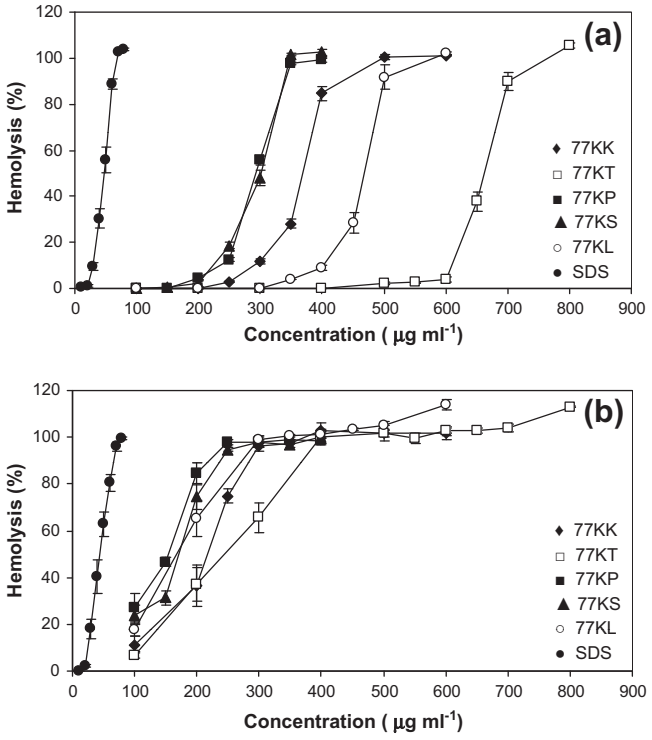


**Fig. 2.** Dependence of rat erythrocyte hemolysis on surfactant concentration: (a) pH 7.4; (b) pH 5.4. Each point represents the mean of three independent experiments ± SE (error bars).

comparisons, as indicated, using SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered to denote significance.

## 3. Results and discussion

### 3.1. Concentration-dependent hemolysis

The hemolytic activity of the lysine surfactants at pH 8.0, 7.4, 6.5 and 5.4 was determined as a function of concentration, and the concentration-dependent curves at pH 7.4 (physiological) and 5.4 (pH at late endosomes) are shown in Fig. 2. In this experiment, hemolysis was determined at a fixed time (after 10 min of incubation) in the presence of a range of surfactant concentrations, which were defined on the basis of the hemolytic potency of each surfactant [26]. At pH 7.4 (Fig. 2a), all the surfactants studied were non-hemolytic or displayed low hemolysis throughout the first points of the concentration curves; however, they exhibited a gradually stronger disruption of membranes as concentration increased, reaching complete hemolysis in the later points of the hemolytic curve. Similar hemolytic activities were obtained at pH 8.0 and 6.5, with only a small shift of the lysis curve to the left for pH 6.5 and to the right for pH 8.0 with respect to that registered at pH 7.4. These observations indicate increased and reduced activity, respectively (data not shown). These profiles show that hemolysis is a relatively complex process, with an initial lag period in all the curves. In contrast, at pH 5.4 (Fig. 2b), the lag period was not observed, and the concentration required to reach 100% hemolysis was considerably lower than that needed at the other pH values tested. Raising concentrations from 100 to 250 $\mu g\ ml^{-1}$ for 77KP and 77KS, from 100 to 300 $\mu g\ ml^{-1}$ for 77KK and 77KL, and from 100 to 400 $\mu g\ ml^{-1}$ for 77KT caused complete disruption of erythrocyte membranes. These results show that the hemolytic activity of all the compounds was strongly influenced by concentration. Similarly, several authors [18,32,33] reported similar results for the membrane–lytic activity of the polymers poly(α-ethylacrylic acid) and poly(L-lysine iso-phthalamide), and for arginine-based gemini surfactants, respectively. In the case of polymers, those authors argued that increasing compound concentration enhances the migration of the polymer molecules to lipid bilayer membranes, thereby resulting in an increase in membrane destabilization. Moreover, it is widely reported that surfactants at high concentrations are hemolytic, and membrane solubilization is often observed [34,35].

At pH 5.4, the surfactants with an inorganic counterion were more hemolytic than the surfactants with an organic counterion, and the $HC_{50}$ values showed statistically significant differences ($p < 0.05$) in comparison with the other pH values tested (Table 1). All the $HC_{50}$ values were below the critical micellar concentration (CMC) of each surfactant [28], thereby indicating that the monomers are responsible for the hemolytic activity, and that the presence of micelles is not a prerequisite for surfactant-mediated cell lysis.

### 3.2. pH-dependent hemolysis

The capacity of amino acid-based surfactants with distinct counterions to disrupt lipid bilayer membranes was studied. For this purpose, a hemolysis assay was used, with the erythrocyte membrane serving as a model for the endosomal membrane [8,17]. The hemolytic activity of the surfactants was studied as a function of pH over a range of concentrations (100–800 $\mu g\ ml^{-1}$) depending on the product tested. At low concentrations (below the $HC_{50}$ determined at pH 7.4 for each surfactant), the surfactants showed negligible hemolytic activity at pH 8.0 and 7.4 (Fig. 3a). When the pH was decreased to 6.5, the surfactants were also almost non-hemolytic, resulting in a maximum hemolysis value of 5.57%. At pH 5.4, the hemolytic activity of the surfactants increased considerably ($p < 0.05$), reaching maxima of between 65.31% and 84.69%, with the following ranking for the $HC_{50}$ values: 77KT < 77KK < 77KS < 77KP < 77KL. Endosomal compartments have a pH of 5.5–6.8, while the pH in the lysosome is ~5.0 [2,21,36]. Therefore, this pH-responsive membrane disruption around pH 5.4, which is in the pH range characteristic of late endosomes, is favorable for intracellular trafficking of therapeutic compounds. Lytic activity at the level of late endosomes should facilitate membrane destabilization, and allow surfactant–drug complexes to escape to the cytoplasm for efficient intracellular drug delivery. Gene delivery vehicles that exhibit specific membrane lytic activity at pH 5.5 have higher transfection efficiency than vehicles that destabilize membranes at pH 6.0–6.5 [21]. Moreover, surfactants with high hemolytic activity at pH 5.4 gave the best siRNA delivery efficiency in U87-luc cells [8]. Furthermore, it was observed that the commercial surfactant SDS showed no pH-sensitive activity, and thus did not facilitate endosomal destabilization.

At concentrations in the range of $HC_{50}$, surfactants presented higher hemolytic activity at pH 8.0 and 7.4, with a concomitant marked rise at pH 6.5, where almost total hemolysis was recorded, demonstrating that at high concentrations the surfactants do not retain pH-responsive behavior (Fig. 3b). These findings show that an increase in surfactant concentrations also caused membrane disruption within the pH range characteristic of early endosomes. However, these concentrations may present toxic effects, as determined by the cytotoxicity assays performed in the 3T3 fibroblast cell line (data shown in Section 3.6), and also as reported in previous cytotoxicity studies in 3T6 fibroblasts and NCTC 2544 keratinocyte cell lines [25].

A strong correlation has been reported between hemolytic activity and endosomal disruption by membrane-disruptive agents [6]. The lysine-based anionic surfactants with pH-sensitive

**Table 1**
$HC_{50}$ values of the surfactants at the different pH under investigation.

| | $HC_{50}$ ($\mu g\ ml^{-1}$) (mean[a] ± SE) | | | |
|---|---|---|---|---|
| Surfactants (MW) | pH 8.0 | pH 7.4 | pH 6.5 | pH 5.4 |
| 77KK (545.7) | 370.99 ± 15.84 | 361.22 ± 9.34 | 344.51 ± 13.51 | 211.00 ± 10.35[b,c,d] |
| 77KT (519.7) | 729.54 ± 15.61 | 651.53 ± 2.55 | 593.96 ± 5.99[c] | 290.32 ± 11.69[b,c,d] |
| 77KP (437.6) | 285.07 ± 0.69 | 269.01 ± 10.51 | 276.51 ± 11.81 | 145.38 ± 11.83[b,c,d] |
| 77KS (421.5) | 298.90 ± 7.79 | 284.10 ± 13.01 | 250.89 ± 6.17 | 158.51 ± 1.43[b,c,d] |
| 77KL (405.6) | 489.22 ± 20.39 | 440.12 ± 10.23 | 382.23 ± 3.81 | 139.68 ± 8.77[b,c,d] |
| SDS (288.4) | 44.82 ± 1.54 | 45.53 ± 1.48 | 43.01 ± 2.31 | 44.93 ± 3.00 |

[a] Mean ± SE of three experiments.
[b] Significantly different from the pH 7.4 condition (Bonferroni's post hoc test, $p < 0.05$).
[c] Significantly different from the pH 8.0 condition (Bonferroni's post hoc test, $p < 0.05$).
[d] Significantly different from the pH 6.5 condition (Bonferroni's post hoc test, $p < 0.05$).

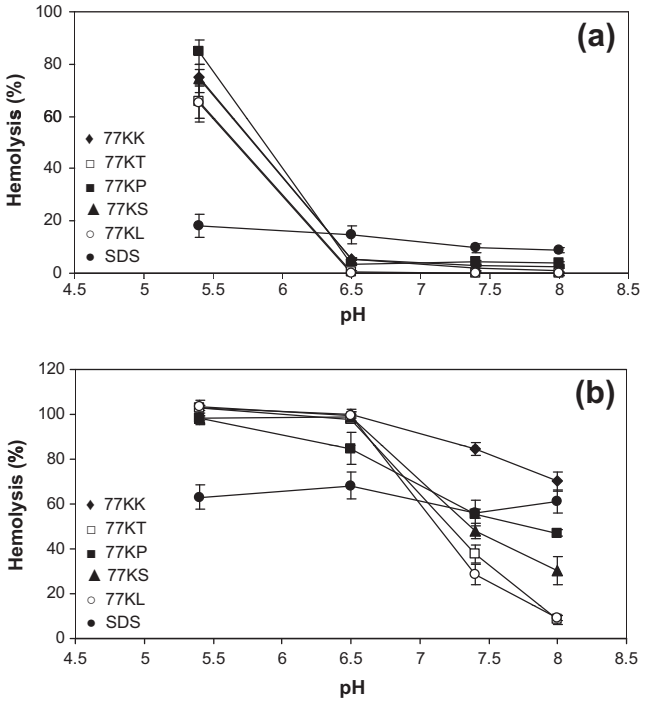


**Fig. 3.** Surfactant-induced hemoglobin release from rat erythrocytes as a function of pH. Surfactants were added from 10 or 1 mg ml$^{-1}$ (SDS) stock solutions to give final concentrations of: (a) 77KK 250 μg ml$^{-1}$, 77KT 300 μg ml$^{-1}$, 77KP, 77KS and 77KL 200 μg ml$^{-1}$, or SDS 30 μg ml$^{-1}$; and (b) 77KK 400 μg ml$^{-1}$, 77KT 650 μg ml$^{-1}$, 77KP and 77KS 300 μg ml$^{-1}$, 77KL 450 μg ml$^{-1}$ or SDS 50 μg ml$^{-1}$. Each point represents the mean of three independent experiments ± SE (error bars).

properties were studied on the basis of the hypothesis that pH-responsive membrane lytic activity facilitates the escape of drug-delivery systems from endosomal compartments. Numerous amphiphilic materials have been found to mediate material transport across cell membranes, such as amphiphilic peptide sequences used by viruses [37], and amphiphilic lipids, such as DOPE [38]. However, virus peptides may have problems of immunogenicity, and lipids may not have the capacity of selective membrane disruption at the endosomal pH. A useful intracellular drug delivery system is required to have low lytic activity at physiological pH and high destabilizing activity in the mildly acidic conditions found in the endosomes, which will cause only selective endosomal membrane disruption [8,15].

Noteworthy is the significant increase in the hemolytic activity observed at pH 5.4 for all the surfactants independently of their counterions. This increase could be explained by a modification in the hydrophobic/hydrophilic balance of these compounds at this pH. The p$K_a$ value of the carboxylic group of the lysine amino acid is reported to be of the order of 2.2 [39] and, therefore, it was initially assumed that, in the pH range tested, there would be no changes in the protonation of this group or in the hydrophobicity of the molecule. However, taking into account the enhanced membrane lysis at pH 5.4, it was assumed that the carboxylic group of the amino acid lysine undergoes an increase in its p$K_a$ value when it is included in the surfactant molecule. This increase could lead to changes in the protonation state of the surfactants in the late endosome pH range, and thus further increase binding to the membrane and enhance the hemolytic activity. This supposition is based on the studies by Pinazo et al. [40], in which the guanidine group of the amino acid arginine was observed to undergo a strong alteration in its original p$K_a$ value when included in the surfactant molecule. Moreover, the enhanced hemolysis at pH 5.4 could also be attributed to modifications in the surface potential of the lipid bilayer after cell exposure to low pH [41]. These altered properties could enhance the interaction of the surfactants with such a modified bilayer.

Moreover, compounds containing counterions differ in their capacity to interact with biological and model membranes [42], and the type of counterion is one of the factors that determine the efficiency of the interaction of a compound with membranes [43,44]. Counterions within the polar domains of phospholipids have been shown to modulate lipid dynamics in numerous models [45,46]. Therefore, given that the lysine surfactants have the same chemical structure, differing only in the type of counterion, the characteristics of the counterion may be crucial to the pH-sensitive membrane disruptive activity. Hemolysis studies of several surfactants reported that hemolytic effects are dependent on both the structure (polar head dimension and alkyl chain length) and form (the type of counterion) of the compound [47]. As demonstrated above, the inorganic counterions facilitated the interaction with the membrane and produced stronger hemolysis in the late endosome pH range. The difference in the effects of the surfactants with inorganic counterions with the same valence could be explained by their different binding to charged surfaces resulting from their distinct polarizability or hydration and mobility [44]. Among the surfactants with organic counterions, the higher hemolytic activity of 77KK could be attributed to the presence of two protonatable amino groups in the lysinium counterion compared with only one in the Tris counterion, which could favor the amphiphilicity of this compound upon reduction of pH below the p$K_a$ ranges. When more amino groups are protonated, the surfactants become more amphiphilic, which results in stronger hemolysis [8,10]. Thus, it has been demonstrated that the pH-sensitive membrane disruptive activity of these surfactants can be tuned by varying the nature of the counterion.

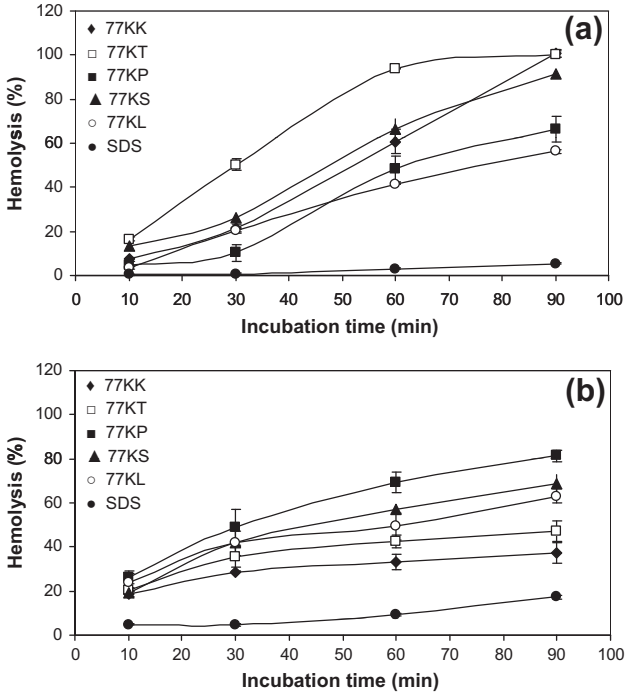


**Fig. 4.** Time dependence of the lysine-based surfactant-induced hemolysis at pH 6.5 and 5.4. Surfactants were added at time zero from 10 or 1 mg ml$^{-1}$ (SDS) stock solutions to give final concentrations of: (a) pH 6.5: 77KK and 77KL 300 μg ml$^{-1}$, 77KT 500 μg ml$^{-1}$, 77KP and 77KS 200 μg ml$^{-1}$ or SDS 20 μg ml$^{-1}$; and (b) pH 5.4: 77KK, 77KP, 77KS and 77KL 150 μg ml$^{-1}$, 77KT 200 μg ml$^{-1}$ or SDS 20 μg ml$^{-1}$. Each point represents the mean of three independent experiments ± SE (error bars).

### 3.3. Time-dependent hemolysis

Induction of hemolysis by the surfactants was also determined as a function of time at the pH range characteristic of early and late endosomes (pH 6.5 and 5.4, respectively). The concentrations evaluated (200–500 μg ml$^{-1}$) were in the range of those that present specific pH-dependent activity, and were slightly adjusted to achieve time-dependent hemolysis. All the surfactants showed improved kinetic hemolytic activity in the endosomal pH range (Fig. 4). At pH 6.5 (Fig. 4a), the presence of the compounds caused a low degree of hemolysis up to 17% after 10 min of incubation, followed by a sharp increase in the degree of hemolysis to a maximum between 56% and 100% after 90 min. At pH 5.4, these concentrations resulted in a high degree of hemolysis after 10 min of incubation. As it was not possible to evaluate the time-dependent hemolysis with the same concentration range, low concentrations were assessed (150–200 μg ml$^{-1}$) (Fig. 4b). Despite the reduced kinetic hemolytic activity, time-dependent hemolysis was also observed, reaching a maximum (between 37% and 82%) after 90 min of incubation. This significant hemolytic kinetics indicates that one or more previous steps are required before the erythrocyte membrane becomes permeable to hemoglobin. Among these previous steps, the most important could be the formation of pores or channels, which lead to the efflux of low molecular weight solutes, as discussed in Section 3.4.1. The commercial surfactant SDS did not show significant time-dependent hemolysis in the endosomal pH range. This observation thus confirms the non-pH-responsive activity of this surfactant.

The molecules taken up by endocytosis are trafficked from early endosomes to lysosomes within several hours [16]. Thus, given the improved kinetics of the hemolytic activity shown by the lysine-based surfactants, these compounds could enable the disruption of endosomal membranes before fusion of the endocytic vesicles with lysosomes, thus avoiding non-productive intracellular trafficking, a critical feature for potential intracellular drug delivery applications.

### 3.4. Mechanism of cell membrane disruption

#### 3.4.1. Osmotic protection

Two processes have been proposed to explain cell lysis by surfactants. Hemolysis may be caused by direct disruption of the membrane through complete or partial solubilization of membrane lipids and proteins by the formation of mixed micelles (which usually occurs at high surfactant/membrane ratios) or by the intercalation of the compounds into the membrane and consequently the change in its permeability (which is characteristic of low surfactant concentrations, under the CMC) [48]. As the concentration range studied here was below the surfactant CMC [28], it was assumed that cell lysis and hemoglobin leakage could occur through the colloid osmotic mechanism. To corroborate this hypothesis, an osmotic protection experiment was conducted in suspensions of PBS solutions containing molecules of varying sizes. The rationale behind this experiment was that, since sufficiently large molecules do not permeate through the cell membrane, their presence would counteract the effect of the macromolecules inside

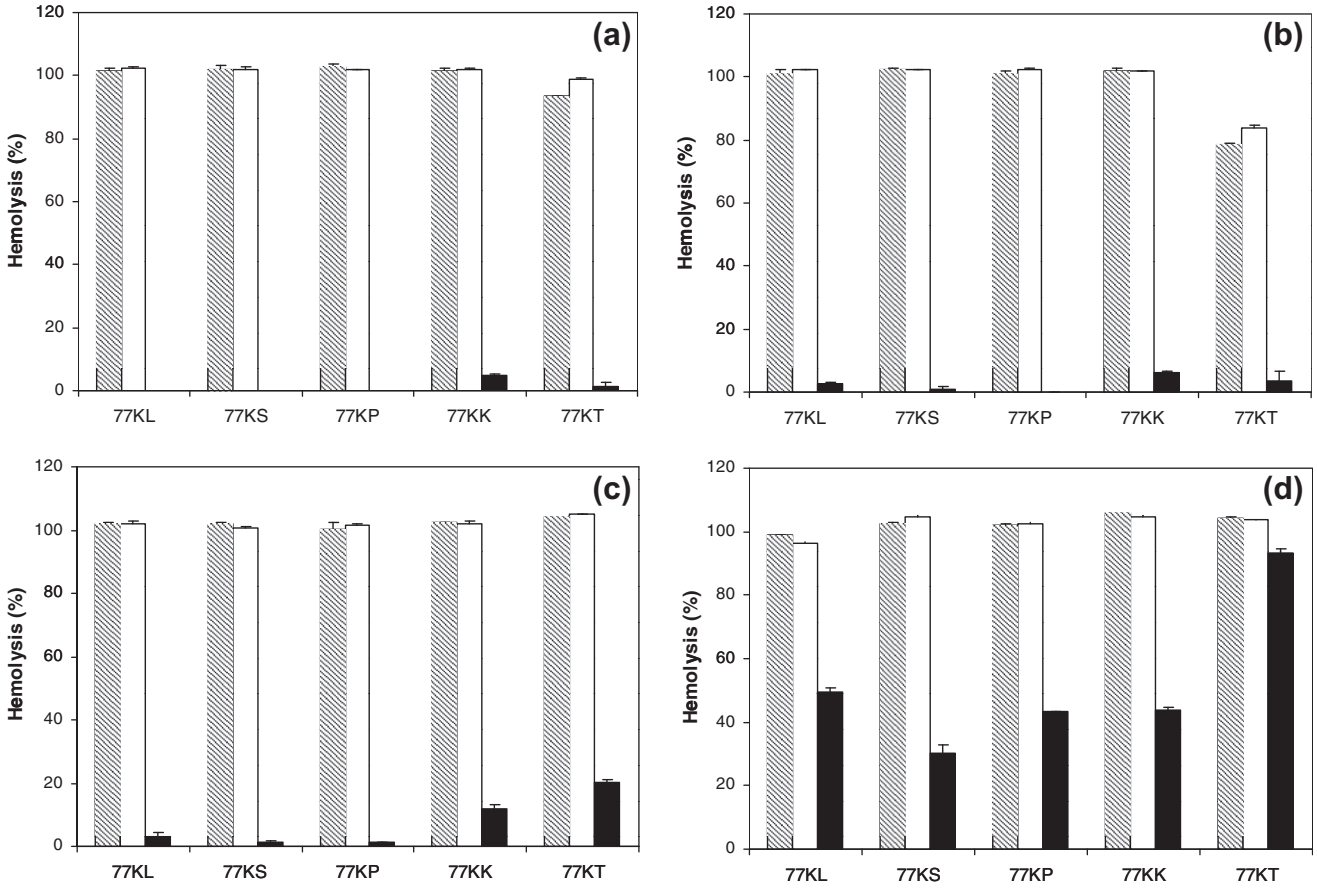


**Fig. 5.** Effects of osmotic protectants PEG-10,000 (10 kDa) and D-glucose (180 Da) on the lysine-based surfactant-induced hemolysis of rat erythrocytes: (a) pH 7.4; (b) pH 8.0; (c) pH 6.5; (d) pH 5.4. PBS alone (striped), PBS + D-glucose (blank) and PBS + PEG-10,000 (black). Surfactants were added at a range of final concentrations: 77KL 600 μg ml$^{-1}$, 77KS 400 μg ml$^{-1}$, 77KP 400 μg ml$^{-1}$, 77KK 600 μg ml$^{-1}$ and 77KT 800 μg ml$^{-1}$. The data represent the mean of three independent experiments. All the hemolysis results obtained in the presence of the osmolyte PEG 10,000 were significantly different from the control in PBS solution alone (Dunnett's post hoc test, $p < 0.05$). The data represent the mean of three independent experiments ± SE (error bars).

the cell (especially hemoglobin) and diminish water penetration into it and, consequently, cell swelling [19].

PEG-10,000 significantly inhibited hemolysis by the surfactants throughout the pH range studied, thereby indicating that defects such as pores or channels are formed in the erythrocyte membrane (Fig. 5). Collapse of transmembrane potential and osmotic swelling are consequences of pore formation and lead to red blood cell lysis [1]. The resulting osmotic imbalance was restored by adding high molecular weight PEG-10,000, which does not diffuse through the pores or channels formed in the membrane, thus leading to a marked decrease in the hemolysis. This decrease was considered evidence of the presence of the osmotic mechanism [49], and as a further indication that membrane lysis is a complex process and that one or more previous steps, such as pore formation and the efflux of low molecular weight solutes, are required before the erythrocyte membrane becomes permeable to hemoglobin. Corroborating the osmotic mechanism, the low molecular weight D-glucose had no effect on the osmotic imbalance caused by the hemoglobin trapped inside the erythrocyte cytoplasm, and therefore no protection was observed, and the degree of hemolysis did not differ significantly ($p > 0.05$) from that caused by the same surfactant concentration in PBS alone. Osmotic protection at pH 5.4 was less significant than at the other pH values tested (Fig. 5d). This observation indicates that, in this case, the osmotic cell swelling is not the only mechanism involved in membrane lysis, and a more complex mechanism is operating. Given the enhanced hemolytic activity observed at pH 5.4, cell membrane disruption could also be due to partial solubilization of membrane lipids and proteins through micellization, caused by extensive surfactant adsorption. Moreover, it is also feasible that the opening of large pores is sufficient to release hemoglobin molecules [49]. Previous studies on the commercial surfactant SDS suggested that this compound opens large pores sufficiently to allow hemoglobin release, or even that it can cause direct disruption of the membranes. Thus, SDS does not present the colloid-osmotic mechanism of hemolysis [48,49].

### 3.4.2. Red blood cell count

Red blood cell counts were performed in order to study whether hemoglobin passes through the cell membrane, or whether it is released into the extracellular medium after rupture of the cell membrane. It has been noted that the overall cell numbers were reduced significantly after 10 min of incubation with a hemolytic concentration of each surfactant throughout the pH range tested, and the percentages of lysed erythrocytes are in agreement with the hemolytic activity results (Fig. 6). These results indicate that hemoglobin is released into the PBS buffer after lysis of cell membranes and is not related to pore formation or increased cell membrane permeability.

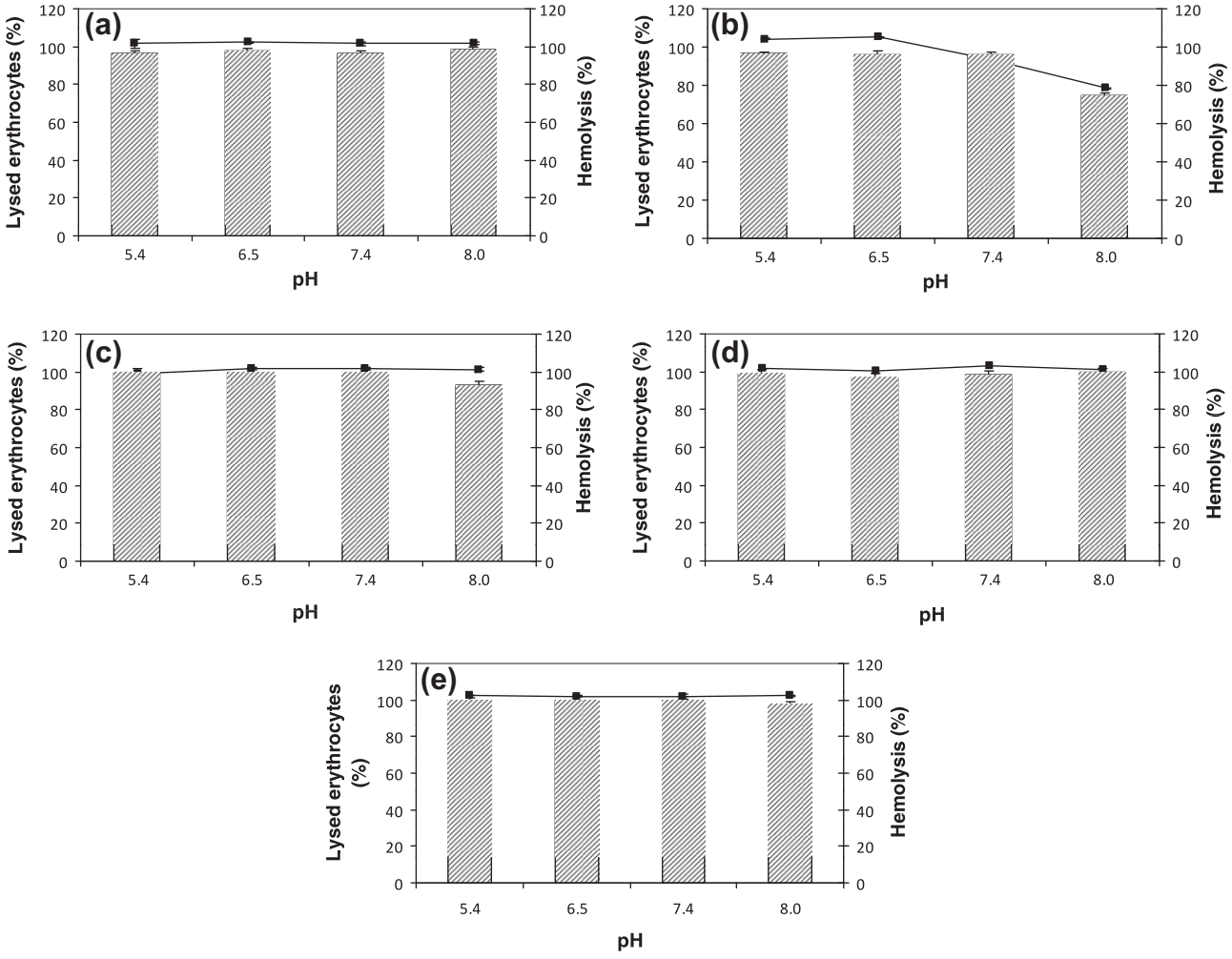


**Fig. 6.** Comparison between the hemolysis (line) and the percentage of lysed rat erythrocytes (column) by the lysine-based surfactants at the pH range tested. Surfactants were added at the following range of final concentrations: (a) 77KK 600 μg ml$^{-1}$; (b) 77KT 800 μg ml$^{-1}$; (c) 77KL 600 μg ml$^{-1}$; (d) 77KP 400 μg ml$^{-1}$; (e) 77KS 400 μg ml$^{-1}$. The data represent the mean of three independent experiments ± SE (error bars).

### 3.5. SEM studies of rat erythrocyte morphology

The capacity of human erythrocytes to maintain their biconcave disk shape is governed by the structural properties of the membrane [41]. A variety of phenomena can induce normal discocytes to undergo transformation to crenated (echinocytic) or cupped (stomatocytic) shapes: for example, ATP depletion, changes in pH, exposure to glass surfaces, $Ca^{2+}$ loading, metabolic depletion or incubation with certain amphiphilic compounds [50]. For better understanding of the effect of the pH of lysine-based surfactants on the erythrocyte membrane structure and cell lysis, SEM studies of rat erythrocytes were performed. Erythrocytes were treated with a

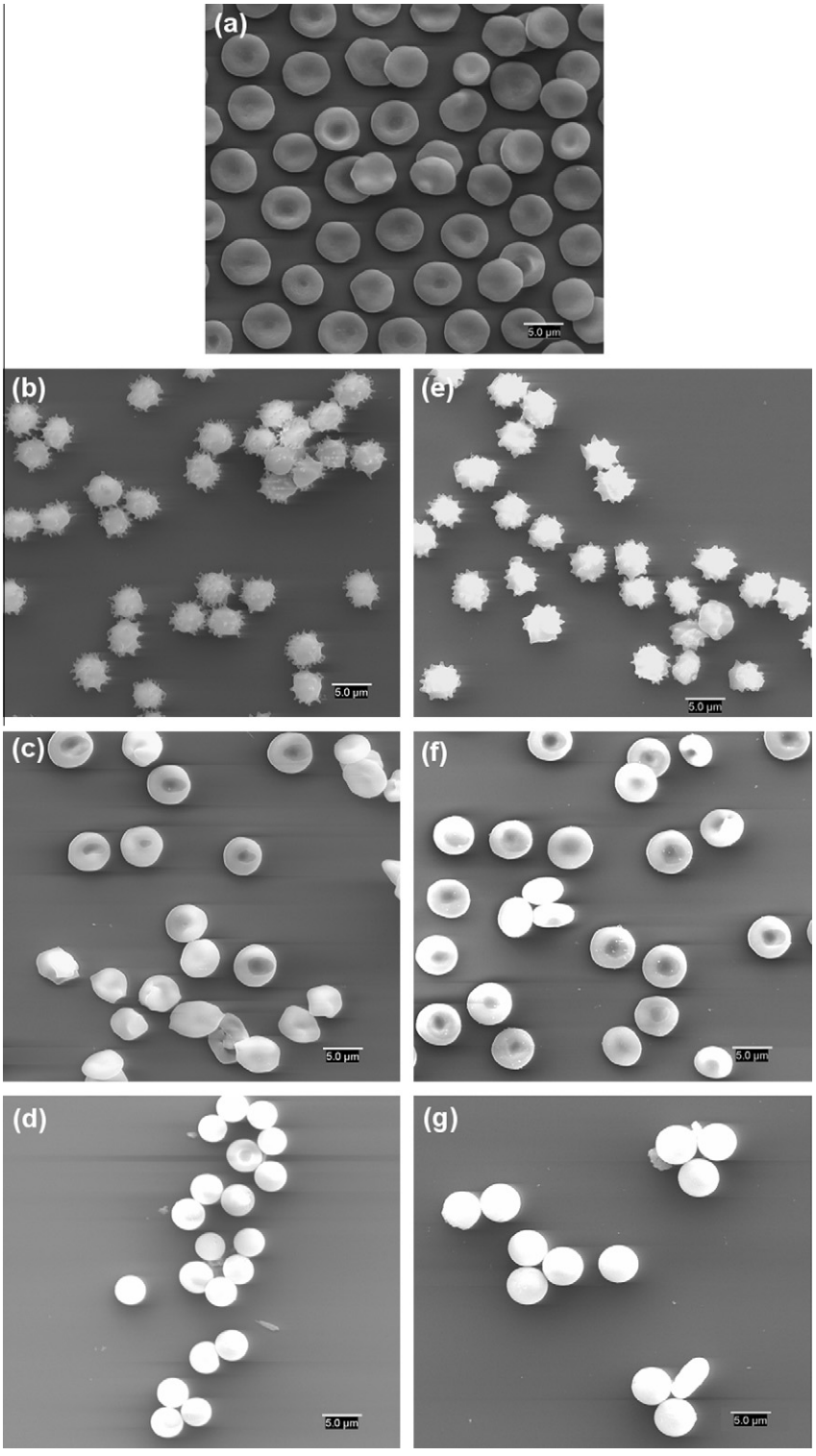


**Fig. 7.** Effect of the lysine-based surfactants on rat erythrocytes morphology. SEM images of control in PBS pH 7.4 (a), and incubated with the surfactant with organic counterion 77KK at (b) pH 7.4, (c) pH 6.5 and (d) pH 5.4; and with the surfactant with inorganic counterion 77KS at (e) pH 7.4, (f) pH 6.5 and (g) pH 5.4. The erythrocytes were incubated for 10 min with a concentration of 100 μg $ml^{-1}$ of each surfactant. Scale bars correspond to 5 μm.

sub-lytic concentration of 100 μg ml$^{-1}$ of the surfactants in the 5.4–8.0 pH range. The results indicated that the surfactants altered the normal biconcave morphology of the cells (Fig. 7). Control erythrocytes incubated in PBS solution at the pH values studied (8.0, 7.4, 6.5 and 5.4) were also evaluated and were found to be discoid or slightly echinocytic. This shape is considered normal in erythrocytes isolated in buffer and in the absence of albumin [51,52]. Fig. 7a shows the control, which consisted of erythrocytes incubated with PBS alone at pH 7.4.

Regardless of the varying capacity of the surfactants to exert membrane lytic activity, both those with organic and those with inorganic counterions induced the same changes in erythrocyte morphology throughout the pH range studied. According to the bilayer couple hypothesis [50], the changes induced in erythrocyte shape by foreign molecules are due to the differential expansion of their two monolayers. The SEM micrographs shown in Fig. 7 correspond to 77KK and 77KS, the surfactants with organic and inorganic counterions, respectively. Morphological analysis revealed that the erythrocytes underwent alteration after treatment with these two surfactants at pH 7.4 (Fig. 7b and e, respectively). These compounds changed the discoid shape of cells (Fig. 7a) to echinocytic, an altered condition in which the erythrocytes show a spiny configuration, exhibiting blebs or protuberances on their surfaces. At pH 8.0, the compounds also induced an echninocyte type of deformation (data not shown). Echinocytes are induced when the compound added is inserted in the outer monolayer of the membrane [50]. This morphological change may be the result of the incapacity of amphipathic molecules to cross the bilayer. These results are consistent with the bilayer hypothesis [50], which proposes that, general speaking, anionic amphiphiles induce echinocytes at physiological pH as a result of the electric repulsion between the negative charge of the molecule and the acidic phospholipids, the latter having negative charges under the physiological conditions and localizing in the inner layer of the lipid bilayer.

In contrast, in mildly acidic media, the surfactants interacted with the phospholipid bilayer in another way. At pH 6.5, they induced a stomatocyte-type deformation, causing the erythrocytes to show a cup-shaped form with evagination of one surface and a deep invagination of the opposite face. These morphological changes are shown in Fig. 7c and f, for 77KK and 77KS, respectively. Stomatocytes arise when the compound accumulates in the inner monolayer. This accumulation may support the slightly increased hemolytic potency shown by the surfactants at this pH, given that a deeper penetration into the membrane took place. Finally, for 77KK and 77KS, respectively, at pH 5.4, most of the cells showed swollen forms (spherocytes), which consisted of echinocytes or stomatocytes, on increasing the incorporation of the products into the membrane [50] (Fig. 7d and g). These findings indicate that these compounds were equally located in the outer and the inner moieties of the red cell membrane, supporting the findings regarding the significantly increased hemolysis at pH 5.4, as a higher interaction (both in the outer and inner moieties of the bilayer) of the surfactants within the cell membrane could be associated with the enhanced hemolytic response. Moreover, one can assume that in mildly acidic conditions the surface potential of the lipid bilayer is positive and favors the electrostatic attraction of the anionic compound, thus also inducing its intercalation in the inner layer of this modified bilayer [41].

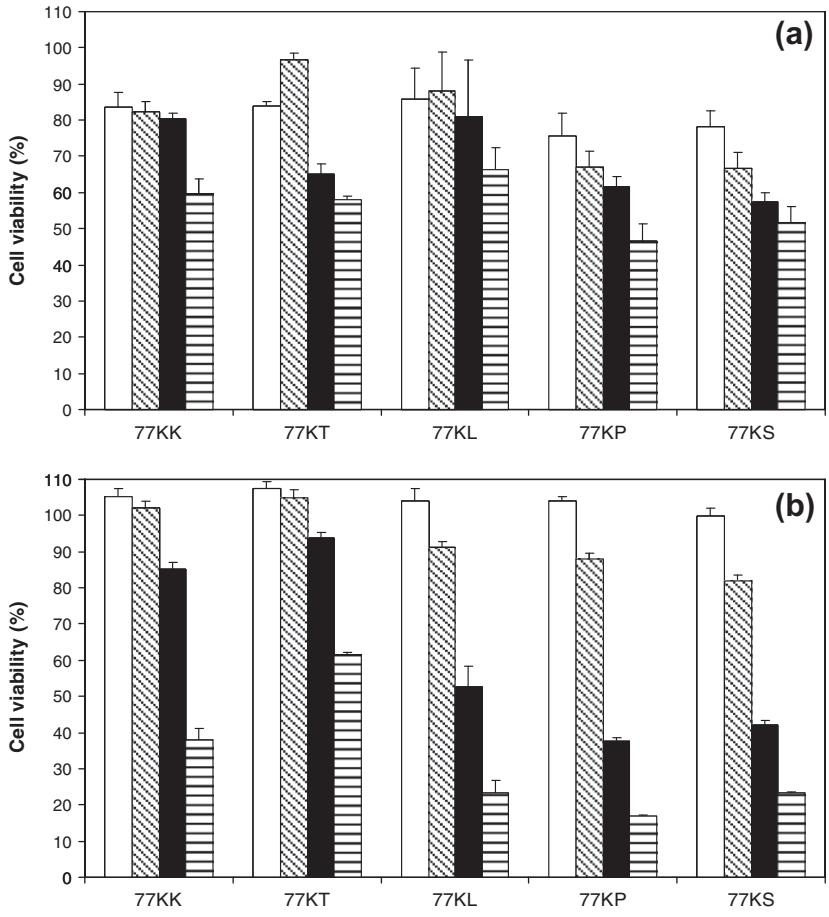


**Fig. 8.** Surfactant cytotoxicity on 3T3 cells as a function of concentration, as determined by (a) MTT and (b) NRU colorimetric assays at (from left to right) 150 μg ml$^{-1}$ (blank), 200 μg ml$^{-1}$ (striped), 250 μg ml$^{-1}$ (black) and 300 μg ml$^{-1}$ (squared). The data represent the mean of three independent experiments ± SE (error bars).

Therefore, in addition to the physico-chemical characteristics of the surfactants, the difference in the transbilayer distribution and mobility are crucial for hemolytic potency. Of note, the increase in negative charges on the lipid bilayer as a result of the distribution of the surfactants may modify the electrostatic and hydration properties of the phospholipid molecules, which determine the correct function of the membrane. The location of the surfactants in the bilayer generates a reorganization of water molecules, which in turn affects phospholipid packing. These observations may also explain the changes in the normal biconcave shape of the red blood cells [53]. The hypothesis that the surfactants bind on the membrane surface was also confirmed by the results obtained by Martínez et al. [54] using fluorescence anisotropy measurements. That study demonstrated that almost all the lysine-based surfactants disturbed the external region of the erythrocyte membrane at physiological pH. In addition to the phospholipid bilayer interactions, surfactants may disrupt the structure of the cytoskeleton, a protein responsible for the maintenance of the biconcave shape of erythrocytes [55]. Finally, it has been proposed that the anion-exchange protein band 3 plays a critical role in determining erythrocyte shape [56,57]. Thus, surfactant-band 3 could be involved in the morphological changes undergone by the erythrocytes in response to surfactants.

### 3.6. Cytotoxicity assays

The cytotoxic effects of the lysine-based surfactants were evaluated with colorimetric assays which measure the capacity of live cells to take up the NR dye (NRU assay) and to metabolize a tetrazolium colorless salt to a blue formazan (MTT assay) as indirect measurements of cell viability. The cytotoxicity assays were performed in the concentration range in which the surfactants presented pH-sensitive membrane lytic activity (150–300 $\mu g\ ml^{-1}$). The compounds showed some cytotoxicity towards 3T3 cells, which displayed viability in the range 96.64–46.44% by the MTT assay (Fig. 8a) and 107.61–16.93% by the NRU assay (Fig. 8b) at the surfactant concentration range tested. The concentrations at which each surfactant presents specific pH-dependent activity were reported in Section 3.2 (Fig. 3a). For 77KK and 77KL, these concentrations (250 and 200 $\mu g\ ml^{-1}$, respectively) induced only mild cytotoxicity (80.51% and 87.87% cell survival by the MTT assay, and 85.03% and 91.34% cell survival by the NRU assay, respectively), and thus could be considered suitable for drug delivery systems. The MTT assay showed that the surfactants 77KT, 77KS and 77KP presented more significant cytotoxic effects, with cell viability falling below 70% within the concentrations that demonstrated marked pH-responsive activity. In contrast, from the NRU assay, it was observed that 77KS and 77KP also displayed viabilities of ~85% with the 200 $\mu g\ ml^{-1}$ concentration, while higher concentrations showed lower cell viabilities in comparison with the MTT assay. These results suggest different toxicity mechanisms for each surfactant, as the two cytotoxicity assays are based on different endpoints. In order to avoid overestimation or underestimation of the toxicity, the combination of cytotoxicity assays are important to increase the reliability of the results.

## 4. Conclusions

The lysine-based surfactants showed concentration-dependent and pH-sensitive hemolytic activity, with a significant increase in hemolysis at pH 5.4. The range of membrane–lytic profiles demonstrated that the counterions make a critical contribution to the interaction of these compounds with the phospholipid bilayer. The improved kinetics of the hemolytic activity of the surfactants supports their capacity to disrupt endosomal membranes before vesicular evolution from endosomes to lysosomes. Furthermore, the decrease in the rate of hemolysis by PEG 10,000 was considered evidence of the colloid osmotic mechanism in cell membrane disruption. SEM studies on rat erythrocytes showed that the surfactants interacted with the phospholipid bilayer to induce shape changes, forming echinocytes at pH 7.4, stomatocytes at pH 6.5, and spherocytes at pH 5.4. The surfactants 77KK and 77KL were also well tolerated by 3T3 cells at surfactant concentrations which present specific pH-dependent activity, and thus appear promising as endosomal disruptive agents with specificity for late endosome stages. This study on anionic lysine-based surfactants provides a foundation for further study of the therapeutic applications of these lysine-based surfactants. On the basis of recent studies which reported the preparation of biocompatible devices with amino acid-based surfactants for the controlled encapsulation and release of DNA [11], the present authors' research is now focused on the development of biocompatible lysine-based surfactant conjugates as potential drug delivery systems for pharmaceutical applications.

## Acknowledgements

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## Artículo 2

**MEMBRANE-DESTABILIZING ACTIVITY OF pH-RESPONSIVE CATIONIC LYSINE-BASED SURFACTANTS: ROLE OF CHARGE POSITION AND ALKYL CHAIN LENGTH**

**(Actividad desestabilizadora de membrana de tensioactivos catiónicos pH-sensibles derivados de lisina: efecto de la posición de la carga y de la longitud de la cadena alquílica)**

**Daniele Rubert Nogueira, Montserrat Mitjans, M. Carmen Morán, Lourdes Pérez, M. Pilar Vinardell**

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# Resumen

*Objetivos*

Estudiar la actividad hemolítica pH-dependiente de tensioactivos catiónicos derivados del aminoácido lisina, utilizando el eritrocito como modelo de membrana endosomal. Además, se propuso valorar el efecto de las características estructurales de los tensioactivos, como hidrofobicidad y posición de la carga catiónica, en su potencia hemolítica.

*Material y métodos*

Se estudió un grupo de tensioactivos catiónicos derivados de lisina del tipo sales de hidrocloruro de $N^{\alpha}$ o $N^{\varepsilon}$-acil lisina metil éster que difieren en cuanto a la longitud de la cadena hidrocarbonada y a la posición de la carga catiónica. También se evaluó el tensioactivo comercial bromuro de hexadecil trimetilamonio (HTAB) con fines comparativos. El ensayo de hemólisis se utilizó para la valoración de la interacción de estos compuestos bioactivos con las membranas celulares. Los eritrocitos se aislaron de sangre de rata y se utilizaron como modelo de la membrana de los endosomas. Se prepararon disoluciones de solución amortiguadora de fosfato (PBS) a diferentes pH (7,4; 6,5 y 5,4), reproduciendo las condiciones fisiológicas y la de los endosomas primarios y secundarios, respectivamente. Se estudió la habilidad de los tensioactivos en romper la membrana de los eritrocitos en función de la concentración, del pH del medio y del tiempo de incubación. Por último, también se evaluaron los mecanismos de interacción de los tensioactivos con la membrana celular, a través del estudio de la morfología de los eritrocitos por microscopía electrónica de barrido (SEM), del efecto de compuestos de diferentes pesos moleculares en el proceso de ruptura de la membrana y del recuento de los eritrocitos por microscopía óptica, como parámetro indicativo de la lisis celular.

*Resultados*

Los tensioactivos que tienen la carga positiva en el grupo α-amino de la lisina presentaron actividad lítica pH-dependiente, mientras que el compuesto con la carga en el grupo ε-amino de la lisina y el HTAB no presentaron actividad hemolítica sensible al cambio de pH del medio. Además, sólo los compuestos con la carga en el grupo α-amino mostraron cinética mejorada de su actividad hemolítica en los valores de pH característico de los endosomas. El aumento en la hidrofobicidad de la molécula del tensioactivos disminuyó su potencial lítico de membrana en todo el rango de pH estudiado. Las evaluaciones del mecanismo de ruptura de la membrana demostraron que el proceso de hinchazón osmótica de la célula no es el único mecanismo involucrado en su lisis. Además, a través del recuento de los eritrocitos, se comprobó que la membrana celular realmente se rompió tras el tratamiento con los tensioactivos. Los estudios mediante SEM mostraron diferentes cambios en la morfología normal de las células tras el tratamiento con los tensioactivos, lo que corrobora que los diferentes grados de interacción de estos compuestos con la membrana celular dependen de sus características estructurales y del pH del medio.

*Conclusiones*

La actividad hemolítica pH-dependiente puede ser ajustada variando las características estructurales de los tensioactivos. Los resultados obtenidos demostraron que los tensioactivos catiónicos derivados de lisina con la carga positiva en el grupo α-amino de la lisina son potenciales excipientes bioactivos en el desarrollo de vehículos para la liberación intracelular de biomoléculas.

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ORIGINAL ARTICLE

# Membrane-destabilizing activity of pH-responsive cationic lysine-based surfactants: role of charge position and alkyl chain length

**Daniele Rubert Nogueira · Montserrat Mitjans · M. Carmen Morán · Lourdes Pérez · M. Pilar Vinardell**

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**Abstract** Many strategies for treating diseases require the delivery of drugs into the cell cytoplasm following internalization within endosomal vesicles. Thus, compounds triggered by low pH to disrupt membranes and release endosomal contents into the cytosol are of particular interest. Here, we report novel cationic lysine-based surfactants (hydrochloride salts of $N^{\varepsilon}$- and $N^{\alpha}$-acyl lysine methyl ester) that differ in the position of the positive charge and the length of the alkyl chain. Amino acid-based surfactants could be promising novel biomaterials in drug delivery systems, given their biocompatible properties and low cytotoxic potential. We examined their ability to disrupt the cell membrane in a range of pH values, concentrations and incubation times, using a standard hemolysis assay as a model of endosomal membranes. Furthermore, we addressed the mechanism of surfactant-mediated membrane destabilization, including the effects of each surfactant on erythrocyte morphology as a function of pH. We found that only surfactants with the positive charge on the α-amino group of lysine showed pH-sensitive hemolytic activity and improved kinetics within the endosomal pH range, indicating that the positive charge position is critical for pH-responsive behavior. Moreover, our results showed that an increase in the alkyl chain length from 14 to 16 carbon atoms was associated with a lower ability to disrupt cell membranes. Knowledge on modulating surfactant-lipid bilayer interactions may help us to develop more efficient biocompatible amino acid-based drug delivery devices.

**Keywords** Lysine-based surfactants · Hemolysis · pH-sensitivity · Membrane disruption · Drug delivery

**Abbreviations**

| | |
|---|---|
| CMC | Critical micellar concentration |
| $HC_{50}$ | Surfactant concentration that induces 50% hemolysis |
| HTAB | Hexadecyl trimethyl ammonium bromide |
| MKM | $N^{\varepsilon}$-myristoyl lysine methyl ester |
| MLM | $N^{\alpha}$-myristoyl lysine methyl ester |
| PBS | Phosphate buffered saline |
| PEG | Polyethylene glycol |
| PKM | $N^{\varepsilon}$-palmitoyl lysine methyl ester |
| SEM | Scanning electron microscopy |
| SEM | Standard error of the mean |

## Introduction

Advances in strategies for treating a wide variety of diseases require the efficient delivery of the active compound into the cytosol or nucleus of target cells (Hu et al. 2007). Therapeutic agents, such as proteins, peptides, DNA and antitumor drugs, act at intracellular sites, thus their therapeutic efficacy depends on efficient intracellular trafficking (Plank et al. 1998). Cells usually take up drug carriers via endocytosis that confines the internalized active compounds to vesicles (endosomes). Therefore, one of the

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challenges for efficient intracellular delivery of therapeutic compounds is to facilitate their release into the cytosol by destabilizing endosomal membranes under mildly acidic conditions (Chen et al. 2009; Stayton et al. 2000). This would manipulate or circumvent the non-productive trafficking from endosomes to lysosomes, thereby avoiding degradation at a pH as low as 4.6 (Mellman 1996).

Over the past two decades, considerable research has focused on delivery systems that specifically destabilize endosomal membranes following endocytic uptake (Christie and Grainger 2003). Carriers based on attenuated viruses have been studied extensively as pH-dependent membrane-disruptive components in gene delivery systems to enhance transport from endosomes to the cytosol. However, clinical use of these carriers is limited by their antigenicity and toxicity (Gordon and Anderson 1994; McTaggart and Al-Rubeai 2002; Temin 1990). Safety issues have prompted the development of synthetic peptides structurally derived from viruses to specifically disrupt endosomal membranes (Plank et al. 1998), but these peptides are also likely to be immunogenic in vivo (Sandhu et al. 1997). To overcome these limitations, a variety of non-viral delivery vectors has been studied, such as synthetic surfactants and polymers. Anionic lysine-based surfactants exhibit pH-responsive membrane-lytic activity in the late endosomal pH range, thus showing promise for intracellular drug delivery systems (Nogueira et al. 2011). Polymerizable surfactants with tunable pH-sensitive amphiphilicity have been designed and tested as multifunctional delivery devices for systemic and targeted delivery of therapeutic siRNA (Wang et al. 2007, 2008, 2009). Moreover, cationic amino acid-based surfactants have been used to prepare biocompatible devices for the controlled encapsulation and release of DNA, where the surfactants form stable complexes with the oppositely charged DNA through electrostatic interactions (Morán et al. 2010). Cationic and anionic polymers with pH-sensitive activity, including derivatives of poly (acrylic acid) (Jones et al. 2003; Kusonwiriyawong et al. 2003; Kyriakides et al. 2002), methacrylic acid copolymers (Yessine et al. 2003), imidazole-containing polymers (Seo and Kim 2010) and pseudo-peptidic polymers (Chen et al. 2008, 2009), have also been developed to promote endosomal release. Cationic polymers have been described to enhance membrane lysis at low pH via electrostatic interactions between protonated amines and the negatively charged membranes (Yessine et al. 2003).

Surfactants are one of the most widely applied excipients in the pharmaceutical industry due to their surface and interface activities (Paulsson and Edsman 2001). Those derived from amino acids usually present biocompatible properties and low cytotoxicity, and are therefore of great interest for pharmaceutical applications, especially in the field of novel non-viral drug delivery devices (Morán et al. 2010; Pérez et al. 2009). As surface properties (hydrophobicity and surface charge) have a major impact on cellular uptake of particulate drug delivery systems, the incorporation of charged surfactants into these carriers might improve targeting to specific cells (Schöler et al. 2001). Furthermore, surfactants with pH-responsive membrane-disruptive activity may further destabilize endosomal compartments (Nogueira et al. 2011; Wang et al. 2007). The physicochemical and biological properties of cationic amino acid-based surfactants, as well as their synthesis, have been widely reported by our group (Colomer et al. 2011a; Lozano et al. 2011; Infante et al. 2010; Pérez et al. 2002, 2009). Therefore, we selected biocompatible cationic surfactants from the amino acid lysine (hydrochloride salts of $N^{\varepsilon}$- and $N^{\alpha}$-acyl lysine methyl ester) since they display lower toxicity potential and are classified as biodegradable (Pérez et al. 2009), and thus suitable for practical applications.

Here, we studied the membrane-destabilizing properties as a function of pH of three cationic lysine-based surfactants that differ in the position of the positive charge and the length of the alkyl chain. To evaluate their potential application in intracellular drug delivery systems, we examined the pH-dependent cell membrane-disruptive activity of these compounds using a standard hemolysis assay of rat erythrocytes as a model of endosomal membranes. The hemolysis dependence on the concentration and the kinetic properties of the surfactants at the endosomal pH range were also evaluated. Furthermore, we investigated the mechanisms involved in cell membrane disruption, including the effects of each surfactant on erythrocyte morphology at varying pH values. To gain insight into the structure-dependent interaction of these compounds with membrane bilayers, the influence of the charge position and alkyl chain length on hemolytic activity was also discussed.

## Materials and methods

### Reagents

All solvents were reagent grade and were used without further purification. NaCl, $Na_2HPO_4$, and $KH_2PO_4$ were supplied by Merck (Darmstadt, Germany). Polyethylene glycol (PEG)-10,000, D-glucose and hexadecyl trimethyl ammonium bromide (HTAB) were from Sigma-Aldrich (St. Louis, MO, USA).

### Surfactants

Three biocompatible amino acid-based surfactants derived from $N^{\varepsilon}$ or $N^{\alpha}$-acyl lysine methyl ester salts with one lysine

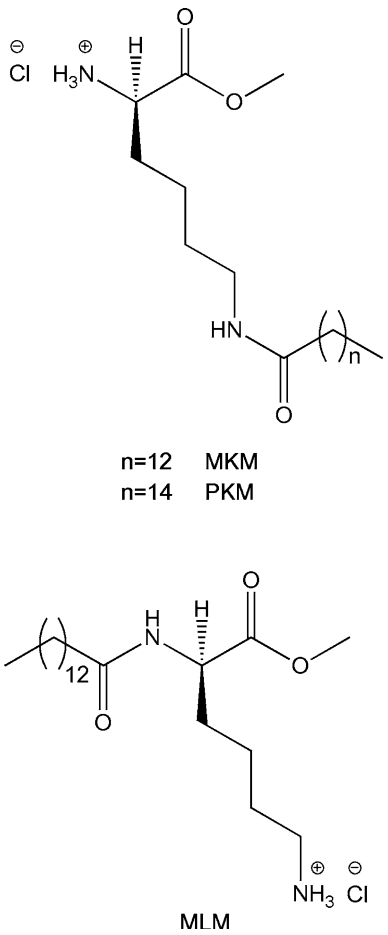


**Fig. 1** Molecular structures of the cationic lysine-based surfactants

as the cationic polar head (one cationic charge) and one alkyl chain were evaluated: $N^{\varepsilon}$-myristoyl lysine methyl ester (MKM) with one alkyl chain of 14 carbon atoms and one positive charge on the α-amino group of the lysine, $N^{\varepsilon}$-palmitoyl lysine methyl ester (PKM) with one alkyl chain of 16 carbon atoms and one positive charge on the α-amino group of the lysine and $N^{\alpha}$-myristoyl lysine methyl ester (MLM) with one alkyl chain of 14 carbon atoms and one positive charge on the ε-amino group of the lysine. MKM and PKM have a hydrophobic chain attached to the ε-amino group of the lysine, while MLM has the hydrophobic chain attached to the α-amino group of the lysine (Fig. 1). The commercial cationic surfactant hexadecyl trimethyl ammonium bromide (HTAB) was used as the reference compound. These lysine-based surfactants were synthesized in our laboratory as previously described (Colomer et al. 2011a; Pérez et al. 2009) and made from natural fatty acid and amino acid organic building blocks. The chemical structure of these compounds was checked by nuclear magnetic resonance and their purity, higher than 99%, was confirmed by elemental analysis and high-performance liquid chromatography. In all cases, all building blocks were linked by amide bonds to form biodegradable molecules. See Table 1 for the physicochemical properties and analytical data.

## Preparation of erythrocyte suspensions

Rat blood was obtained from anesthetized animals by cardiac puncture and drawn into tubes containing EDTA. The procedure was approved by the institutional ethics committee on animal experimentation. Red blood cells were isolated by centrifugation at 3,000 rpm at 4°C for 10 min and washed three times in an isotonic phosphate buffered saline (PBS) solution containing 123.3 mM NaCl, 22.2 mM $Na_2HPO_4$ and 5.6 mM $KH_2PO_4$ in distilled water (pH 7.4; 300 mOsmol/l). The cell pellets were then suspended in PBS solution at a cell density of $8 \times 10^9$ cells/ml.

## Hemolysis assay

The membrane lytic activity of the surfactants was examined by hemolysis assay. PBS buffers in the pH range of 5.4–7.4 were prepared to be isosmotic inside the erythrocyte and cause negligible hemolysis. 25-µl aliquots of erythrocyte suspension were exposed to different surfactant

**Table 1** Physicochemical properties and analytical data for the cationic lysine-based surfactants

| Surfactant | MW (g/mol) | CMC[a] (µg/ml) | p*Ka*[a] | Number of alkyl chains | Length of alkyl chain | Elemental analysis (%) (% calculated)[b] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | C | H | N |
| MKM | 406.66 | 650[c] | 5.3[c] | 1 | C14 | 61.77 (62.02) | 10.78 (10.56) | 6.73 (6.88) |
| PKM | 434.66 | 260[c] | 4.5[c] | 1 | C16 | 63.61 (63.49) | 11.20 (10.81) | 6.37 (6.44) |
| MLM | 406.66 | 765[d] | 8.1[d] | 1 | C14 | 61.76 (62.02) | 10.42 (10.56) | 6.62 (6.88) |

*MW* Molecular weight

[a] Determined in water

[b] Pérez et al. (2009)

[c] Colomer et al. (2011b)

[d] Determined as described in "Hemolysis assay" and "pH-dependent hemolysis" sections

concentrations based on preliminary studies (from 50 to 500 μg/ml for MKM and PKM, 10 to 60 μg/ml for MLM and 2.5 to 20 μg/ml for HTAB) and dissolved in PBS solution in a total volume of 1 ml. The samples were incubated at room temperature for 10 min. Two controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). The kinetic experiments were performed with concentrations associated with initial low hemolytic in the pH range of endosomal compartments (pH 6.5 and 5.4). The samples were incubated at room temperature under constant shaking for various periods up to 90 min. In all these hemolysis experiments, the samples were centrifuged at 10,000 rpm for 5 min at the end of each incubation time. Absorbance of the hemoglobin release in supernatants was measured at 540 nm using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan) and the percentages of hemolysis were determined by comparison with the positive control samples completely hemolyzed with distilled water. Concentration-response curves were obtained from the hemolysis results and the concentration inducing 50% hemolysis ($HC_{50}$) was calculated.

### Osmotic protection experiments

To gain further insight into the mechanism of cell membrane disruption, the effect of osmolytes with different molecular weights on the membrane-disruptive activity of the surfactants was investigated. For this, PBS buffers at the pH range studied were prepared by adding D-glucose (180 Da) or polyethylene glycol (PEG)-10,000 (10 kDa) at a concentration of 10 mM, which is below the range at which these molecules alone induce hemolysis as a result of osmotic pressure (Murthy et al. 1999). Red blood cells were incubated with these buffers and exposed to a concentration of each surfactant that achieved significant hemolysis in PBS buffer alone (500 μg/ml for MKM and PKM, and 60 μg/ml for MLM). Hemolysis was determined after incubating the cells for 10 min at room temperature, following the procedure described above. Controls with only the osmolytes were prepared to ensure that no hemolysis occurred without the surfactants.

### Erythrocyte count

Erythrocytes were counted in a Zeiss Axioskop optical microscope (Zeiss, Jena, Germany) using a Bürker counting chamber (Brand, Wertheim, Germany). The percentages of lysed erythrocytes at the required concentration of each surfactant (500 μg/ml for MKM and PKM, and 60 μg/ml for MLM) were determined relative to the total cell number in PBS buffer alone. The hemolysis experiment was performed following the procedure above at the same pH range and each sample was diluted four times for cell counts.

### Studies of rat erythrocyte morphology by scanning electron microscopy (SEM)

Interaction of the surfactants with the erythrocyte membrane in the pH range under study was determined by incubating intact cells with a sub-lytic concentration (10 μg/ml) of each surfactant. After a 10-min incubation, samples were fixed by adding 1 ml of 2.5% glutaraldehyde in PBS solution and incubated at 4°C for 2 h. The samples were then centrifuged (1,500 rpm for 5 min), the supernatant was discarded, and 500 μl of 1.25% glutaraldehyde in PBS was added. Fixed samples were washed with PBS solution, postfixed with 1% osmium tetroxide, placed on a glass coverslip, dehydrated in an ascending series of ethyl alcohol (50–100%), air-dried by the critical point drying method using a CPD 7501 apparatus (Polaron, Watford, UK), and finally mounted on an aluminium stub and gold-coated by an SEM coating system SC 510 (Fisons Instruments, East Grinstead, UK). Resulting specimens were examined under a Zeiss DSM 940A scanning electron microscope (Carl Zeiss SMT AG, Jena, Germany).

### Statistical analyses

Each hemolysis experiment was performed at least three times using three replicate samples for each surfactant concentration tested. Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using Student's $t$ test or one-way analysis of variance (ANOVA) to determine the differences between the datasets, followed by Bonferroni's or Dunnett's post hoc tests for multiple comparisons using the SPSS® software (SPSS Inc., Chicago, IL, USA). $P < 0.05$, $P < 0.01$ and $P < 0.005$ were considered significant. Pearson's correlation coefficients ($r$) between the $HC_{50}$ and CMC values were also calculated by linear regression analysis.

## Results and discussion

### Hemolysis assay

We studied the disruption of lipid bilayer membranes by cationic lysine-based surfactants. Erythrocytes are considered a simple cellular model and are therefore used as a convenient cell membrane system to study surfactant–membrane interactions (Sánchez et al. 2007). We used a hemolysis assay with the erythrocyte membrane as a model of the endosomal membrane (Chen et al. 2009; Wang et al. 2007). Early endosomal compartments have a pH from 6.5

to 6.8, while the lumen of late endosomes has a lower pH, of about 5.5 (Moore et al. 2008; Stayton et al. 2000). The lysosome has a pH as low as 4.6 to 5.0 (Mellman 1996). Therefore, we explored the membrane lytic activity of the class of compounds at the pH range of 5.4–7.4, mimicking the environment that the surfactant molecules are expected to encounter when incorporated into a drug delivery device translocating through the endocytic pathway.

The hemolytic activity of the surfactants was determined as a function of concentration at pH 5.4, 6.5, and 7.4. The concentration-dependent curves in Fig. 2 demonstrate that the membrane-disruptive activity of the surfactants was strongly influenced by concentration. At pH 7.4 (Fig. 2a), all the surfactants displayed almost a linear increase in hemolysis as a function of concentration. Moreover, the surfactants MKM and PKM were considerably less hemolytic than MLM and the commercial surfactant HTAB. MLM and HTAB (used as the reference compound) were nearly 9 and 30-fold more active in disrupting cell membranes at physiological conditions, respectively, as demonstrated by the $HC_{50}$ values calculated from these curves (Table 2).

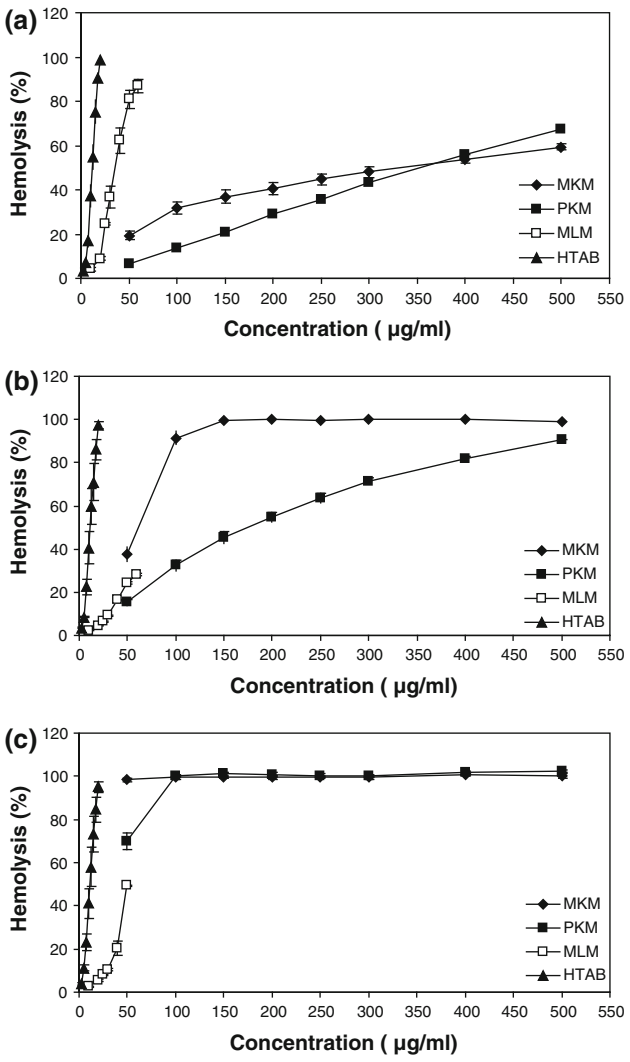


**Fig. 2** Dependence of rat erythrocyte hemolysis on surfactant concentration. **a** pH 7.4, **b** pH 6.5 and **c** pH 5.4. Each point represents the mean of three independent experiments ± SEM (*error bars*)

When the effect of concentration on hemolytic potency was evaluated at pH 6.5 (a very early stage of endosomal acidification), the surfactants showed different profiles (Fig. 2b). MKM and PKM displayed higher degrees of hemolysis than at physiological conditions (pH 7.4) throughout the concentration range tested, and the differences in $HC_{50}$ values were significant ($P < 0.05$) (Table 2). MKM was much more potent in disrupting the plasma membrane at pH 6.5, showing 100% membrane lysis at 150 µg/ml, while only 36.91% hemolysis was observed at pH 7.4. PKM was 3.5-fold less efficient than MKM in disrupting plasma membrane on the basis of $HC_{50}$, since almost complete disruption of erythrocyte membranes was only achieved gradually with an increasing concentration from 50 to 500 µg/ml. In contrast, MLM was 2.5-fold less hemolytic at pH 6.5 than at pH 7.4, reaching maximum hemolysis of only 28.24% at 60 µg/ml while a maximum of 86.86% was reached at physiological conditions.

Finally, at pH 5.4 (a late stage of endosomal acidification) (Fig. 2c), the membrane lytic activity of MKM and PKM increased dramatically, reaching 100% hemolysis at low concentrations of 50 and 100 µg/ml, respectively. In contrast, MLM did not show increased activity at pH 5.4. Hemolysis was greater here than at pH 6.5 but still lower than in physiological conditions (Table 2). The commercial surfactant HTAB displayed the same concentration-dependent hemolytic activity in the endosomal and physiological pH range and differences in $HC_{50}$ values were not significant ($P > 0.05$).

**Table 2** $HC_{50}$ values of the surfactants in the pH range studied

| Surfactants | $HC_{50}$ (µg/ml) (mean[a] ± SEM) | | |
|---|---|---|---|
| | pH 7.4 | pH 6.5 | pH 5.4 |
| MKM | 340.86 ± 14.03 | 56.45 ± 12.10[b] | <50[c] |
| PKM | 356.27 ± 13.68 | 199.84 ± 24.54[b] | <50[c] |
| MLM | 38.88 ± 3.51 | 98.58[d] ± 1.53[e] | 75.51[d] ± 20.73 |
| HTAB | 11.61 ± 0.88 | 11.48 ± 1.57 | 11.49 ± 1.79 |

[a] Mean ± SEM of three experiments

[b] Significantly different from the pH 7.4 condition (Student *t* test, $P < 0.05$)

[c] The lowest concentration tested displayed more than 50% hemolysis

[d] Estimated value (out of experimental curve range)

[e] Significantly different from the pH 7.4 condition (ANOVA followed by Bonferroni's post hoc test, $P < 0.05$)

Despite the individual differences within the pH range tested, the hemolytic activity of all the compounds was strongly affected by concentration. In previous studies, we reported similar results for the membrane lytic activity of arginine-based gemini surfactants and anionic lysine-based surfactants (Mitjans et al. 2003; Nogueira et al. 2011). These results were as expected, since it has been widely reported that surfactants at high concentrations are hemolytic and membrane solubilization is often observed (Jones 1999; Maher and Singer 1984). Here, the enhanced hemolysis with increasing surfactant concentration could also be due to increased ionic interactions between the negatively charged lipid membranes and positively charged amino groups of the surfactant molecule (Seo and Kim 2010). Furthermore, surfactant properties, such as alkyl chain length, position of the cationic charge and head group hydrophobicity can significantly affect surfactant interaction with cell membranes (Colomer et al. 2011a). Our results here showed that the compounds with the positive charge in the α-amino group (MKM and PKM) had lower disruptive potency at physiological pH and higher membrane lytic activity in the late endosomal pH range. Regarding the surfactants with the charge in the same position (MKM and PKM), concentration-dependent hemolytic activity decreased with increasing length of the hydrophobic tail. Likewise, several authors have reported similar results for the hemolysis of hydrogels of amino acid-based cationic amphiphiles (Roy and Das 2008) and of partially fluorinated pyridinium bromides (Vyas et al. 2006). This behavior contrasts with the typically hemolytic activity described for cationic surfactants, where longer alkyl chains are associated with higher membrane lytic activity (Rasia et al. 2007; Benavides et al. 2004).

It is worth mentioning that the $HC_{50}$ values of MKM and MLM were below the critical micellar concentration (CMC) across the entire pH range studied here, indicating that the monomers are responsible for hemolytic activity and that micelles are not needed for surfactant-mediated cell lysis (Table 1). In contrast, PKM disrupted membranes at micellar concentrations in physiological conditions and in its monomer form in the pH range of endosomal compartments. The CMC was determined by conductivity measurements at 25°C of each surfactant aqueous solution at the adequate concentration range, following the procedure described by Colomer et al. (2011a). The CMC values of MKM and PKM were previously reported by our research group (Colomer et al. 2011b). We found no significant correlations ($P > 0.05$) between the CMC and the $HC_{50}$ values at pH 7.4 ($r = 0.7209$) and 6.5 ($r = 0.8756$). Several studies have been performed to demonstrate a correlation between hemolytic activity and the CMC of surfactants; however, there are no clear conclusions in the literature (Pérez et al. 2009; Preté et al. 2002; Spengler et al. 2011).

## pH-dependent hemolysis

To corroborate the pH-dependent hemolytic activity of MKM and PKM in contrast to MLM and HTAB, specific studies on the effects of pH were performed. At low concentrations (Fig. 3a), the surfactants showed low or negligible hemolytic activity at pH 7.4. When the pH was decreased to 6.5, MKM had increased membrane lytic activity and so did PKM but to a lesser extent, resulting in a maximum hemolysis of 37.51 and 15.23%, respectively. At pH 5.4, the hemolytic activity of MKM and PKM increased considerably ($P < 0.01$), reaching maxima of 98.26 and 69.92%, respectively. The pK50 (pH at which 50% hemolysis is obtained) was also calculated for each surfactant from the pH-response curves showed in Fig. 3a (Lee et al. 2010), with a value of approximately 6.2 for MKM, while the hemolysis response curve of PKM shifted toward lower pH and had an apparent pK50 of 5.6. These results support the early lytic activity of MKM at mildly acidic conditions (pH 6.5). MLM and HTAB showed no pH-sensitive activity throughout the endosomal pH range and thus, did not facilitate endosomal destabilization.

At concentrations near the $HC_{50}$ values (Fig. 3b), MKM and PKM exhibited a concomitant rise in hemolytic activity at pH 7.4 and 6.5, reaching approximately 40–50% hemolysis at physiological conditions, while 71.5 and 100%

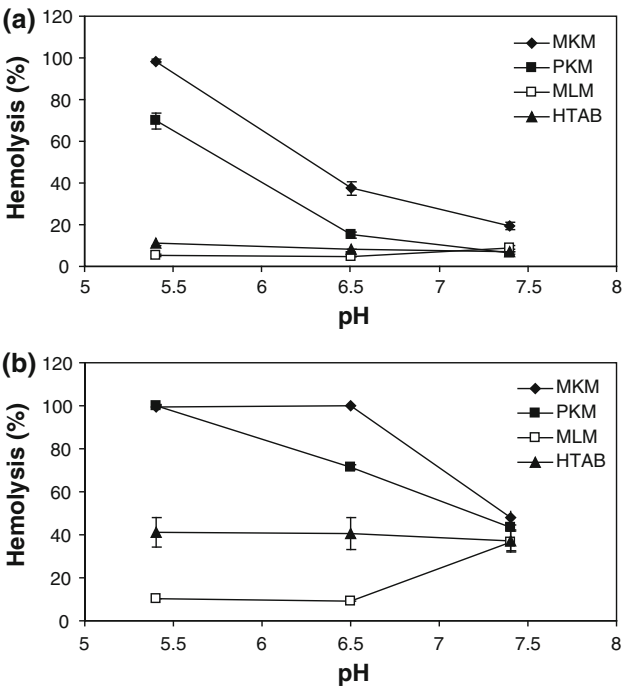


**Fig. 3** Surfactant-induced hemoglobin release from rat erythrocytes as a function of pH. Surfactants were added from concentrated solutions to give final concentrations of **a** 50 μg/ml MKM and PKM, 20 μg/ml MLM, or 5 μg/ml HTAB; and **b** 300 μg/ml MKM and PKM, 30 μg/ml MLM, or 10 μg/ml HTAB. Each point represents the mean of three independent experiments ± SEM (*error bars*)

hemolysis were recorded at pH 6.5 for PKM and MKM, respectively. At pH 5.4, MKM maintained the approximately 100% lysis observed at lower concentrations and PKM also reached total hemolysis. These observations demonstrated that surfactants at high concentrations do not retain the specific pH-responsive behavior, as significant hemolysis was also observed at physiological conditions. Surprisingly, higher concentrations of MLM displayed lower membrane-disruptive activity in acidic conditions than at physiological pH, while HTAB did not show any significant changes in its hemolytic potency throughout the pH range evaluated, confirming the inability to disrupt endosomal membranes.

Noteworthy was the significant rise in the hemolytic activity of MKM and PKM at pH 5.4 and to a lesser extent at pH 6.5, while MLM demonstrated no pH-dependent responses. The difference between MKM and MLM lies in the polar group (position of the cationic charge), while MKM and PKM differ in the length of the hydrophobic tail. It has been suggested that protonation and pH-dependent activity may be governed by the overall p*K*a of the head group of the surfactants (Wang et al. 2007). Lysine is a dibasic amino acid consisting of two amino groups with different basicity since the α-amino group has a p*K*a = 8.9 and the ε-amino group a p*K*a = 10.5. However, to estimate the protonation state of the surfactant molecule in the pH range studied, we determined the apparent p*K*a values of the polar groups when they were included in the surfactant molecule. The p*K*a value of each compound was estimated by potentiometric titration, as described by Tabohashi et al. (2001). The titration was carried out at 25°C by adding increments of the NaOH solution to the surfactant solution with constant magnetic stirring, and the p*K*a value was obtained from the inflection point in the titration curve. The experimental p*K*a values obtained from pH measurements in aqueous medium were found to be considerably lower than those of the original lysine amino acid: 5.3 and 4.5 for MKM and PKM (cationic charge in the α-amino group of the lysine), respectively (Colomer et al. 2011b), and 8.1 for MLM (cationic charge in the ε-amino group of the lysine). Given that the only difference between MKM and MLM is the position of the cationic charge, the p*K*a associated with the protonated amino group may be crucial for pH-sensitive membrane-disruptive activity. Consistent with these values, the lack of pH-responsive activity of MLM is related to the fact that no significant changes in the protonation state of the molecule took place in our experimental conditions, as the polar head should remain almost completely protonated throughout the studied pH range (which means an average charge of approximately 1). In contrast, MKM and PKM have p*K*a values lower or in the order of the low pH range studied and therefore different protonation states can be achieved. The increased membrane-disruptive activity of these compounds in acidic conditions could be explained by a modification in the hydrophobic/hydrophilic balance. At pH 5.4, the lysine in MKM is approximately 50% protonated (which means an average charge of 0.5), while PKM has a protonation state lower than 50% (average charge $< 0.5$) and hence, a lower ability to disrupt the lipid bilayer. This higher lytic activity of MKM is in accordance with the study reported by Chen et al. (2003), in which pH-sensitive acyloxyalkylimidazoles reached the maximum rate of hemolysis when about 50% of the molecule was protonated. The same reasoning is valid for the surfactant lytic activity at pH 6.5, in which MKM has a more protonated polar group and is thus more hemolytic than PKM (even though both compounds are less than 50% protonated). More protonable amino groups add more charges to the polar head, which consequently increases membrane-lytic activity. At neutral pH, these compounds predominantly exist as unprotonated species, which explains the lower hemolysis achieved at physiological conditions. To summarize, surfactant membrane lytic activity changes with the pH because of the variation in the net charge at the studied pH range. Furthermore, concerning the surfactants that have the charge in the same position but differ in the alkyl chain length (MKM and PKM), our results showed that increasing the number of carbon atoms from 14 to 16 did not affect the pH-responsive behavior, but decreased membrane lytic activity in the endosomal pH range. In conclusion, the pH-sensitive membrane-disruptive activity of these surfactants can be fine-tuned mainly by varying the position of the cationic charge (by adjusting the p*K*a) and, to a lesser extent, by altering the length of the alkyl chain.

A strong correlation has been reported between hemolytic activity and endosomal disruption by membrane-disruptive agents (Plank et al. 1994). The cationic lysine-based surfactants with pH-sensitive properties were studied based on the hypothesis that pH-responsive membrane lytic activity in endosomes should facilitate membrane destabilization and allow surfactant-drug complexes to escape to the cytoplasm for efficient intracellular drug delivery. Numerous amphiphilic materials can mediate material transport across cell membranes, such as amphiphilic peptide sequences used by viruses (Janshoff et al. 1999) and amphiphilic lipids (Wasungu and Hoekstra 2006). However, virus peptides may have problems of immunogenicity and lipids may not selectively disrupt membranes at the endosomal pH. Our results demonstrated that MKM and PKM have specific pH-responsive membrane disruption at pH 5.4, the final most acidic endosomal pH, which may help to circumvent the non-productive trafficking of therapeutic compounds from endosomes to lysosomes, where degradation may occur. In summary, a useful intracellular drug delivery system should have low lytic activity at physiological pH and high destabilizing

activity in the mildly acidic conditions found in the endosomes to elicit only selective endosomal membrane disruption (Wang et al. 2007; Yessine et al. 2003). Low concentrations of PKM and MKM induced membrane lysis only at the endosomal pH range and there was no significant hemolysis at blood stream pH (7.4), supporting these surfactants as potential biocompatible materials in novel non-viral drug delivery systems.

### Kinetics of hemolytic activity

Surfactant-mediated hemolysis was also determined as a function of time in the pH range characteristic of early and late endosomes (pH 6.5 and 5.4, respectively). The concentrations evaluated (25 μg/ml MKM, 50 μg/ml PKM, 20 μg/ml MLM and 5 μg/ml HTAB) were in the range of those that displayed specific pH-dependent activity and/or low degree of hemolysis after the initial 10 minutes of incubation. MKM and PKM showed improved kinetics of hemolytic activity at both stages of endosomal acidification. At pH 6.5 (Fig. 4a), MKM caused 100% of hemolysis after 30 min of incubation, which corresponded to a membrane-disruptive activity approximately 17-fold higher than that observed after 10 min of incubation. PKM was less effective than MKM, as it showed a lag time of 30 min and achieved around 80 and 95% hemolysis only after 60 and 90 min of incubation, respectively. The presence of a lag time implies the requirement of a minimum threshold amount of surfactant that has to accumulate in the lipid bilayer before hemoglobin leakage (Chen et al. 2003). At pH 5.4 (Fig. 4b), both compounds caused relatively weak hemolysis after 10 minutes of incubation, followed by a sharp increase to a maximum of about 80% (MKM) and 100% (PKM) after 90 minutes. The membrane lytic activity of MLM and the reference surfactant HTAB did not show any dependence on incubation time at the two pH values assessed, confirming their inability to disrupt endosomal membranes.

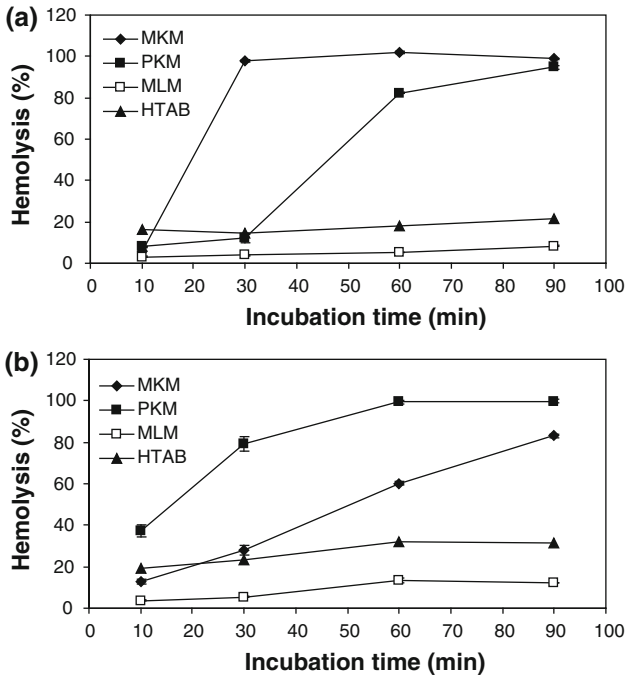


**Fig. 4** Kinetics of hemolytic activity of the lysine-based surfactants at **a** pH 6.5 and **b** pH 5.4. Surfactants were added at time zero from concentrated solutions to give final concentrations of 25 μg/ml MKM, 50 μg/ml PKM, 20 μg/ml MLM and 5 μg/ml HTAB. Each point represents the mean of three independent experiments ± SEM (*error bars*)

The significant hemolytic kinetics shown by MKM and PKM indicate that one or more previous steps are required before the erythrocyte membrane becomes permeable to hemoglobin. Among these steps, the most important could be the formation of pores or channels that lead to the efflux of low molecular weight solutes (see "Osmotic protection"). The molecules taken up by endocytosis are trafficked from early endosomes to lysosomes within several hours (Pack et al. 2005). Thus, timely permeabilization of the endosomal membrane is a prerequisite for cytosolic translocation of drugs in order to exert their pharmacological effect (Asokan and Cho 2005). Given the improved hemolytic kinetics and pH-responsive membrane activity of PKM and MKM, these compounds could disrupt endosomal membranes before fusion of the endocytic vesicles with lysosomes, thus avoiding non-productive intracellular trafficking, a critical feature for potential intracellular drug delivery applications.

### Mechanism of cell membrane disruption

#### *Osmotic protection*

To assess whether membrane lysis occurs through the colloid osmotic mechanism (after formation of transient defects or pores in the membrane due to surfactant treatment), an osmotic protection experiment was conducted in PBS solutions that contained molecules of varying sizes. The rationale behind this was that since sufficiently large molecules do not permeate through the cell membrane, their presence would counteract the osmotic pressure of the macromolecules inside the cell (especially hemoglobin), diminishing water penetration and consequently cell swelling (Murthy et al. 1999).

PEG-10,000 significantly ($P < 0.05$) inhibited surfactant-mediated hemolysis throughout the pH range studied, thereby indicating that defects such as pores or channels are formed in the erythrocyte membrane (Fig. 5). In the presence of PKM and MKM at pH 7.4, and MLM at pH 6.5, PEG-10,000 produced less pronounced inhibition of

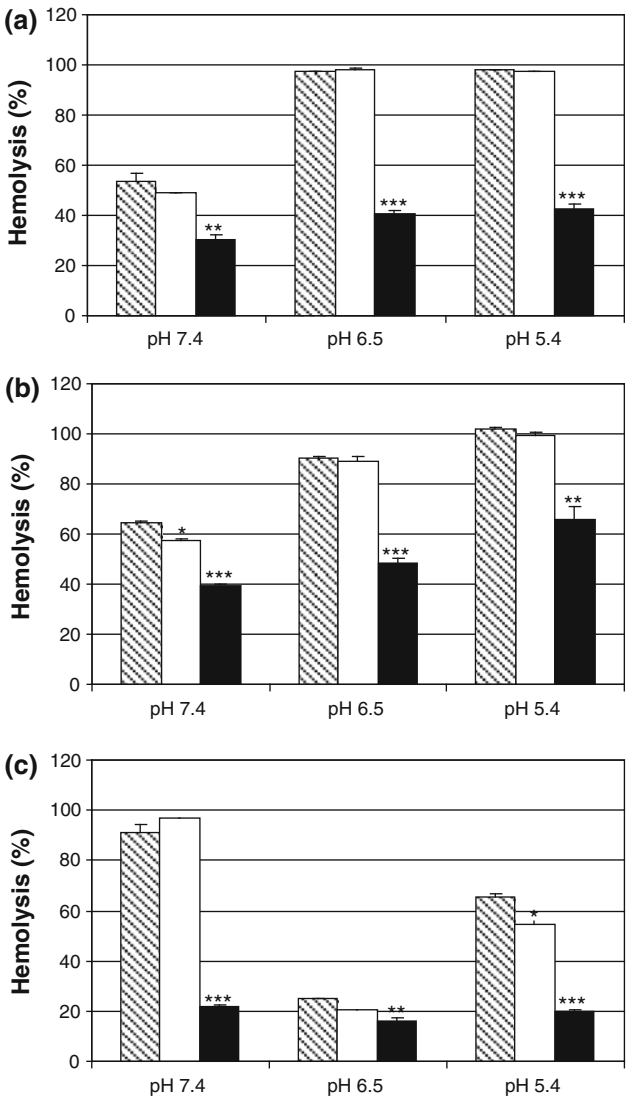


**Fig. 5** Effects of the osmotic protectant PEG-10,000 (10 kDa) and D-glucose (180 Da) on lysine-based surfactant-induced hemolysis of rat erythrocytes. Surfactants were added at the following range of final concentrations **a** 500 μg/ml MKM, **b** 500 μg/ml PKM, and **c** 60 μg/ml MLM. PBS alone (*striped bars*), PBS + D-glucose (*blank bars*) and PBS + PEG-10,000 (*black bars*). The hemolysis results obtained in the presence of the osmolytes D-glucose and PEG-10,000 were compared to the control in PBS solution alone by ANOVA followed by Dunnett's post hoc test. $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$ denote significant differences. The data represent the mean of three independent experiments ± SEM (*error bars*)

hemolysis, achieving maximum protection of 39.15, 43.26, and 35.96%, respectively. The highest protection degrees mediated by PEG-10,000 addition were 46.33, 58.19, and 75.83% for PKM and MKM at pH 6.5, and MLM at pH 7.4, respectively. Despite the significant degrees of protection against hemolysis after the addition of PEG-10,000, this osmolyte only partially avoided cell membrane disruption (generally by less than 50%) within the pH range studied. This means that the surfactants induced pores in the lipid bilayer that were smaller than the hydrodynamic radius of PEG-10,000 (hence, not permeable to this osmolyte, thereby decreasing hemolysis), as well as larger pores that were permeable to this protectant. The osmotic imbalance resulting from the diffusion of low molecular weight solutes out of the cell cannot be balanced when these large pores are formed and consequently, cell lysis occurs. The overall observations of this experiment indicated that osmotic cell swelling was not the only mechanism involved in membrane lysis and other more complex mechanisms may also contribute. It is also feasible that cell membrane disruption could be due to partial solubilization of membrane lipids and proteins through micellization caused by extensive surfactant adsorption (Chernitsky and Senkovich 1998). Regardless of the partial inhibition of cell lysis and the complexity of the mechanisms involved, the significant protection against hemolysis ($P < 0.05$) can be considered as evidence of the presence of an osmotic mechanism (Chernitsky and Senkovich 1998). Corroborating the osmotic mechanism, the low molecular weight D-glucose had no effect on the osmotic imbalance caused by the hemoglobin trapped in the erythrocyte cytoplasm (Fig. 5). Negligible or very small protection was observed and the degree of hemolysis did not differ significantly ($P > 0.05$) from that caused by the same surfactant concentration in PBS alone, except for PKM at pH 7.4 ($P = 0.018$) and MLM at pH 5.4 ($P = 0.01$). For comparison, our previous studies on anionic lysine-based surfactants showed that PEG-10,000 almost completely protected against cell lysis at pH 7.4 and 6.5, while partial protection was only observed at pH 5.4 (Nogueira et al. 2011).

### *Erythrocyte count*

Erythrocyte counts were performed to study whether hemoglobin passes through the cell membrane or whether it is released into the extracellular medium after cell membrane rupture (Chen et al. 2009; Nogueira et al. 2011). The overall cell numbers declined significantly after 10 minutes of incubation with the hemolytic concentration of each surfactant throughout the pH range tested and the percentages of lysed erythrocytes were in agreement with the hemolytic activity results (Fig. 6). These observations indicate that hemoglobin is released into the PBS buffer after cell membrane lysis and is not related to increased cell membrane permeability or the opening of large pores sufficient to release hemoglobin molecules.

### *SEM studies of rat erythrocyte morphology*

To better understand the interaction of cationic lysine-based surfactants with the lipid bilayer, we assessed

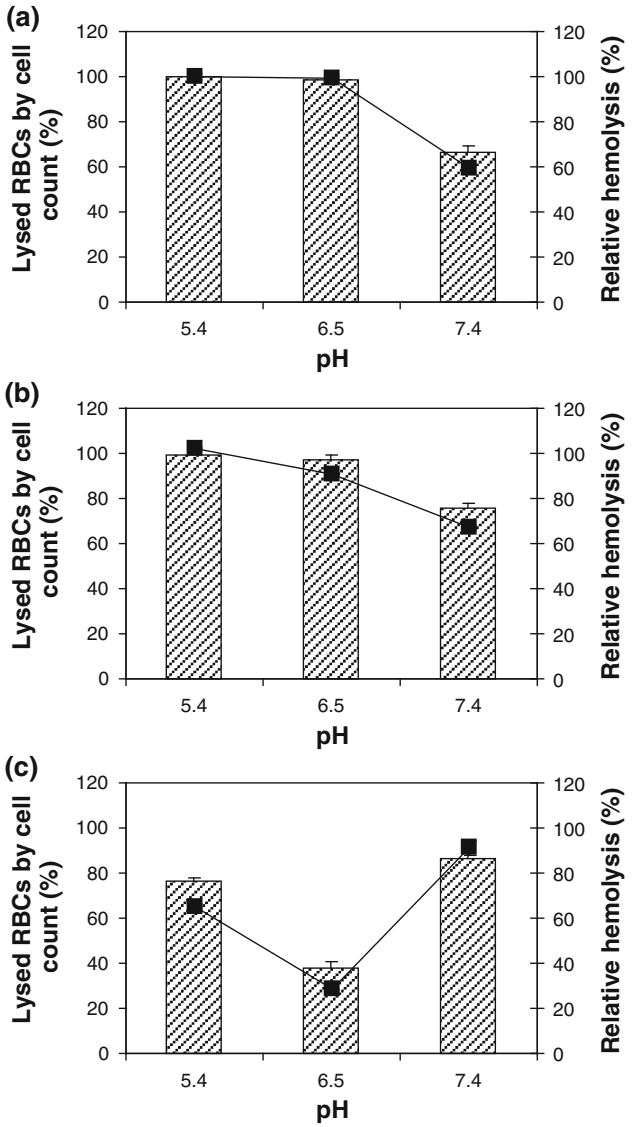


**Fig. 6** Comparison between the hemolysis (*line*) and the percentage of lysed rat erythrocytes (*columns*) caused by the cationic lysine-based surfactants at the pH range tested. Surfactants were added at the following range of final concentrations **a** 500 μg/ml MKM, **b** 500 μg/ml PKM, and **c** 60 μg/ml MLM. The data represent the mean of three independent experiments ± SEM (*error bars*)

morphological changes produced in rat erythrocytes by SEM after treatment with the surfactants at the sub-lytic concentration of 10 μg/ml at pH ranging from 5.4 to 7.4. The results indicated that the surfactants interacted with the lipid bilayer and altered the normal biconcave morphology of the cells (Fig. 7). Control erythrocytes incubated in PBS solution at the pH values studied (7.4, 6.5, and 5.4) were also evaluated and found to be discoid or slightly echinocytic (Fig. 7a). This shape is considered normal in erythrocytes isolated in buffer and in the absence of albumin (Dubnicková et al. 2000; Rasia et al. 2007).

Despite varying membrane lytic activity of MKM and MLM, erythrocytes underwent similar morphological alterations after treatment with these surfactants throughout the pH range studied, indicating that morphological changes are not affected by the position of the cationic charge, unlike hemolytic activity, which is strongly affected. MKM and MLM changed the discoid shape of cells at pH 7.4 to stomatocytes (Fig. 7b, e, respectively), producing a cup-shaped form. This morphological change was induced when the compound was inserted into the inner monolayer of the membrane, expanding it relative to the outer layer. Our results are consistent with the bilayer hypothesis (Sheetz and Singer 1974), which proposes that cationic amphiphiles induce stomatocytes at physiological pH as a result of the electrostatic attraction between the positive polar head group of the molecule and the acidic phospholipids, the latter having negative charges under physiological conditions and localizing in the inner layer of the lipid bilayer. Furthermore, in mildly acidic media (pH 6.5), these two surfactants induced a spherostomatocyte-type deformation (Fig. 7c, f, respectively). The spherostomatocytes are formed from stomatocytes increasing the incorporation of compounds into the membrane, and are considered to be the previous stage before transformation to spherocytes. Finally, at pH 5.4, the cells displayed swollen forms (spherocytes), which can be assumed to be the last stage of the morphological change with maximum accumulation of the compound at the membrane before cell lysis (Fig. 7d, g, respectively). This increased accumulation at both pH 6.5 and 5.4 supports the significantly increased membrane lytic activity of MKM, since more interactions of the surfactants with the cell membrane could enhance hemolysis (Nogueira et al. 2011). In contrast, the higher incorporation of MLM into the erythrocyte membrane did not reflect increased hemolysis, suggesting that the accumulation of this compound in the lipid bilayer may play a protective role against hemolysis.

In contrast, PKM (with a longer alkyl chain) interacted with the phospholipid bilayer in a slightly different way. At physiological pH, it induced a leptocyte-type deformation (Fig. 7h), with the erythrocyte having a larger surface area than normal cells. This morphological change could be attributed to the higher incorporation of this surfactant into the lipid bilayer, thus giving an appearance of "excess" plasma membrane. A possible explanation is that the most common phospholipid in the bilayer is 16–18 carbons long, which could favor the incorporation of this compound (with 16 carbon atoms) into the membrane (Martínez et al. 2007). This hypothesis of increased incorporation was not reflected in its hemolytic activity, possibly because this surfactant may rearrange the lipid bilayer and thus exert a protective effect at low concentrations. At pH 6.5, PKM also induced stomatocytes (Fig. 7i), but at an earlier stage

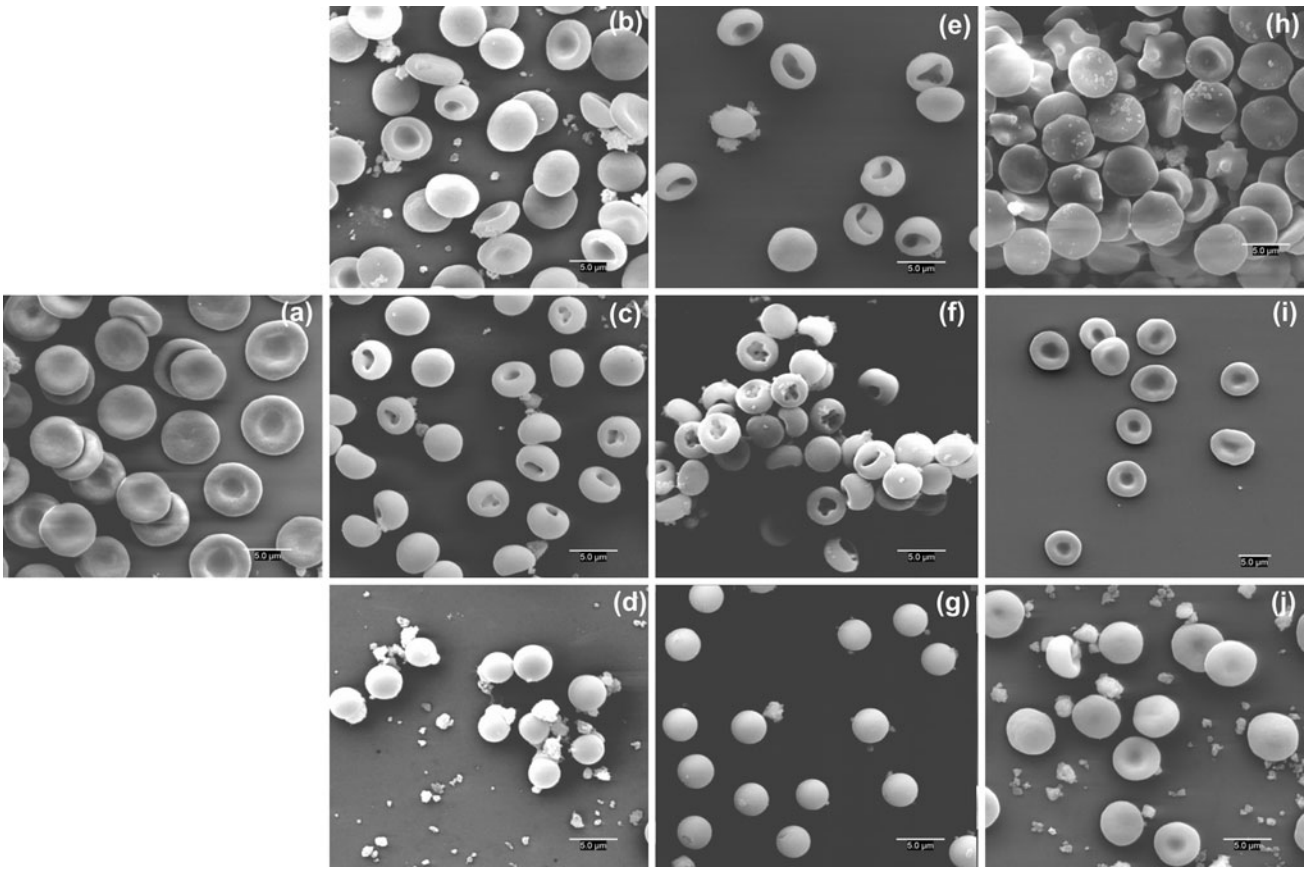


**Fig. 7** Effect of the cationic lysine-based surfactants on rat erythrocyte morphology. SEM images of **a** control in PBS pH 7.4, and after incubation with the surfactants: MKM at **b** pH 7.4, **c** pH 6.5 and **d** pH 5.4; MLM at **e** pH 7.4, **f** pH 6.5 and **g** pH 5.4; and PKM at **h** pH 7.4, **i** pH 6.5 and **j** pH 5.4. The erythrocytes were incubated for 10 min at a concentration of 10 μg/ml of each surfactant. *Scale bars* correspond to 5 μm

than that observed with MKM and MLM. The less pronounced stomatocyte-type of deformation was consistent with the lower hemolytic activity of PKM at mildly acidic conditions. At the pH range of late endosomes (pH 5.4), PKM caused spherocytes and some spherostomatocytes (Fig. 7j), which could also explain the significant rise in its membrane lytic activity (see discussion above).

The same set of experiments was carried out across the pH range of 5.4–7.4 in the presence of PEG 10,000, an osmotic protectant against surfactant-induced hemolysis. We observed that PEG only partially protected against cell lysis (generally less than 50% protection), which is in agreement with the effects observed on the morphology of erythrocytes (data not shown). PEG did not recover the initial discocyte shape and only small changes were observed at pH 5.4 from spherocytes to stomatocytes for PKM and MKM, and at pH 7.4 from leptocytes to an early stage of stomatocytes for PKM. Therefore, no clear protective role of PEG was observed, corroborating the fact that osmotic cell swelling is not the only mechanism involved in the surfactant's membrane-disruptive activity. In contrast to our results, some authors have reported that the presence of PEG induced significant changes in cell morphology (Zaragoza et al. 2010).

The overall results showed that in addition to the physicochemical characteristics of the surfactants, differences in the transbilayer distribution and mobility are crucial for hemolytic potency. Of note, the increase in positive charges at the lipid bilayer due to the distribution of the surfactants might modify phospholipid packing, which determines the correct function of the membrane. This observation may also explain the changes in the normal biconcave shape of the red blood cells (Manrique-Moreno et al. 2010).

## Conclusions

The membrane-disruptive activity of novel cationic lysine-based surfactants was assessed using erythrocytes as a model of an endosomal membrane. Although all the surfactants enhanced hemolysis with increasing concentrations, only the compounds with the positive charge on the α-amino group of lysine (MKM and PKM) showed

pH-responsive hemolytic activity. Moreover, an increase in the alkyl chain length from 14 to 16 carbon atoms lowered the ability to disrupt cell membranes. Hence, the pH-dependent membrane-disruptive activity of these surfactants can be fine-tuned mainly by varying the position of the cationic charge and, to a lesser extent, by altering the length of the alkyl chain. The overall hemolysis results suggest that MKM and PKM might achieve maximum membrane lytic activity in the late endosomes, and the improved hemolytic kinetics demonstrate their ability to disrupt endosomal membranes before vesicular evolution from endosomes to lysosomes. The partial decrease in the rate of hemolysis by PEG-10,000 implies that osmotic cell swelling is not the only mechanism involved in membrane lysis and that a more complex mechanism may operate. SEM studies on rat erythrocytes showed that the surfactants interacted with the phospholipid bilayer and induced shape changes, forming mainly stomatocytes at pH 7.4 and 6.5, and spherocytes at pH 5.4. Based on our results, we conclude that the biocompatible surfactants MKM and PKM, but not MLM, have potential applications as a new class of bioactive excipients in drug delivery systems. These insights into the membrane-disruptive properties and mechanisms by which pH-sensitive surfactants facilitate the delivery of membrane-impermeant molecules into the cell cytoplasm may help in the design of specific endosome-destabilizing compounds. Furthermore, knowledge on the modulation of the physicochemical properties of novel surfactants may lead to the development of many more efficient amino acid-based drug carriers. Current studies are focusing on the development of lysine-based surfactant conjugates as potential biocompatible drug delivery systems for pharmaceutical applications.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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## Artículo 3

**PHOSPHOLIPID BILAYER-PERTURBING PROPERTIES UNDERLYING LYSIS INDUCED BY pH-SENSITIVE CATIONIC LYSINE-BASED SURFACTANTS IN BIOMEMBRANES**

**(Propiedades perturbadoras de la bicapa fosfolipídica subyacentes a la lisis inducida por tensioactivos catiónicos pH-sensibles derivados de lisina en biomembranas)**

**Daniele Rubert Nogueira, Montserrat Mitjans, M. Antonia Busquets, Lourdes Pérez, M. Pilar Vinardell**

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  Nogueira DR, Mitjans M, Pérez L, Vinardell MP. 'Membrane perturbing properties of biocompatible cationic lysine-based surfactants'.
  Publicado en *Toxicology Letters* 2011; 205: S169.

# Resumen

*Objetivos*

Evaluar las propiedades de interacción de tensioactivos catiónicos derivados del aminoácido lisina con las membranas celulares y estudiar los mecanismos involucrados en el proceso de lisis de la bicapa lipídica en función del pH del medio.

*Material y métodos*

Se estudiaron tres tensioactivos catiónicos derivados de lisina del tipo sales de hidrocloruro de $N^{\alpha}$ o $N^{\varepsilon}$-acil lisina metil éster que difieren en cuanto a la longitud de la cadena hidrocarbonada y a la posición de la carga catiónica. También se evaluó el tensioactivo comercial bromuro de hexadecil trimetilamonio (HTAB) con propósito comparativo. Se utilizaron eritrocitos humanos y de rata como modelo de membrana celular. Se estudiaron los mecanismos involucrados en los procesos de interacción y lisis de los tensioactivos con la bicapa lipídica utilizando técnicas como: ensayo estándar de hemólisis, ensayo de antihemólisis en medio hipotónico, mediciones por anisotropía de fluorescencia para detectar cambios en la fluidez de la membrana, análisis por electroforesis en gel de poliacrilamida para valorar se hay alteraciones en el perfil proteico de la bicapa lipídica, y por último estudios por microscopía electrónica de barrido (SEM) para evaluar el grado de interacción con la membrana y su cambio morfológico resultante.

*Resultados*

Los tensioactivos con la carga positiva en el grupo α-amino de la lisina fueron considerablemente menos hemolíticos que el tensioactivo con la carga en el grupo ε-amino y el tensioactivo comercial HTAB. Además, los tensioactivos con la carga en α-amino protegieron los eritrocitos de la lisis hipotónica en un amplio rango de concentraciones, mientras que la potencia antihemolítica de estos compuestos se correlacionó inversamente con su hidrofobicidad. Los experimentos de anisotropía de fluorescencia mostraron que los tensioactivos con actividad pH-dependiente (que tienen la carga positiva en el grupo α-amino de la lisina) aumentaron de modo significativo la fluidez de la membrana a los valores de pH característicos de los compartimentos endosomales. Los análisis por electroforesis demostraron que todos los compuestos estudiados, independientemente de la posición de la carga catiónica, indujeron degradación significativa de algunas proteínas de la membrana en condiciones hipotónicas y a pH 5,4. Los estudios por SEM corroboraron la interacción e incorporación de los tensioactivos en diferentes regiones de la bicapa lipídica.

*Conclusiones*

Las alteraciones en las características estructurales de los tensioactivos pueden modular su grado de interacción con las membranas celulares, sea en función de la tonicidad del medio o sea en función de su pH. Los tensioactivos con actividad pH-dependiente perturbaron de modo significativo la membrana en condiciones ácidas, lo que justifica su potencial lítico aumentado en los valores de pH representativos de los endosomas. Además, los resultados obtenidos sugieren estos compuestos como una nueva clase de excipientes bioactivos multifuncionales con capacidad de promover una liberación intracelular eficiente de moléculas biológicamente activas.

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# Phospholipid Bilayer-Perturbing Properties Underlying Lysis Induced by pH-Sensitive Cationic Lysine-Based Surfactants in Biomembranes

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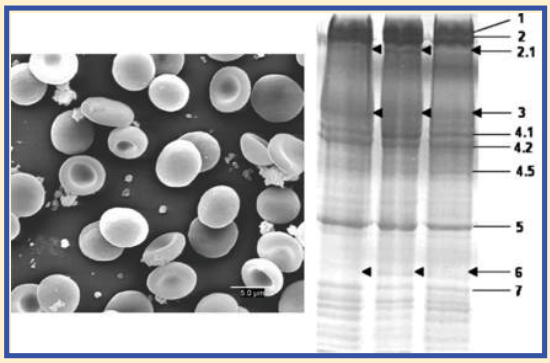
**ABSTRACT:** Amino acid-based surfactants constitute an important class of natural surface-active biomolecules with an unpredictable number of industrial applications. To gain a better mechanistic understanding of surfactant-induced membrane destabilization, we assessed the phospholipid bilayer-perturbing properties of new cationic lysine-based surfactants. We used erythrocytes as biomembrane models to study the hemolytic activity of surfactants and their effects on cells' osmotic resistance and morphology, as well as on membrane fluidity and membrane protein profile with varying pH. The antihemolytic capacity of amphiphiles correlated negatively with the length of the alkyl chain. Anisotropy measurements showed that the pH-sensitive surfactants, with the positive charge on the $\alpha$-amino group of lysine, significantly increased membrane fluidity at acidic conditions. SDS-PAGE analysis revealed that surfactants induced significant degradation of membrane proteins in hypo-osmotic medium and at pH 5.4. By scanning electron microscopy examinations, we corroborated the interaction of surfactants with lipid bilayer. We found that varying the surfactant chemical structure is a way to modulate the positioning of the molecule inside bilayer and, thus, the overall effect on the membrane. Our work showed that pH-sensitive lysine-based surfactants significantly disturb the lipid bilayer of biomembranes especially at acidic conditions, which suggests that these compounds are promising as a new class of multifunctional bioactive excipients for active intracellular drug delivery.




## 1. INTRODUCTION

Surfactants are one of the most widely used excipients in the pharmaceutical industry.[1] Their surface and interface activities in systems involving interaction with membranes have been the subject of intense study.[2−6] Surfactants derived from amino acids constitute an important class of natural surface-active biomolecules that usually have biocompatible properties and multifunctional capabilities, which make them extremely relevant for pharmaceutical applications, especially in the field of novel nonviral drug delivery devices.[4,7,8] Our group has considerable experience in the synthesis of surfactants derived from amino acids. Indeed, we recently developed new families of lysine-based surfactants,[8,9] and some of them were deeply studied here to find out some important characteristics of surfactant−membrane interaction. Cationic lysine-based surfactants have the advantage of offering a wide range of possibilities for structure modulation.[8,9] This feature allows the design of surfactants with low toxicity, high biodegradability, and pH-sensitive activity, which make them highly suitable for practical applications in comparison to the current commercial surfactant systems.

The field of surfactant−membrane interactions is becoming a rate-limiting step of biomedical and pharmacological progress.[10] As the way surfactants interact with biological membranes is not clearly understood, various research groups have made efforts to clarify the molecular processes involved in surfactant-induced cell membrane lysis.[2,3,11,12] The extent of interaction of a surfactant with a membrane is a complex phenomenon, and the properties of both the membrane and the surfactant are involved. The physicochemical properties of a biomembrane are rather constant and defined for a physiological condition. In contrast, the factors relating to the surfactant include its affinity for the membrane, its free monomer concentration in the aqueous environment adjacent to the membrane, and its structure, which allows it to accumulate at a given membrane surface area.[13]

Cationic compounds, such as lipids, peptides, polymers, and surfactants, have great potential as carriers of drugs or DNA across cell membranes.[7,14,15] This underlines the great importance of the discovery of new bioactive excipients for the development of efficient drug delivery devices: biocompatible amino acid-based surfactants could be a promising choice

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for application in this field.[4,7] Previous studies by our group showed that one novel class of cationic surfactants derived from the amino acid lysine (hydrochloride salts of $N^{\varepsilon}$-acyl lysine methyl ester) has pH-sensitive membrane-lytic activity, which is dependent on the fine-tuning of the cationic charge position, and thus is promising as a bioactive excipient in intracellular drug delivery systems.[16] This knowledge, together with a deeper study of the interaction properties of these compounds with the phospholipid bilayer, is key to the design of efficient intracellular drug carriers with specific endosome-destabilizing activity.

Therefore, to gain a better mechanistic understanding of surfactant-induced biomembrane destabilization and to complete our previous study,[16] here we assessed the phospholipid bilayer-perturbing properties of three novel cationic lysine-based surfactants derived from the salts, $N^{\varepsilon}$ and $N^{\alpha}$-acyl lysine methyl ester. We chose rat and human erythrocytes and their membranes as in vitro models to study the hemolytic activity of surfactants and their effects on osmotic resistance and morphology of cells, as well as on membrane fluidity and the membrane protein profile with varying pH. Because erythrocytes have no internal organelles and because they are the simplest cell models obtainable, they are the most popular cell membrane systems for studying surfactant−membrane interaction.[17] Moreover, to gain insight into the specific interaction of these compounds with the membrane, we also examined their structure−activity relationship. Knowledge of the membrane-destabilizing properties of the bioactive excipients is of great importance in attempting to develop efficient drug carriers with specific physicochemical and biological properties, in line with the pharmaceutical industry's needs.

## 2. MATERIALS AND METHODS

**2.1. Reagents.** All solvents were reagent grade and were used without further purification. NaCl, $Na_2HPO_4$, and $KH_2PO_4$ were supplied by Merck (Darmstadt, Germany). Hexadecyl trimethyl ammonium bromide (HTAB), sodium dodecyl sulfate (SDS), methanol, and glycine were from Sigma-Aldrich (St. Louis, MO). Fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-(4-trimethylammonium phenyl)-6-phenyl-1,3,4-hexatriene *p*-toluenesulfonate) were purchased from Molecular Probes (Eugene, OR). Acrylamide PAGE 40%, methylenebisacrylamide 2%, TEMED, mercaptoethanol, ammonium persulfate, and bromophenol blue used for SDS-PAGE were obtained from GE Healthcare Amersham Biosciences (Uppsala, Sweden). Finally, Precision Plus Unstained Standard was purchased at Bio-Rad (Hercules, CA).

**2.2. Surfactants.** Three novel biocompatible amino acid-based surfactants derived from $N^{\varepsilon}$ or $N^{\alpha}$-acyl lysine methyl ester salts with one lysine as the cationic polar head (one cationic charge) and one alkyl chain were evaluated: $N^{\varepsilon}$-myristoyl lysine methyl ester (MKM) with one alkyl chain of 14 carbon atoms and one positive charge on the $\alpha$-amino group of the lysine, $N^{\varepsilon}$-palmitoyl lysine methyl ester (PKM) with one alkyl chain of 16 carbon atoms and one positive charge on the $\alpha$-amino group of the lysine, and $N^{\alpha}$-myristoyl lysine methyl ester (MLM) with one alkyl chain of 14 carbon atoms and one positive charge on the $\varepsilon$-amino group of the lysine. MKM and PKM have a hydrophobic chain attached to the $\varepsilon$-amino group of the lysine, while MLM has the hydrophobic chain attached to the $\alpha$-amino group of the lysine (Figure 1). The commercial cationic surfactant HTAB was used as the reference compound. These lysine-based surfactants were synthesized in our laboratory, as described elsewhere,[8,9] and made from natural fatty acid and amino acid organic building blocks. In all cases, all building blocks were linked by amide bonds to form biodegradable molecules.

**2.3. Preparation of Erythrocyte Suspensions.** Rat blood was obtained from anesthetized animals by cardiac puncture and drawn into tubes containing EDTA. The procedure was approved by the institutional ethics committee on animal experiments. Human blood was obtained from adult healthy donors after informed consent, according to the approved institutional protocol for blood sample acquisition by venipuncture. Erythrocytes were isolated by centrifugation at 3000 rpm at 4 °C for 10 min and washed three times in an isotonic phosphate buffered saline (PBS) solution containing 123.3 mM NaCl, 22.2 mM $Na_2HPO_4$, and 5.6 mM $KH_2PO_4$ in distilled water (pH 7.4; 300 mOsmol/L). The cell pellets were then suspended in PBS solution at a cell density of $8 \times 10^9$ cells/mL.

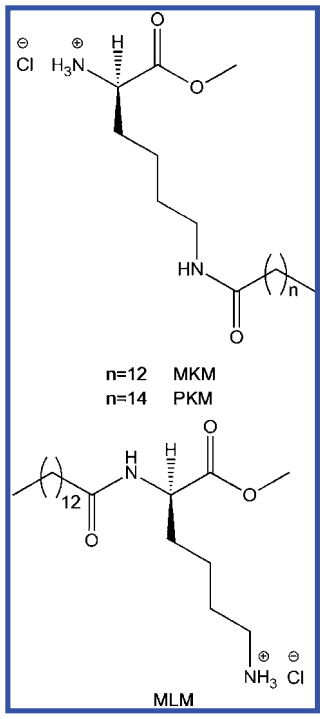


**Figure 1.** Molecular structures of the cationic lysine-based surfactants.

**2.4. Hemolysis Assay.** The membrane-lytic activity of the surfactants was examined by hemolysis assay. 25 $\mu$L aliquots of rat or human erythrocyte suspension were exposed to different surfactant concentrations based on the hemolytic potency of each compound (from 50 to 500 $\mu$g/mL for MKM and PKM, 10 to 60 $\mu$g/mL for MLM, and 2.5 to 20 $\mu$g/mL for HTAB) and dissolved in PBS buffer in a total volume of 1 mL. The samples were incubated at room temperature for 10 min and then centrifuged at 10 000 rpm for 5 min. Two controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). Absorbance of the hemoglobin release in supernatants was measured at 540 nm by a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan), and the hemolysis percentages were determined by comparison with the positive control samples completely hemolyzed with distilled water. Concentration−response curves were obtained from the hemolysis results, and the concentration inducing 50% hemolysis ($HC_{50}$) was calculated.

**2.5. Protection against Hypotonic Hemolysis.** The antihemolysis activity of the surfactants was evaluated with both rat and human erythrocytes. A volume of erythrocyte suspension (25 $\mu$L) was incubated with different concentrations of each surfactant (from 25 to 125 $\mu$g/mL for MKM, 50 to 210 $\mu$g/mL for PKM, and 10 to 50 $\mu$g/mL for MLM), dissolved in hypotonic solution of PBS in a total volume of 1 mL. The PBS buffer was diluted to the osmolarity provoking about 80−90% hemolysis of untreated samples. The osmotic pressure of the solutions was calculated by the freezing point method, using a cryoscopic osmometer (Osmomat 030). Antihemolysis was determined after incubating the cells for 10 min at room temperature, following the procedure described above for the


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hemolysis assay. The concentrations resulting in maximum protection against hypotonic hemolysis ($cAH_{max}$) were estimated from concentration−response curves. The antihemolytic potency of the surfactants was expressed as the percentage of hemolysis reduction from the level in samples not treated with surfactants.

**2.6. Volume Expansion Calculation.** To determine the percentage of cell volume expansion induced by surfactants, a 25 $\mu$L aliquot of rat or human erythrocyte suspension was incubated with the $cAH_{max}$ of each compound dissolved in PBS solutions of different osmolarities. The degree of hemolysis was determined by the same procedure described above. Concentration−response curves were constructed from hemolysis results, and the osmolarities inducing 50% hemolysis ($C_{50\%}$) were then calculated. To avoid differences in the hemolysis ratios resulting from the different erythrocyte suspensions, a hemolysis control curve was performed without adding surfactant.

Theoretical calculations of the volume expansion induced by surfactants were carried out in line with Ponder,[18] who proposed that the association between the critical hemolytic volume ($V_h$) and the osmotic concentration inducing 50% hemolysis ($C_{50\%}$) is described by the equation:

$$V_h = V_{na} + V_a(C_{iso}/C_{50\%}) \quad (1)$$

where $V_{na}$ is the osmotically nonactive volume representing 30% of the normal erythrocyte volume ($V_o$ = 98 fL), $V_a$ is the osmotically active part of the erythrocyte volume representing 70% of $V_o$, and $C_{iso}$ is the iso-osmotic concentration. The results were $V_{na}$ = 29.4 fL; $V_a$ = 68.6 fL; $C_{iso}$ = 0.300 Osmol/L; $C_{50\%}$ = value in Osmol/L determined for the control or for the surfactant. The relationship between the $V_h$ of the control and $V_h$ of treated cells was calculated and expressed as a percentage.[19]

**2.7. Fluorescence Anisotropy Measurements.** To determine cell membrane fluidity by fluorescence anisotropy, DPH and TMA-DPH fluorescent probes were used. Treated and nontreated rat cell suspensions (hematocrit of 0.01%) in PBS were labeled with the fluorescent probes (final concentration in samples $10^{-6}$ M) during incubation in the dark at room temperature for 1 h. The changes in membrane fluidity were evaluated in samples assayed in hypotonic condition, as well as in isotonic medium with increasing concentration range and varying pH (5.4, 6.5, and 7.4). DPH is located within the hydrophobic region of the bilayer membrane, while TMA-DPH is incorporated near the surface of the cell membrane.[20] The fluorescence anisotropy values are inversely proportional to cell membrane fluidity. A high degree of fluorescence anisotropy represents a high structural order and/or low cell membrane fluidity.[21] Steady-state anisotropy was measured with a SLM-Aminco AB-2 spectrofluorometer, using polarizers in the L configuration in a quartz cuvette at room temperature. Samples were illuminated with linearly (vertically or horizontally) polarized monochromatic light ($\lambda_{ex}$ = 365 nm), and the fluorescence intensities emitted ($\lambda_{em}$ = 425 nm) parallel or perpendicular to the direction of the excitation beam (slit-widths: 8 nm) were recorded. Fluorescence anisotropy ($r$) was calculated automatically by the software provided with the instrument, according to the following equation:

$$r = (I_{vv} - I_{vh}\times G)/(I_{vv} + 2I_{vh}\times G) \quad (2)$$

where $I_{vv}$ and $I_{vh}$ represent the components of the light intensity emitted, respectively, parallel and perpendicular to the direction of the vertically polarized excitation light. The factor $G = I_{hv}/I_{hh}$ was used to correct the inequality of the detection beam to horizontally and vertically polarized emission.[21]

**2.8. Erythrocyte Ghost Preparation.** After surfactant treatment at room temperature for 10 min, human erythrocyte ghost membranes were prepared following the procedure of Fairbanks et al.[22] The packed erythrocytes were hemolyzed by hypotonic lysis in 5 mM phosphate buffer at pH 8.0. The pellet obtained by centrifugation subsequent to hemolysis was resuspended and washed several times (by suspension in the buffer and centrifugation at 14 800 rpm for 15 min) until white ghost membranes were obtained. The protein content of erythrocyte ghosts resuspended in PBS was measured by the Bio-Rad assay (Bio-Rad, Hercules, CA), which is based on the dye-binding procedure of Bradford,[23] using bovine serum albumin (BSA) as a protein standard.

**2.9. Electrophoretic Analysis of Membrane Proteins.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the membrane proteins was performed under reducing conditions, according to the procedure of Laemmli,[24] using a Mini-PROTEAN Tetra Cell unit (Bio-Rad, Hercules, CA). The slab gel consisted of a 7.5% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. The prepared human erythrocyte ghost membranes were mixed with 2× SDS sample buffer, heated at 95 °C for 5 min, and then loaded on the gel in a concentration of 15 $\mu$g of protein. Electrophoresis was carried out for 10 min at 60 V followed by 35 min at 200 V. Protein bands were viewed by staining with Coomassie brilliant blue R-250 for an hour under gentle shaking and destained with a mixture of 7.5% methanol and 10% acetic acid. The molecular weight of the membrane proteins was estimated from the molecular size marker (Bio-Rad Precision Plus Unstained Standard), ranging from 10 to 250 kDa. Densitometry analysis of Coomassie stained gel was performed using IMAT software (developed by the Scientific-Technical Services of the University of Barcelona). Actin (band 5) was chosen as the "internal standard" for quantitative calculations.[25]

**2.10. Studies of Erythrocyte Morphology by Scanning Electron Microscopy (SEM).** Cell morphology changes derived from the interaction of the surfactants with the erythrocyte membrane were determined by incubating intact cells with a sublytic concentration (10 $\mu$g/mL) of each surfactant. Samples were prepared as described elsewhere,[4] and the resulting specimens were examined under a Zeiss DSM 940A scanning electron microscope (Carl Zeiss SMT AG, Jena, Germany).

**2.11. Statistical Analyses.** Each isotonic and hypotonic hemolysis experiment was performed at least three times using three replicate samples for each surfactant concentration tested. All anisotropy fluorescence values were also the means of three independent experiments. Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using Student's $t$-test or one-way analysis of variance (ANOVA) to determine the differences between the data sets, followed by Dunnett's or Tukey's post hoc tests for multiple comparisons using the SPSS software (SPSS Inc., Chicago, IL). $p < 0.05$ and $p < 0.005$ were considered significant. Pearson's correlation coefficients were also calculated, as indicated, by linear regression analysis using the SPSS software.

## 3. RESULTS AND DISCUSSION

Surfactant−biomembrane interaction studies were performed with the aim to gain insights into the mechanisms of membrane lysis. Low concentrations of the amphiphiles (below the CMC) were chosen to determine their interaction properties; as at nonsolubilizing concentrations, surfactants serve useful purposes in biological experimentation as agents that permeabilize or perturb membrane structure in various ways.[6] Here, erythrocytes were chosen as biomembrane model, whereas artificial membranes such as solid-supported lipid bilayers[26] and phosphatidylcholine vesicles[27,28] are also widely used to perform such kind of studies. Each membrane model presents its advantages and disadvantages, which makes it difficult in the election of the most appropriate model. Studies using solid-supported lipid bilayers can give accurate results about corrosion of the membrane caused by surfactants at determined concentrations,[26] while phosphatidylcholine vesicles are used as model systems in search of specific biophysical mechanisms of surfactant−membrane interaction.[27,28] Erythrocytes are also largely used as biomembrane model[2−4,11,29] and, thus, might be a suitable approach to complete, correlate, and/or corroborate the results obtained with the former models. Erythrocytes have enough functions in common with more specialized cells, and the given results may be good indicators of the biological

Table 1. Hemolysis, Antihemolysis, and Theoretical Calculation of the Volume Expansion Induced by the Surfactants

| surfactant (MW[a] g/mol) | CMC[b] ($\mu$g/mL) | $HC_{50}$[c] ($\mu$g/mL) | $cAH_{max}$[d] ($\mu$g/mL) | antihemolytic potency (%) | volume expansion (%) |
|---|---|---|---|---|---|
| Rat Erythrocytes | | | | | |
| MKM (406.66) | 650[e] | 340.86 ± 14.03[f] | 37.5 | 41.06 | 10.19 ± 2.22 |
| PKM (434.66) | 260[e] | 356.27 ± 13.68[f] | 50 | 26.55 | 0.79 ± 0.34 |
| MLM (406.66) | 765[f] | 38.88 ± 3.51[f] | 15 | 44.63 | 5.03 ± 0.17 |
| HTAB (598.40) | 400 | 11.61 ± 0.88[f] | 5 | 57.09 | 4.06 ± 0.13 |
| Human Erythrocytes | | | | | |
| MKM (406.66) | 650[e] | 411.74 ± 8.78[g] | 100 | 63.20 | 11.86 ± 3.21 |
| PKM (434.66) | 260[e] | 348.26 ± 4.89 | 150 | 37.94 | 0.79 ± 0.45 |
| MLM (406.66) | 765[f] | 71.89 ± 2.56[g] | 20 | 58.27 | 13.90 ± 2.12 |
| HTAB (598.40) | 400 | 11.60 ± 0.1 | 5 | 45.28 | 5.46 ± 1.26 |

[a]Molecular weight. [b]Critical micelle concentration; determined in water. [c]Concentration of surfactant inducing 50% hemolysis (mean ± SEM). [d]Concentration resulting in maximum protection against hypotonic hemolysis. [e]Colomer et al.[9] [f]Nogueira et al.[16] [g]Significantly different from rat erythrocytes (Student's $t$ test, $p < 0.05$).

activity of the surfactants at physiological conditions.[29,30] Therefore, these characteristics justify our choice of this model as a natural cell membrane system.

**3.1. Surfactants Induced Hemolysis and Antihemolysis.** The measurement of hemoglobin released from erythrocytes is a reliable experimental approach to studying plasma membrane permeabilization by various chemical compounds. Characterization of the biological interactions of the surfactants was conducted first by hemolysis studies. The lysine-based surfactants had hemolytic activity in isotonic medium against rat erythrocytes, with $HC_{50}$ values[16] dependent mainly on the position of the cationic charge and, to a lesser extent, on the length of the alkyl chain (Table 1). The surfactants MKM and PKM have the lowest $pK_a$ values (5.3 and 4.5, respectively) and consequently have the minor densitiy of cationic charge at physiological pH, whereas MLM, with a $pK_a$ = 8.1, is highly protonated. We found that MKM and PKM were considerably less hemolytic than MLM,[16] which means that the surfactant−membrane interactions in isotonic conditions were first affected by the charge density rather than by the hydrophobicity of the compounds. These results are in line with a previous report of our group,[31] which showed that other monocatenary lysine-based surfactants that differ in the alkyl chain length increased the hemolysis when the cationic charge density increases. Furthermore, we previously demonstrated that the charge density was directly related to the pH-sensitive activity of MKM and PKM: more protonable amino groups at acidic conditions add more charges to the polar head and consequently raise the membrane-lytic activity.[16] For these surfactants, the variation of pH promotes a variation in the protonation state that would lead to changes in their amphiphilicity and aggregation shape,[31] which, in turn, changes their interaction properties with the cell membrane. On comparing results with rat erythrocytes and with human erythrocytes, significantly higher resistance ($p < 0.05$) of the human cells to MKM and MLM was found. Our findings corroborate previous studies, in which erythrocytes from different species (having specific phospholipid composition and mechanical properties of the cytoskeleton[32,33]) also had varied hemolytic behavior in the presence of surfactants[34,35] and of other different substances.[36,37]

The effect of surfactants on the resistance of erythrocytes on hypotonically induced lysis was also assessed with the aim to gain insight into the mechanisms of interaction. At low concentrations, well below those inducing lysis of erythrocytes, the lysine-based surfactants protected cells against their lysis in hypo-osmotic medium. The surfactants MKM and MLM showed similar antihemolytic potency, whereas PKM was the least active (Table 1). In rat erythrocytes, MLM reduced the extent of osmotically induced cell lysis by ∼45%, whereas the least active compound PKM reduced it by ∼25%. In human erythrocytes, MKM was the most active compound, inhibiting more than 60% of hypotonic lysis, whereas PKM was the least active as also observed with rat erythrocytes, reducing the hemolysis by less than 40% (Table 1). This means that, differently from the hemolytic activity, the antihemolytic potency was first affected by the hydrophobicity rather than by the charge density of the compounds.

The dose−response curves representing the antihemolytic profile of the surfactants in rat and human erythrocytes are reported in Figure 2a and b, respectively. It is worth noting that, with both human and rat erythrocytes, MLM and HTAB rapidly increased the hemolysis rate beyond their $cAH_{max}$ values, whereas the compounds MKM and PKM had a wide range of protective concentrations under hypotonic conditions. This behavior can be supported by the higher increase in the membrane fluidity at hypotonic medium induced by MKM and PKM in comparison to MLM and HTAB (see section 3.2). This might indicate that the former compounds incorporated in a higher extent into the bilayer, which in turn may support the maintenance of their antihemolytic activity over a wide concentration range. Surprisingly, we found no direct correlation between the antihemolytic profile (Figure 2) and antihemolytic potency (Table 1): PKM showed protective activity over various concentrations and the lowest antihemolytic potency, whereas MLM displayed higher antihemolytic potency and promptly lost its protective activity beyond the $cAH_{max}$. We can state that the antihemolytic potency depended especially on the hydrophobicity of the compound, while the antihemolytic profile is directly affected by the position of the cationic charge. Small differences in the structure of molecules can affect their capacity to adsorb to erythrocyte membrane and, thus, their protective effects.[38] Finally, we found one meaningful correlation: the surfactants MKM and PKM that protect against hypotonic hemolysis over a wide range of surfactant concentration are the ones with the higher $HC_{50}$ values, while the surfactants with the lower values of $HC_{50}$ (MLM and HTAB) are those that displayed rapid loss of antihemolytic protection with increasing concentration.

The effects of surfactants on the osmotic fragility of both rat and human erythrocytes, evaluated from the curves of hemolysis as a function of PBS solution osmolarities, are

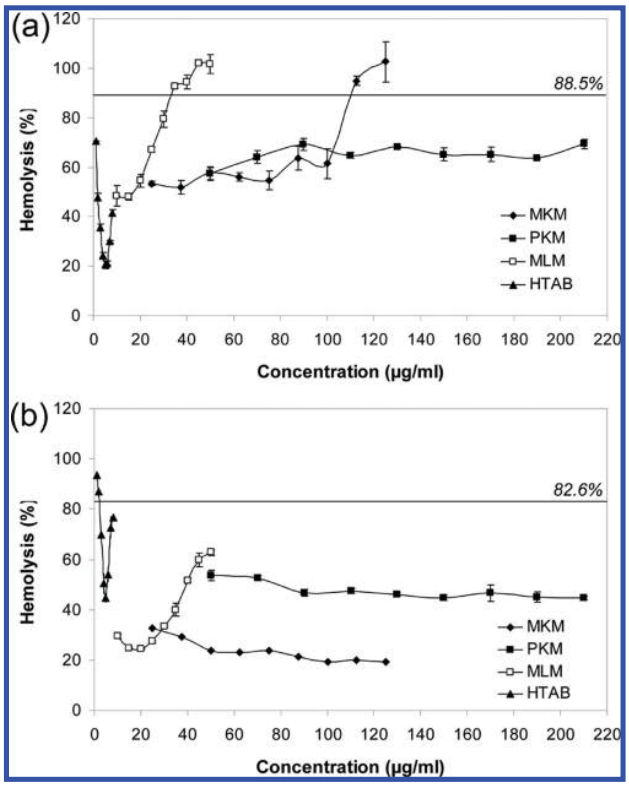


**Figure 2.** Dependence of the extent of hypo-osmotically induced hemolysis on surfactant concentration: (a) rat erythrocytes and (b) human erythrocytes. The straight solid line in each graph corresponds to the percentage of hemolysis reached by untreated erythrocytes in hypotonic medium (88.5% and 82.6% for rat and human erythrocytes, respectively). Each point represents the mean of three independent experiments ± SEM (error bars).

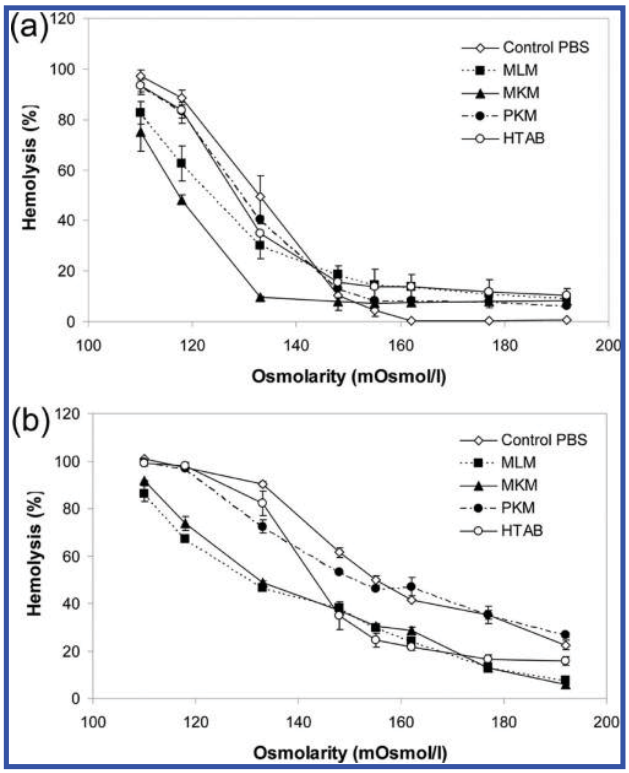


**Figure 3.** Osmotic lysis of (a) rat erythrocytes and (b) human erythrocytes in serial dilutions of PBS solution that reach different osmolarities at room temperature. Each point represents the mean of three independent experiments ± SEM (error bars).

shown in Figure 3. The overall results indicated that the addition of the surfactants MKM, MLM, and HTAB caused a shift in lysis curve to the left, revealing that these surfactants increased the resistance of erythrocytes to hypotonic osmotic lysis. In contrast, the compound PKM barely shifted the osmotic fragility curve and so had little effect on cells' osmotic resistance. These results are consistent with the low antihemolytic potency and cell volume expansion shown by PKM (Table 1). Noteworthy is the bifunctional behavior of HTAB with human erythrocytes (Figure 3b) that was not observed with the other surfactants: it induced lower hemolysis than the untreated control cells at high osmolarities, while at low osmolarities the % of hemolysis was similar to the control cells. This indicated that HTAB has the ability to improve the cell osmotic resistance up to a critic osmolarity value; beyond that, it becomes able to reach a molecular ratio in the membrane sufficient to enhance hemolysis.

In an attempt to find a correlation between antihemolytic potency and the volume changes induced by the surfactants to the cell,[2] we calculated the cell volume expansion (Table 1) using eq 1 and the data from the concentration–response curves at different osmolarities (Figure 3). We found no significant correlations between antihemolytic potency and cell volume expansion ($r$ = 0.9459 and $r$ = 0.7196, $p$ > 0.05, for human and rat erythrocytes, respectively). However, the trend that antihemolytic potency is related to cell volume expansion is in general followed by the surfactants even when the correlation obtained was not significant. For example, the compound PKM showed the lowest antihemolytic potency (Table 1), which can be related to negligible cell volume expansion. In contrast, MKM and MLM showed higher potency to protect against hypotonic lysis as well as enhanced cell volume expansion.

To explain the antihemolytic activity of surfactants, some mechanisms have been suggested: (i) It could be attributed to a phospholipid rearrangement,[39] which can lead to an increase in the ionic permeability of membrane and, thus, decrease the osmotic difference existing between the interior and exterior of the cell.[40] (ii) The insertion of surfactant molecules into the lipid bilayer increases either the cell surface area/volume ratio or the stretching capacity of the membrane, thereby allowing the cell to swell more and to attain a critical hemolytic volume before the leakage.[11,41,42] (iii) It could be related to the improved elastic, mechanical, and cohesive properties of the membrane resulting from the insertion of amphiphiles.[11] In our case, one individual or simultaneous mechanisms may be involved in the antihemolytic activity of the surfactants. The increased membrane fluidity observed in hypotonic condition (see section 3.2), together with the cell volume expansion, might support the mechanism that the surfactant molecules can increase the stretching capacity of the membrane, while the loss of band 3 protein (see section 3.3) might be related to changes in its ionic permeability.

Several studies have attempted to demonstrate a correlation between hemolytic activity and the CMC of amphiphiles, but there are no clear conclusions in the literature.[8,11,43] In our previous study, we found no significant correlation between the


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$HC_{50}$ and the CMC of the surfactants.[16] Here, we have studied the potential correlation between antihemolytic activity and the CMC. We found that the $cAH_{max}$ values were lower than the CMC (Table 1), but no significant correlations were obtained. This indicates that the surfactant-mediated hemolysis protection depends on its monomer form and not on its micellar structures. In contrast, significant correlation was found between antihemolytic potency and the CMC of the lysine-based surfactants in rat erythrocytes ($r = 0.9995$, $p < 0.05$), but not in human cells ($r = 0.9195$, $p > 0.05$). Moreover, the CMC also correlated with the cell volume expansion in human erythrocytes ($r = 0.9973$, $p < 0.05$), but not in rat erythrocytes ($r = 0.6974$, $p > 0.05$). We can conclude from these results that the ability of this group of lysine-based surfactants to protect against hypotonic hemolysis correlates, in general, directly with the CMC and inversely with the alkyl chain length of the product. We found that the less lipophilic amphiphiles (with shorter alkyl chain and higher CMC values) were the most protective, which was in contrast to the expected behavior: the more hydrophobic is the surfactant, the greater is its tendency to interact with the membrane and, thus, to have a strong antihemolytic potency. This contradictory behavior might be explained by the cutoff effect,[44] which could be caused by a decrease in the achievable concentration of the long-chained compound at the site of action due to its limit solubility, or by a complication in the intercalation of such amphiphile into the lipid bilayer due to its critical chain length, resulting, thus, in a decrease in the partition coefficient. In line with our findings, this latter model has been proposed to explain the cutoff in protection against hypotonic hemolysis of erythrocytes exposed to single chain amphiphiles at concentrations well below the CMC.[45]

**3.2. Effect of Surfactant Treatment on Membrane Fluidity.** To determine whether surfactants disturb the phospholipid bilayer across its thickness, steady-state fluorescence anisotropy ($r$) was measured to test membrane fluidity, because the latter is a sensitive indicator for monitoring fluorophore binding to regions of biomembranes whose movement is constrained.[46] Membrane fluidity is an important parameter relating to the structure and functional state of the cell membrane.[21] The fluorescent probes, DPH and TMA-DPH, were incorporated into the rat erythrocyte membranes to detect the changes in membrane fluidity in the region near the center of the bilayer and in the outer leaflet of the membrane, respectively. It is known that the accumulation of surfactants on the membrane may, in consequence, cause a change in its function. Depending on the magnitude of interaction, they may cause a change in membrane permeability by alteration of lipid order, orientation, and fluidity.[13]

Fluorescence anisotropy showed a significant decrease, along with the increase in surfactant concentration in isotonic conditions (Figure 4). This suggests that the lysine-based surfactants directly increase the fluidity of the phospholipid bilayer in a concentration-dependent manner. In general, the most marked decrease in anisotropy was found with the TMA-DPH probe, indicating that the external region of the bilayer is more affected by the compounds. We can conclude that, for MKM and PKM, the increase in membrane fluidity at increasing concentration might correlate directly with the increased hemolytic activity observed at the same concentration range.[16] PKM showed lower effects on packing constraints respect to MKM, which indicates inverse correlation between the alkyl chain length and the membrane fluidity. This finding is

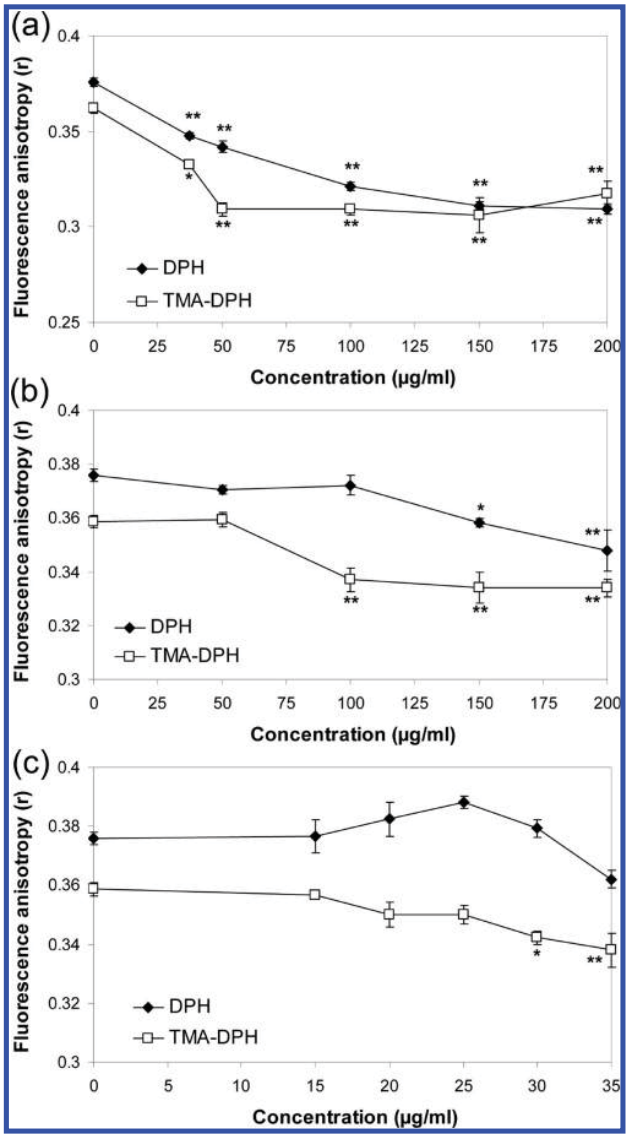


**Figure 4.** The effect of surfactants on the membrane fluidity of rat erythrocytes as a function of concentration. (a) MKM, (b) PKM, and (c) MLM. The fluorescence anisotropy ($r$) values determine the fluidity in both the external region (TMA-DPH) and the core (DPH) of the lipid bilayer. The ($r$) values were compared to the control (untreated cells) by ANOVA followed by Dunnett's post hoc test. *$p < 0.05$ and **$p < 0.005$ denote significant differences. Each point represents the mean of three independent experiments ± SEM (error bars).

in line with the cutoff effect[44] and also with a previous report, which showed that longer tail amphiphiles lead to the formation of more ordered bilayer.[5] Surprisingly, although MLM was the most hemolytic surfactant, it displayed the minor effects on membrane fluidity even with increasing concentrations. This behavior suggests that this compound might induce bilayer solubilization due to its elevated hemolytic potency, and, therefore, the increase in the bilayer fluidity might not be the main mechanism underlying its membrane-lytic activity.

The effect of the surfactants on membrane fluidity was also assessed in isotonic conditions when the pH of the medium was varied. It was found that the penetration of the surfactants in


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bilayers depends on the pH of the medium (Figure 5). The core and the external region of the membrane were significantly

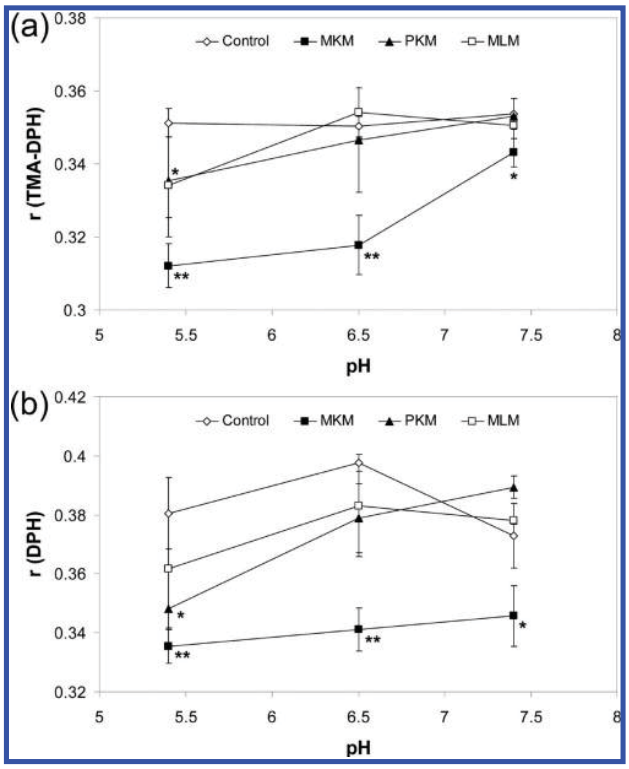


**Figure 5.** The effect of surfactants on the membrane fluidity of rat erythrocytes as a function of pH. (a) TMA-DPH and (b) DPH fluorescence probes. Surfactants were added at the following final concentrations: 25 $\mu$g/mL MKM and PKM, 15 $\mu$g/mL MLM. The fluorescence anisotropy ($r$) values were compared to the control (untreated cells) by ANOVA followed by Dunnett's post hoc test. *$p$ < 0.05 and **$p$ < 0.005 denote significant differences. The data represent the mean of three independent experiments ± SEM (error bars).

altered by MKM ($p$ < 0.005) at pH 6.5 and 5.4 and by PKM ($p$ < 0.05) at pH 5.4, as demonstrated by the DPH and TMA-DPH anisotropy values, respectively. In contrast, MLM showed no significant effect with varying pH in the structural order of lipids across the entire thickness of the bilayer. Although MKM and PKM were less lipophilic (more protonated) at acidic conditions, their disturbing properties into the lipid bilayer might be attributed to the increased electrostatic attraction between the positive polar headgroup (higher charge density at low pH) and the negatively charged cell membrane (see section 3.1). In turn, the lack of effects of MLM could be attributable to its tendency to solubilize the bilayer (see discussion above), which might also be due to its high charge density across the entire pH range. These results support our findings regarding the pH-responsive membrane-disruptive activity of the surfactants.[16] We found that MKM was much more potent in disrupting the plasma membrane at pH 6.5 and 5.4, while PKM also showed significantly higher lytic activity at pH 5.4. In contrast, MLM did not show pH-responsive activity. Therefore, we can conclude that the pH-sensitive activity of these compounds could be directly related to the order of the lipids in the membrane: at low pH, the incorporation of MKM and PKM into the membrane might increase, and, in consequence, the membrane becomes more fluid, which leads to lysis. The higher incorporation of MKM and PKM into the membrane can be also substantiated by the SEM studies of erythrocyte morphology at acidic conditions.[16] These compounds interacted intensively with the lipid bilayer and induced a spherocyte-type deformation at pH 5.4, which can be assumed to be the last stage of the morphological change with maximum accumulation of the compound into both the outer and the inner layers of the erythrocyte membrane before cell lysis.[47] In summary, the dependence of surfactant penetration into the membrane on the position of the cationic charge is clear: compounds with the positive charge on the $\alpha$-amino group of the lysine are deeply embedded in the lipid bilayer in acidic conditions.

The effect of the surfactants on membrane fluidity was also evaluated in the hypo-osmotic medium, and the results were compared to those obtained in isotonic medium with the same final concentrations. The concentrations assessed were those that showed the greatest antihemolytic activity in rat erythrocytes. Surfactant treatment in hypotonic conditions increased membrane fluidity significantly ($p$ < 0.005) in both the core and the external region of the bilayer (Table 2).

**Table 2. Steady-State Fluorescence Anisotropy of the Probes DPH and TMA-DPH Incorporated into Erythrocyte Membrane in Isotonic and Hypotonic Conditions[a]**

| samples ($\mu$g/mL) | ($r$) DPH (mean ± SE) | ($r$) TMA-DPH (mean ± SE) |
|---|---|---|
| control | 0.3772 ± 0.0081 | 0.3593 ± 0.0038 |
| | Isotonic Medium | |
| MKM (37.5) | 0.3480 ± 0.0005[b] | 0.3251 ± 0.0104[b] |
| PKM (50) | 0.3683 ± 0.0005 | 0.3546 ± 0.0022 |
| MLM (15) | 0.3594 ± 0.0059 | 0.3509 ± 0.0014 |
| HTAB (5) | 0.3708 ± 0.0005 | 0.3541 ± 0.0024 |
| | Hypotonic Medium | |
| MKM (37.5) | 0.2980 ± 0.0061[b,d] | 0.2982 ± 0.0053[b,c] |
| PKM (50) | 0.3211 ± 0.0094[b,d] | 0.3147 ± 0.0036[b,d] |
| MLM (15) | 0.3420 ± 0.0075[b] | 0.3305 ± 0.0013[b,c] |
| HTAB (5) | 0.3328 ± 0.0036[b,d] | 0.3362 ± 0.0040[b] |

[a] $r$ values = anisotropy measurements. [b] $p$ < 0.005 when compared to control PBS (Dunnett's post hoc test). [c] $p$ < 0.05 when compared to isotonic medium (Tukey's post hoc test). [d] $p$ < 0.005 when compared to isotonic medium (Tukey's post hoc test).

Moreover, significant changes ($p$ < 0.05 and $p$ < 0.005) in the lipid bilayer fluidity were observed in comparison to the same surfactant concentration assessed in physiological condition (with the exception of MLM (DPH) and HTAB (TMA-DPH)), corroborating that the hypotonicity of the medium enhances the effect of the surfactants in the membrane fluidity. These results can be directly correlated to the antihemolytic activity of the surfactants and might be one of its mechanisms (see section 3.1).

**3.3. Electrophoresis of Membrane Proteins.** SDS-PAGE experiments were run to assess whether surfactant-induced membrane lytic activity is associated with alterations in the erythrocyte membrane proteins and damage to them. We performed the electrophoresis experiments with human erythrocytes, as the preparation of ghost membranes was not very effective with rat erythrocytes. Moreover, this technique is widely performed for the same purpose with human erythrocytes.[46,48,49]


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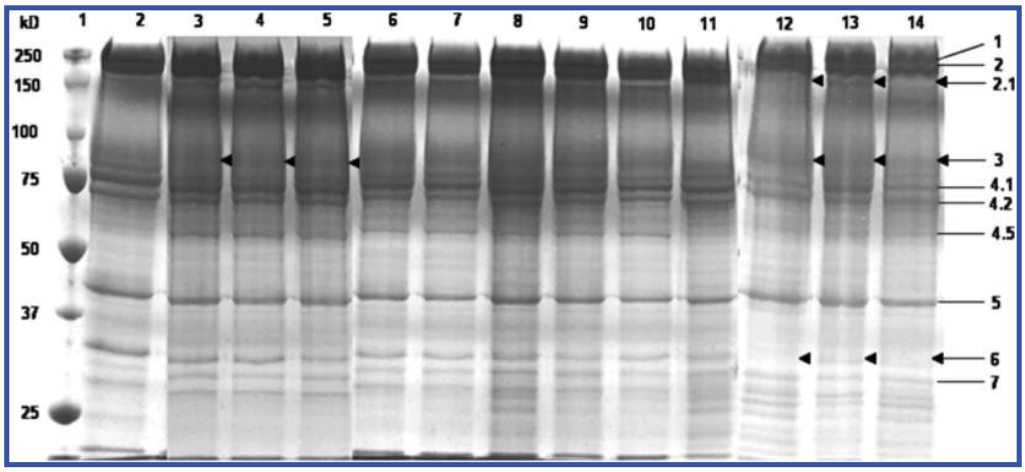


**Figure 6.** Effect of surfactants on human erythrocyte membrane skeletal proteins, as determined by SDS-PAGE. Following the preincubation of the erythrocytes for 10 min in the absence or presence of the surfactants, membranes were separated and washed as described in section 2.8. The names of the major cytoskeletal proteins follow the classification of Fairbanks et al.[22] and are given on the right of the gel. Arrowheads indicate the differences in the protein banding pattern between control and surfactant treatments. Each lane corresponds to a different treatment: (1) protein standard; (2) untreated membrane proteins (control); treatment of surfactants in hypo-osmotic medium at their $cAH_{max}$, (3) 100 $\mu$g/mL MKM, (4) 150 $\mu$g/mL PKM, (5) 20 $\mu$g/mL MLM; treatment of surfactants at concentrations about their $HC_{50}$, (6) 300 $\mu$g/mL MKM at pH 7.4, (7) 300 $\mu$g/mL PKM at pH 7.4, (8) 35 $\mu$g/mL MLM at pH 7.4, (9) 50 $\mu$g/mL MKM at pH 6.5, (10) 200 $\mu$g/mL PKM at pH 6.5, (11) 100 $\mu$g/mL MLM at pH 6.5, (12) 50 $\mu$g/mL MKM at pH 5.4, (13) 50 $\mu$g/mL PKM at pH 5.4, and (14) 75 $\mu$g/mL MLM at pH 5.4.

Figure 6 shows the electrophoretic profile of the erythrocyte membrane proteins. The well-established normal distribution of the major membrane cytoskeletal proteins is shown in lane 2, which contains untreated erythrocytes ghosts. The effect of the surfactants on the membrane proteins was evaluated after treatment under hypotonic conditions (at the $cAH_{max}$ concentrations) and in isotonic conditions varying the pH of the medium (at concentrations of about $HC_{50}$ for each pH). The treatments with surfactants in hypotonic medium led to a significant loss of band 3 (36.21%, 39.89%, and 11.88% for MKM, PKM, and MLM, respectively, as determined by densitometry analysis) (Figure 6; lanes 3−5). This corroborates a previous report that suggested that band 3, an anion exchange protein in human erythrocytes, participates in hypotonic hemolysis.[50] In contrast, as shown in Figure 6 and revealed by the densitometric analysis, the surfactants did not cause noticeable changes in the electrophoretic pattern of erythrocyte membrane proteins when the treatments were in isotonic medium at pH 7.4 and 6.5 (Figure 6, lanes 6−11). These results indicated minimal interaction with the bilayer proteins during surfactant-induced membrane lysis under physiological conditions and at the pH range characteristic of the early endosomes, respectively. However, when the treatments were performed at pH 5.4, all tested surfactants induced significant loss of band 3 (34.10%, 44.55%, and 40.34% for MKM, PKM, and MLM, respectively), ankyrin (band 2.1) (73.53%, 56.14%, and 45.66% for MKM, PKM, and MLM, respectively), and band 6 (81.80%, 68.53%, and 74.44% for MKM, PKM, and MLM, respectively) (Figure 6, lanes 12−14), indicating that these compounds caused degradation of some membrane proteins before the onset of cell lysis or that the proteins were segregated from the mother cell into shed microvesicles.[51,52] These microvesicles are enriched with membrane proteins,[53] and their release might depend on the level of membrane fluidity.[54] In summary, the alterations observed in some membrane proteins indicate that the surfactants change lipid−protein interactions in the bilayer, which can be a biophysical mechanism directly related to membrane lysis. These data suggest that an efficient bioactive compound in an intracellular drug delivery system might have the ability to interact with both membrane lipids and proteins to prompt the destabilization of the endosome membrane and release of the active content inside the cell.

**3.4. SEM Studies of Erythrocyte Morphology.** SEM experiments were conducted to better understand the interaction of cationic lysine-based surfactants with the lipid bilayer. From the changes in cell morphology, we assessed how the surfactants interact with the membrane, whether it is with the outer or inner layer of the lipid bilayer. Rat erythrocytes were treated with each surfactant in isotonic medium and physiological pH at the sublytic concentration of 10 $\mu$g/mL.[16] The effect of pH on erythrocytes' shape changes induced by these surfactant treatments was described previously by our group,[16] results that are complementary and correlate directly with the membrane-related properties studied here.

SEM examinations corroborated that the surfactants interacted with the lipid bilayer by altering the normal biconcave morphology of the cells (Figure 7). Control erythrocytes incubated in PBS solution were found to be discoid (Figure 7a). Despite varying membrane lytic activity of MKM and MLM, erythrocytes underwent similar morphological alterations after treatment with these surfactants, indicating that morphological changes are not affected by the position of the cationic charge, unlike hemolytic activity, which is strongly affected. MKM and MLM changed the discoid shape of cells at pH 7.4 to stomatocytes (Figure 7b and c, respectively), which is consistent with the bilayer hypothesis.[47] Stomatocytes are formed when the compounds interact with the inner layer of the bilayer, and thus, for MKM, the increased membrane fluidity in the core of the bilayer (see section 3.2) could also be related to this type of cell deformation due to the deeper incorporation of the surfactant. The stomatocyte-type deformation induced by MLM (Figure 7c) seems to show also endovesicle formation in the membrane, which can be related to torocyte-like endovesicles.[55] Torocyte endovesicles seem to be formed in a process in which an initially stomatocyte

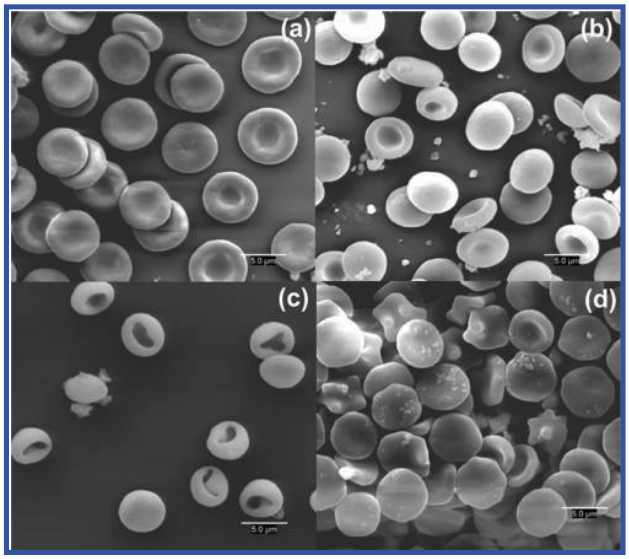


**Figure 7.** Effect of the cationic lysine-based surfactants on rat erythrocyte morphology. SEM images[16] of (a) control in PBS pH 7.4, and after incubation with the surfactants at pH 7.4: (b) MKM, (c) MLM, and (d) PKM. The erythrocytes were incubated for 10 min at the sublytic concentration of 10 $\mu$g/mL of each surfactant. Scale bars correspond to 5 $\mu$m.

invagination loses volume while maintaining a large surface area. Moreover, this type of deformation might be attributable to the interaction of the surfactant with both the outer and the inner layers of the lipid bilayer,[56] which could be directly related to the greater hemolytic activity of MLM than of MKM that we found.

However, PKM (with a longer alkyl chain) induced in general a leptocyte-type deformation (Figure 7d), but also prompted the morphological change of some cells to the shape characteristic of the first stage of echinocytosis. Echinocytes are induced when the compound added is inserted in the outer monolayer of the membrane, which, therefore, might substantiate the greater effect of PKM on the membrane fluidity in the external leaflet of the bilayer (see section 3.2). Leptocyte is a flattened cell with decreased volume that also has an unusually large membrane in proportion to its contents, which could be attributed to the greater incorporation of PKM into the lipid bilayer, thus giving an appearance of "excess" plasma membrane. This hypothesis of enhanced incorporation did not lead to increased hemolytic activity or membrane fluidity (see sections 3.1 and 3.2, respectively), possibly because PKM may increase the packing degree of the lipid bilayer and thus exert a protective effect at sublytic concentrations.[5]

Finally, as we have demonstrated no noticeable change in the membrane proteins' profile after surfactant treatments at physiological pH, the surfactant−protein interaction might not be involved in the morphological changes undergone by the erythrocytes in this condition. Our findings contradict studies of some authors[35,57] that reported the proteins of the membrane skeleton and integral membrane proteins as responsible for the shape of erythrocytes. However, our results obtained under acidic conditions are in accordance with these reports, as we demonstrated significant alterations in the membrane proteins' profile (see section 3.3) and the change in cell morphology to spherocytes.[16]

**3.5. Structure−Activity Relationship.** Studies on surfactant-induced biomembrane lysis were performed in an attempt to gain some mechanistic understanding and insights into the structure−activity relationship of this new class of cationic lysine-based amphiphiles. The main found structure−activity relationships were summarized in Table 3, in which is shown that, for molecules with similar hydrophobic character, the

**Table 3. Summary of the Structure−Activity Relationship of the Cationic Lysine-Based Surfactants[a]**

| surfactant | MKM | | PKM | | MLM | |
|---|---|---|---|---|---|---|
| charge position | $\alpha$-amino | | $\alpha$-amino | | $\varepsilon$-amino | |
| alkyl chain length | 14C | | 16C | | 14C | |
| hemolysis | | | | | | |
| pH 7.4 | + | | + | | +++ | |
| pH 6.5[b] | +++ | | ++ | | + | |
| pH 5.4[b] | +++ | | +++ | | + | |
| antihemolytic potency | +++ | | + | | +++ | |
| volume expansion | ++ | | − | | ++ | |
| membrane fluidity | DPH | TMA-DPH | DPH | TMA-DPH | DPH | TMA-DPH |
| increasing concentrations | +++ | +++ | ++ | +++ | − | ++ |
| pH 7.4 | ++ | + | − | − | − | − |
| pH 6.5 | ++ | ++ | − | − | − | − |
| pH 5.4 | +++ | +++ | ++ | + | − | − |
| hypotonic medium | +++ | +++ | ++ | ++ | + | + |
| membrane proteins | | | | | | |
| pH 7.4 | − | | − | | − | |
| pH 6.5 | − | | − | | − | |
| pH 5.4 | +++ | | +++ | | +++ | |
| hypotonic medium | + | | + | | + | |
| cell morphology | | | | | | |
| pH 7.4 | stomatocyte (+) | | leptocyte (+) | | stomatocyte (+) | |
| pH 6.5[b] | spherostomatocyte (++) | | Sstomatocyte (+) | | spherostomatocyte (++) | |
| pH 5.4[b] | spherocyte (+++) | | spherocyte (+++) | | spherocyte (+++) | |

[a]Effect level: (+) low, (++) medium, (+++) high, (−) no significant effect. [b]Nogueira et al.[16]


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position of the charged group and, thus, the density of charge play an important role in the overall surfactant's activity. The cationic charge on the $\alpha$-amino group of lysine determines the pH-sensitive membrane-lytic activity of the amphiphiles.[16] This specific activity can be explained by the increasing protonation state of the surfactants with decreasing pH and, beyond that, can be mechanistically evidenced by the increased disturbance in the packing of lipid bilayer and by the prominent changes on the membrane protein pattern at acidic environment. To substantiate this proposed mechanism, it is worth noting that the normal lipid−protein interactions are required for the maintenance of the overall membrane function.[44] In turn, the positive charge on the $\varepsilon$-amino group of lysine gives to the surfactant a greater hemolytic activity at physiological pH (due to the increased charge density), together with a prominent antihemolytic potency. This behavior might be because the highly hemolytic compounds immediately insert themselves into the erythrocyte lipid bilayer, likely increasing its permeability or even its lateral expansion ability, and hence initially conferring hypotonic protection.[58] Moreover, for this specific class of lysine-based surfactants, the longer is the alkyl chain, the lower is the overall membrane-disturbing activity. Surprisingly, the enhanced hydrophobicity did not directly increase the phospholipid bilayer-perturbing effects. Finally, we can conclude that the combination of structural parameters such as shorter alkyl chain (C14) and positive charge on the $\alpha$-amino group of lysine gives to the surfactant the overall much greater effects on membrane.

## 4. CONCLUSIONS

This study revealed some important characteristics of surfactant−biomembrane interactions. The mechanism of biomembrane lysis was shown to be associated with lipid bilayer disorganization, through interaction with the lipids and proteins of the membrane. We also showed that the phospholipid bilayer-perturbing properties of the surfactants depend on their structural features. A tendency might be established: the longer is the alkyl chain, the lower is its power to protect the cell against lysis in hypotonic medium. In addition, the compounds having the positive charge on the $\alpha$-amino group of the lysine (MKM and PKM) prompted higher disturbance in the lipid bilayer packing at acidic conditions. The SDS-PAGE experiments showed that the main interaction of the surfactants with the membrane proteins occurred at pH 5.4, while minimal interaction was at physiological pH. These findings can be interpreted as necessary steps in the overall process of pH-sensitive membrane-lytic activity of MKM and PKM, which might be directly attributable to their higher charge densitiy at low pH. Furthermore, SEM studies corroborated the interaction of the surfactants with the lipid bilayer, as demonstrated by the changes in the cell shape. On the basis of our overall results, we conclude that the pH-sensitive surfactants affect the structural and dynamic properties of the biomembranes especially at acidic environment. At the molecular level, they operate mainly by increasing the fluidization of the phospholipid bilayer and, to a lesser extent, by interacting with the proteins of the membrane. Finally, the results obtained here, which mechanistically corroborated the pH-sensitive activity of MKM and PKM previously demonstrated by our group,[16] suggested that these amphiphiles could be promising as a new class of multifunctional bioactive excipients for active intracellular drug delivery. Substantial efforts are still needed to fully understand the functions of surfactant-like biomolecules and to optimize the numerous biomedical and technical applications of these compounds rationally. Therefore, the insights into the biological processes of surfactant-induced changes in membrane pattern given in this Article are of great importance in increasing our knowledge of the phospholipid bilayer-perturbing properties of novel bioactive excipients and, thus, in developing new efficient and specific drug delivery devices.

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### Notes

The authors declare no competing financial interest.

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## Artículo 4

**COMPARATIVE SENSITIVITY OF TUMOR AND NON-TUMOR CELL LINES AS A RELIABLE APPROACH FOR *IN VITRO* CYTOTOXICITY SCREENING OF LYSINE-BASED SURFACTANTS WITH POTENTIAL PHARMACEUTICAL APPLICATIONS**

**(Sensibilidad comparativa de líneas celulares tumorales y no tumorales como un método confiable para el cribado *in vitro* de la citotoxicidad de tensioactivos derivados de lisina con potencial aplicación farmacéutica)**

**Daniele Rubert Nogueira, Montserrat Mitjans, M. Rosa Infante, M. Pilar Vinardell**

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# Resumen

*Objetivos*

Estudiar de modo comparativo el potencial citotóxico *in vitro* de una familia de tensioactivos aniónicos derivados de lisina que difieren en cuanto a su contraión, utilizando diferentes líneas celulares de origen tumoral y no tumoral y dos ensayos de viabilidad que valoran diferentes mecanismos de citotoxicidad.

*Material y métodos*

Se estudió una familia de tensioactivos aniónicos de doble cadena derivados de la $N^{\alpha},N^{\varepsilon}$-dioctanoil lisina (77K), comprendiendo 5 tipos de contraiones: tres inorgánicos (sodio, 77KS; litio, 77KL; y potasio, 77KP) y dos orgánicos (lisina, 77KK; y tris (hidroximetil) amino metano, 77KT). Se evaluó la sensibilidad de seis líneas celulares, dos tumorales (HeLa y MCF-7) y cuatro no tumorales (3T3, 3T6, HaCaT y NCTC 2544), a la exposición a los diferentes tensioactivos. Para ello, se utilizaron dos ensayos de viabilidad como marcadores de citotoxicidad: el ensayo de reducción de la sal de tetrazolio (MTT) y el ensayo de captación del rojo neutro (NRU).

*Resultados*

Se obtuvieron valores de citotoxicidad ($IC_{50}$) muy variables. Las líneas celulares 3T6 y NCTC 2544 fueron las más sensibles a los efectos tóxicos de los tensioactivos, mientras que los fibroblastos 3T3 y ambas las células tumorales fueron las más resistentes. Entre los ensayos de viabilidad, el MTT fue el más sensible para detectar la citotoxicidad, independientemente de la línea celular y del tensioactivo estudiado. De un modo general, los compuestos con contraión orgánico mostraron una tendencia a ser menos citotóxicos que los de contraión inorgánico. Sin embargo, el tensioactivo 77KL mostró citotoxicidad inferior en comparación con los otros tensioactivos con contraión inorgánico y, en algunos casos, también presentó menor efecto tóxico que los compuestos con contraión orgánico.

*Conclusiones*

Los diferentes contraiones tienen contribución directa en la respuesta citotóxica de los tensioactivos. Este estudio comparativo puede servir como una propuesta fiable para el cribado de nuevos productos con potencial aplicación farmacéutica y/o cosmética.

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# Comparative sensitivity of tumor and non-tumor cell lines as a reliable approach for *in vitro* cytotoxicity screening of lysine-based surfactants with potential pharmaceutical applications

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ABSTRACT

Surfactants are used as additives in topical pharmaceuticals and drug delivery systems. The biocompatibility of amino acid-based surfactants makes them highly suitable for use in these fields, but tests are needed to evaluate their potential toxicity. Here we addressed the sensitivity of tumor (HeLa, MCF-7) and non-tumor (3T3, 3T6, HaCaT, NCTC 2544) cell lines to the toxic effects of lysine-based surfactants by means of two *in vitro* endpoints (MTT and NRU). This comparative assay may serve as a reliable approach for predictive toxicity screening of chemicals prior to pharmaceutical applications. After 24-h of cell exposure to surfactants, differing toxic responses were observed. NCTC 2544 and 3T6 cell lines were the most sensitive, while both tumor cells and 3T3 fibroblasts were more resistant to the cytotoxic effects of surfactants. $IC_{50}$-values revealed that cytotoxicity was detected earlier by MTT assay than by NRU assay, regardless of the compound or cell line. The overall results showed that surfactants with organic counterions were less cytotoxic than those with inorganic counterions. Our findings highlight the relevance of the correct choice and combination of cell lines and bioassays in toxicity studies for a safe and reliable screen of chemicals with potential interest in pharmaceutical industry.



## 1. Introduction

Surfactants represent one of the most widely applied excipients in the pharmaceutical and cosmetic industry due to their surface and interface activities. In order to minimize adverse reactions derived from the toxic potential of surfactants, the type of surfactant and concentration used should be considered when designing products for preformulation trials (Benassi et al., 2003; Paulsson and Edsman, 2001). Our previous research into new surfactants with low toxicity and a wide range of applications led to the development of a range of biocompatible surfactants derived from amino acids (Benavides et al., 2004a,b; Martinez et al., 2006; Mitjans et al., 2003). In this context, amino acid-based surfactants constitute a promising choice for applications in topical pharmaceutical products, as well as in novel biocompatible drug delivery devices (Morán et al., 2010; Nogueira et al., 2011). As the surface properties (hydrophobicity and surface charge) have a major impact on cellular uptake of particulate drug delivery systems, the incorporation of charged surfactants in these carriers may improve the targeting to specific cells and tissues, e.g. in cancer therapy (Schöler et al., 2001). Before this class of compounds can be approved for these purposes, however, accurate information about their toxicity is required. Thus, a complete toxicological evaluation of their effects should be performed by comparing a battery of complementary *in vitro* bioassays (Fisher et al., 2003).

Safety evaluation of new products or ingredients destined for human use is crucial prior to exposure. Therefore, rapid, sensitive and reliable bioassays are required in order to examine the toxicity of these substances. Established cell lines are useful alternative test systems for toxicological studies of this kind (Crespi, 1995); however, they must be chosen with care with regard to their origin (Jondeau et al., 2006). Moreover, cytotoxicity assays are among the most common *in vitro* endpoints used to predict the potential toxicity of a substance in a cell culture (Martinez et al., 2006). Cell damage is manifested in several ways, including mitochondrion and plasma membrane dysfunction and, fluctuating intracellular reduction capacity (Kim et al., 2009). Current standard approaches to gauge the degree of cell damage include assays that measure various aspects of cell viability, such as metabolic activity and plasma membrane integrity. The MTT reduction assay, which

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determines cell metabolic activity, is among the most commonly used endpoints. This method measures the reduction of MTT salt to a colored insoluble formazan in active mitochondria in viable cells and also, in certain cases, outside the mitochondria (Berridge et al., 2005; Liu et al., 1997). The neutral red uptake (NRU) assay, which is also widely used in biomedical applications, measures the uptake of neutral red dye by viable cells with intact plasma membrane, and its concentration in lysosomes (Repetto et al., 2008). Differences in the sensitivity of endpoints, together with the type of cell model and the nature of the chemicals being tested, may explain inconsistencies in the results reported (Schröterová et al., 2009). Therefore, complementary endpoint assays based on various mechanisms, as well as comparative analysis of the sensitivity of several cell types, are strongly recommended to increase the reliability of results (Fisher et al., 2003; Schröterová et al., 2009).

Here we studied the sensitivity of two tumor and four non-tumor cell lines of different origins to the toxic effects of five anionic lysine-based surfactants that differ in the nature of their counterions. The choice of dermal and tumor cells as model systems is based on the wide use of surfactants in topical pharmaceuticals and more recently in drug delivery devices (e.g. in cancer therapy), respectively. The knowledge about the cytotoxicity and potential mechanisms of surfactant interaction with healthy and tumor cells may help on the development of specific and effective devices for cancer therapy. In previous studies, we identified a number of toxic effects of this class of surfactants (Sanchez et al., 2004, 2006a,b). Nevertheless, given that no single *in vitro* assay has the capacity to mimic all events that occur *in vivo*, and in order to complete these toxicological studies, here we performed a comparative evaluation using six cell types and two cytotoxicity assays, MTT and NRU. These two assays evaluate different cell physiological mechanisms and are considered to be the most common methods applied to study cell viability after exposure to toxic substances (Fotakis and Timbrell, 2006). To gain insight into structure-dependent toxicity, we also discuss the influence of the counterions on the cytotoxic effects of the surfactants. This comparative study performed using six cell lines and two *in vitro* endpoints can be considered a suitable approach for toxicological screening of chemical compounds prior to pharmaceutical applications.

## 2. Materials and methods

### 2.1. Chemicals and reagents

L-Lysine monohydrochloride, L-lysine, Tris, and the bases NaOH, LiOH and KOH were purchased from Merck (Darmstadt, Germany). 2,5-Diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye and dimethylsulphoxide (DMSO) were from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin–EDTA solution (170,000 U/l trypsin and 0.2 g/l EDTA) and penicillin–streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) were purchased from Lonza (Verviers, Belgium). The 75 $cm^2$ flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland).

### 2.2. Surfactants tested

Five anionic amino acid-based surfactants derived from $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine and with counterions of distinct chemical nature were studied: two salts with organic counterions – lysine salt (77KK) and Tris (hydroxymethyl) aminomethane salt (77KT); and three salts with inorganic counterions – sodium salt (77KS), lithium salt (77KL) and potassium salt (77KP) (Fig. 1). These surfactants were synthesized in our laboratory as previously described (Sanchez et al., 2006a; Vives et al., 1999).

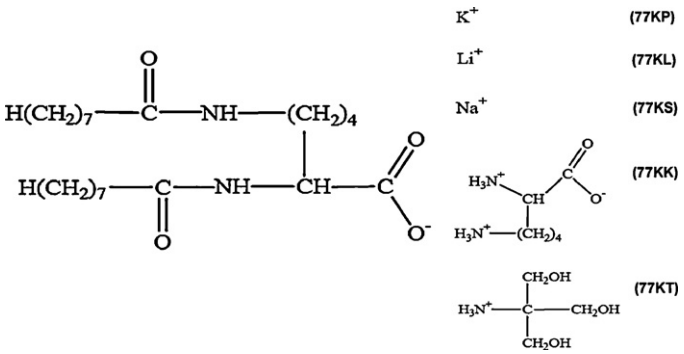


**Fig. 1.** Molecular structure of anionic lysine-based surfactants with distinct counterions. The codes P, L, S, K and T represent potassium, lithium, sodium, lysine and Tris, respectively.

### 2.3. Cell cultures

Two tumor cell lines (HeLa, human epithelial cervical cancer and MCF-7, human breast cancer) and four non-tumor cell lines (3T3, murine Swiss albino fibroblasts; 3T6, spontaneously transformed 3T3 murine Swiss albino fibroblasts; HaCaT, spontaneously immortalized human keratinocytes and NCTC 2544, normal human undifferentiated keratinocytes) were used. The 3T3, HeLa and MCF-7 cell lines were grown in DMEM medium (4.5 g/l glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The HaCaT cell line was cultured under the same conditions as described above, except for supplementation with 10 mM Hepes buffer. The NCTC 2544 and 3T6 cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were routinely grown in 75 $cm^2$ culture flasks and maintained at 37 °C in a humidified 5% $CO_2$ atmosphere. Cells were trypsinized using trypsin–EDTA when they reached approximately 80% confluence.

### 2.4. Experimental design

Cells were seeded into the central 60 wells of 96-well cell culture plates in 100 μl of complete culture medium at the following initial densities (cells/ml): $1 \times 10^5$ for MCF-7, $8.5 \times 10^4$ for 3T3 and HeLa, $5 \times 10^4$ for 3T6, HaCaT and NCTC 2544. Cells were incubated for 24 h under 5% $CO_2$ at 37 °C and the medium was then replaced with 100 μl of fresh medium supplemented with 5% FBS containing 0.22-μm filter-sterilized surfactant solution at the required concentration (serial dilutions between 7.8–500 μg/ml). Each concentration was tested in triplicate and control cells were exposed to medium with 5% FBS only.

### 2.5. Cytotoxicity assays

#### 2.5.1. MTT assay

The MTT assay is based on the protocol first described by Mosmann (1983). In this assay, living cells reduce the yellow tetrazolium salt MTT to insoluble purple formazan crystals. After cell incubation for 24 h, the surfactant-containing medium was removed, and 100 μl of MTT in PBS (5 mg/ml) diluted 1:10 in FBS-free medium without phenol red was then added. Plates were further incubated for 3 h, after which time the medium was removed, and cells were washed in PBS. The purple formazan product was then dissolved by adding 100 μl of DMSO to each well.

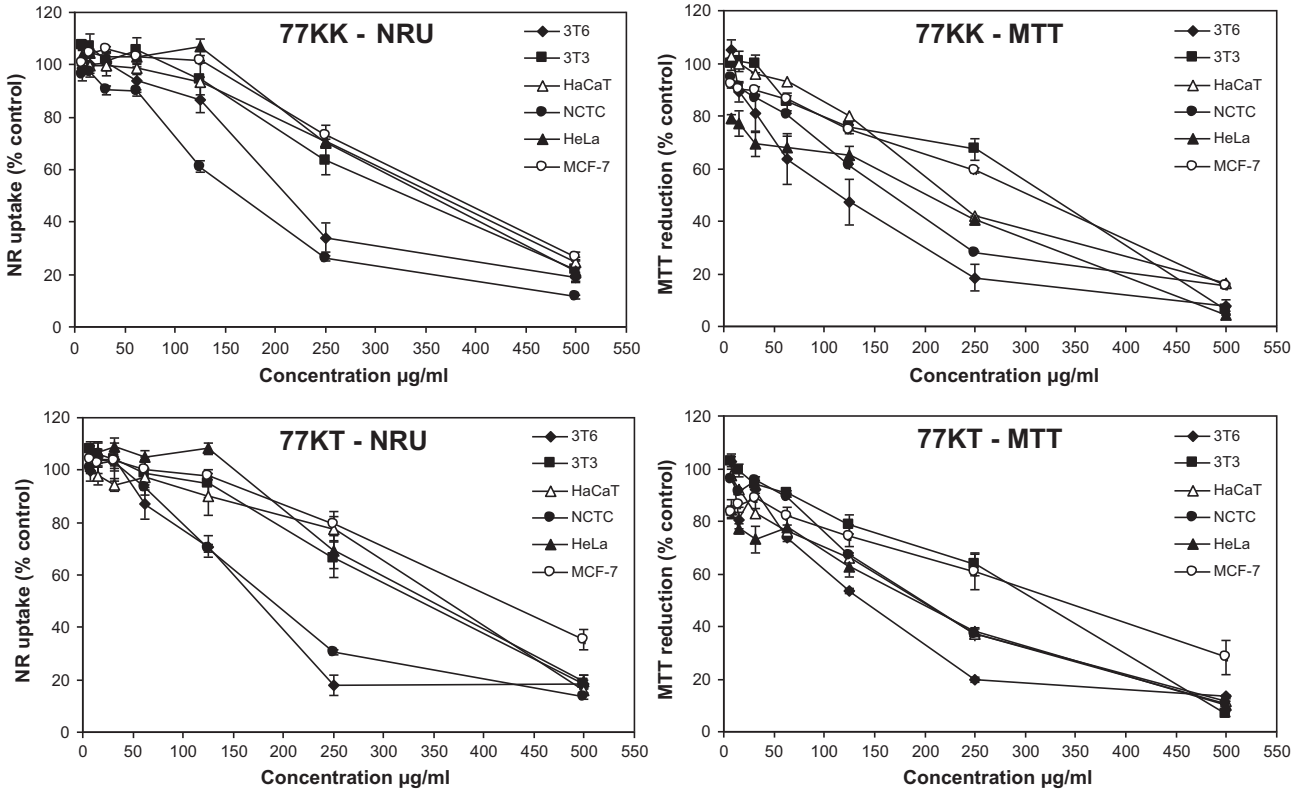


**Fig. 2.** Representative concentration–response curves from 24-h exposure of the two tumor and four non-tumor cell lines to surfactants with organic counterions: 77KK and 77KT. Values were obtained from NRU and MTT assays. Data are expressed as mean ± S.E.M. of three independent experiments, performed in triplicate.

Plates were then placed in a microtitre-plate shaker for 10 min at room temperature and the absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of tetrazolium salt reduction by viable cells against the untreated cell control (cells with medium only).

### 2.5.2. NRU assay

Based on the protocol described by Borenfreund and Puerner (1985), the NRU assay determines the accumulation of the NR dye in the lysosomes of viable, undamaged cells. Following exposure to the surfactants solutions, cells were incubated for 3 h with NR dye solution (50 μg/ml) dissolved in medium without FBS and phenol red. Cells were then washed with PBS, followed by the addition of 100 μl of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water to extract the dye. Plates were gently shaken for 10 min to ensure complete dissolution. We then measured the absorbance of the extracted solution at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of uptake of NR dye by lysosomes against the untreated cell control (cells with medium only).

### 2.6. Statistical analyses

The cytotoxicity of each surfactant was expressed as percentage of viability with regard to untreated control wells (the mean optical density of untreated cells was set at 100% viability) in terms of its $IC_{50}$ (concentration causing 50% death of the cell population), calculated from concentration–response curves. Results are expressed as mean ± standard error of the mean (S.E.M.) of at least three independent experiments.

Statistical analyses of the individual $IC_{50}$-values were performed using Student's *t*-test or one-way analysis of variance (ANOVA) to determine the differences between the sets of data. Tukey's *post hoc* multiple comparison test was also used to further identify significant differences between cell lines, as indicated, using the SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$, $p < 0.01$, $p < 0.005$ or $p < 0.001$ were considered to denote significance. Pearson's correlation coefficients between the $IC_{50}$-values obtained from the cytotoxic assays and cell lines were also calculated by linear regression analysis.

## 3. Results

### 3.1. In vitro cytotoxicity of the compounds

Dose–response curves for each surfactant obtained from NRU and MTT assays in all six cell lines are given in Figs. 2 and 3, for the surfactants with organic and inorganic counterions, respectively. These cytotoxicity assays revealed that a 24 h-exposure of the tumor and non-tumor cell lines to different concentrations (ranging from 7.8 to 500 μg/ml) of the five anionic lysine-based surfactants produced a dose-dependent reduction in the number of viable cells. Differences between cell lines as well as various sensitivities to the surfactants were demonstrated by calculating the half maximal inhibitory concentrations ($IC_{50}$) (Fig. 4). $IC_{50}$-values ranged from 103.67 μg/ml (77KL with 3T6 cells) to 468.53 μg/ml (77KL with MCF-7 cells), highlighting the wide cell sensitivity to these compounds.

Observation of $IC_{50}$-values shows that some cells lines were markedly less sensitive to the cytotoxic effects of the surfactants. In general, the tumor cell lines HeLa and MCF-7 and 3T3 fibroblasts were more resistant to the deleterious effects of the surfactants, while 3T6 and NCTC 2544 cell lines showed greater sensitivity, with $IC_{50}$-values lower than 200 μg/ml (Fig. 4). By scoring the sensitivity of the cells to the five surfactants, we can rank the

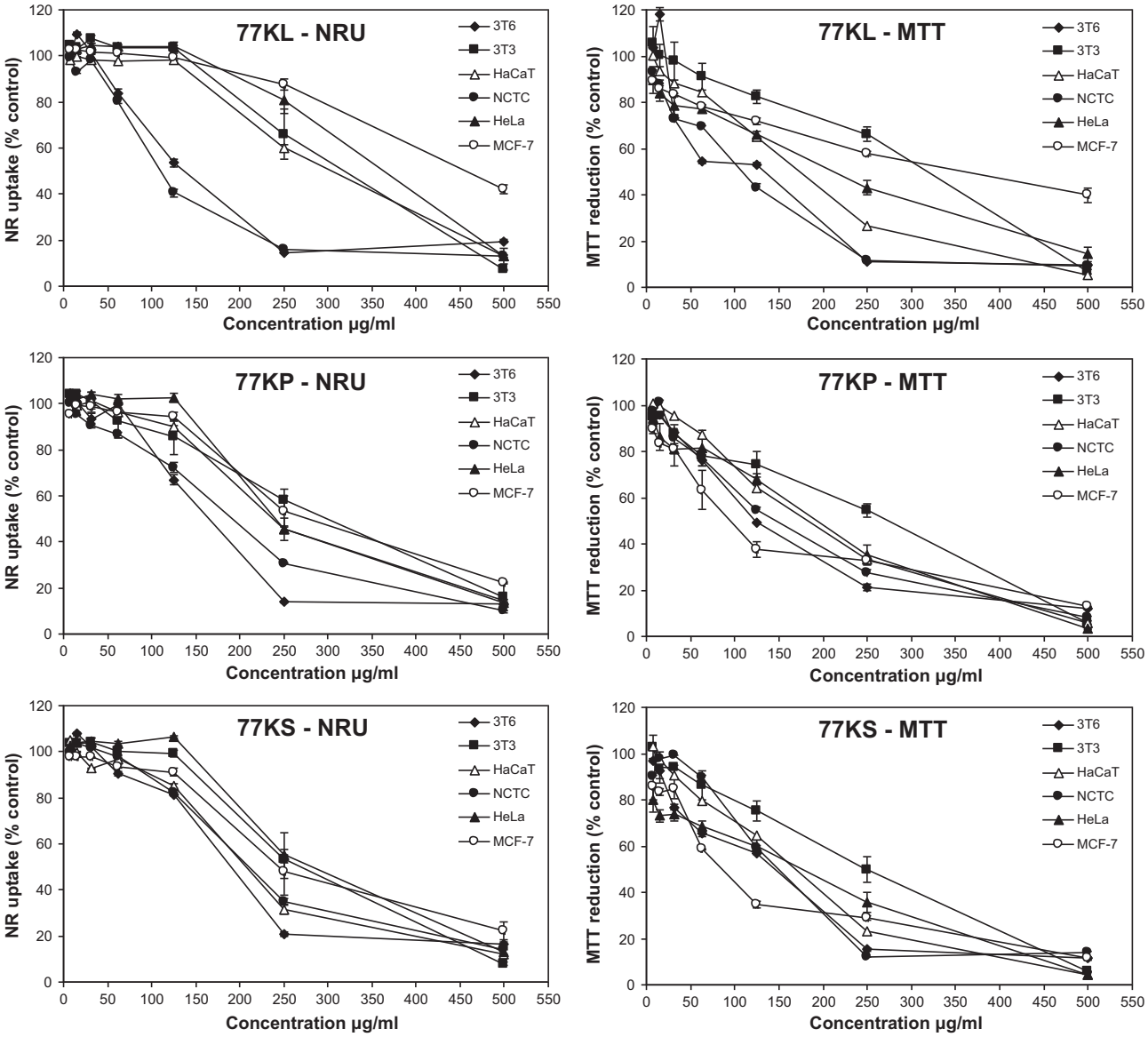


**Fig. 3.** Representative concentration–response curves from 24-h exposure of the two tumor and four non-tumor cell lines to surfactants with inorganic counterions: 77KL, 77KP and 77KS. Values were obtained from NRU and MTT assays. Data are expressed as mean ± S.E.M. of three independent experiments, performed in triplicate.

cell lines from the most resistant to the most sensitive as follows: MCF-7 > 3T3 > HeLa > HaCaT > NCTC 2544 > 3T6 by the NRU assay, and 3T3 > MCF-7 > HeLa > HaCaT > NCTC 2544 > 3T6 by the MTT assay.

The levels of toxicity varied depending not only on cell line and substance tested, but also on the viability assay used. Regardless of the compound or cell line, cytotoxicity was detected and observed earlier by the MTT assay. In contrast, the NRU assay was less sensitive and discriminative. The MTT assay (Figs. 2 and 3) showed significant decrease in the metabolic activity of cells in a concentration-dependent manner and, in general, the lowest concentration of each surfactant that significantly reduced cell viability in all six cell lines ranged from 31.25 to 125 μg/ml. In contrast, no apparent cytotoxicity was detected when the same concentration range was assessed by the NRU assay (Figs. 2 and 3). In this assay, significant loss of cell viability was detected for all the surfactants only at the highest concentrations tested (250 and 500 μg/ml), except for 3T6 and NCTC 2544 cell lines, which showed greater sensitivity to the surfactants in both assays.

The surfactants differed only in the type of counterion, and the overall results showed that those with organic counterions (77KK and 77KT) were less cytotoxic than those with inorganic counterions (77KL, 77KP and 77KS). The $IC_{50}$-values for 77KK and 77KT ranged from 129.07 to 404.37 μg/ml, thereby indicating that they were less cytotoxic than the surfactants with inorganic counterions. The relatively low $IC_{50}$-values for 77KS and 77KP, ranging from 113.97 μg/ml to 298.67 μg/ml, indicate higher cytotoxicity. In contrast, the surfactant 77KL with an inorganic lithium counterion was the most cytotoxic to 3T6 and NCTC 2544 cells ($IC_{50}$-values ranging from 103.67 to 146.23 μg/ml), while it had only mild toxic effects on 3T3 and HaCaT cells, with $IC_{50}$-values ranging from 143.07 to 316.97 μg/ml. Moreover, 77KL was the least cytotoxic surfactant on the tumor cell lines, showing a cytotoxic effect ($IC_{50}$) only at 468.53 and 350.07 μg/ml for MCF-7 cells, and 351.23 and 244.10 μg/ml for HeLa cells, by the NRU and MTT assays, respectively. On the basis of the mean cytotoxicity of the surfactants on each cell line, as established by the MTT and NRU assays, we ranked the compounds from the least to the most cytotoxic as follows: 77KK < 77KT < 77KL < 77KP < 77KS.

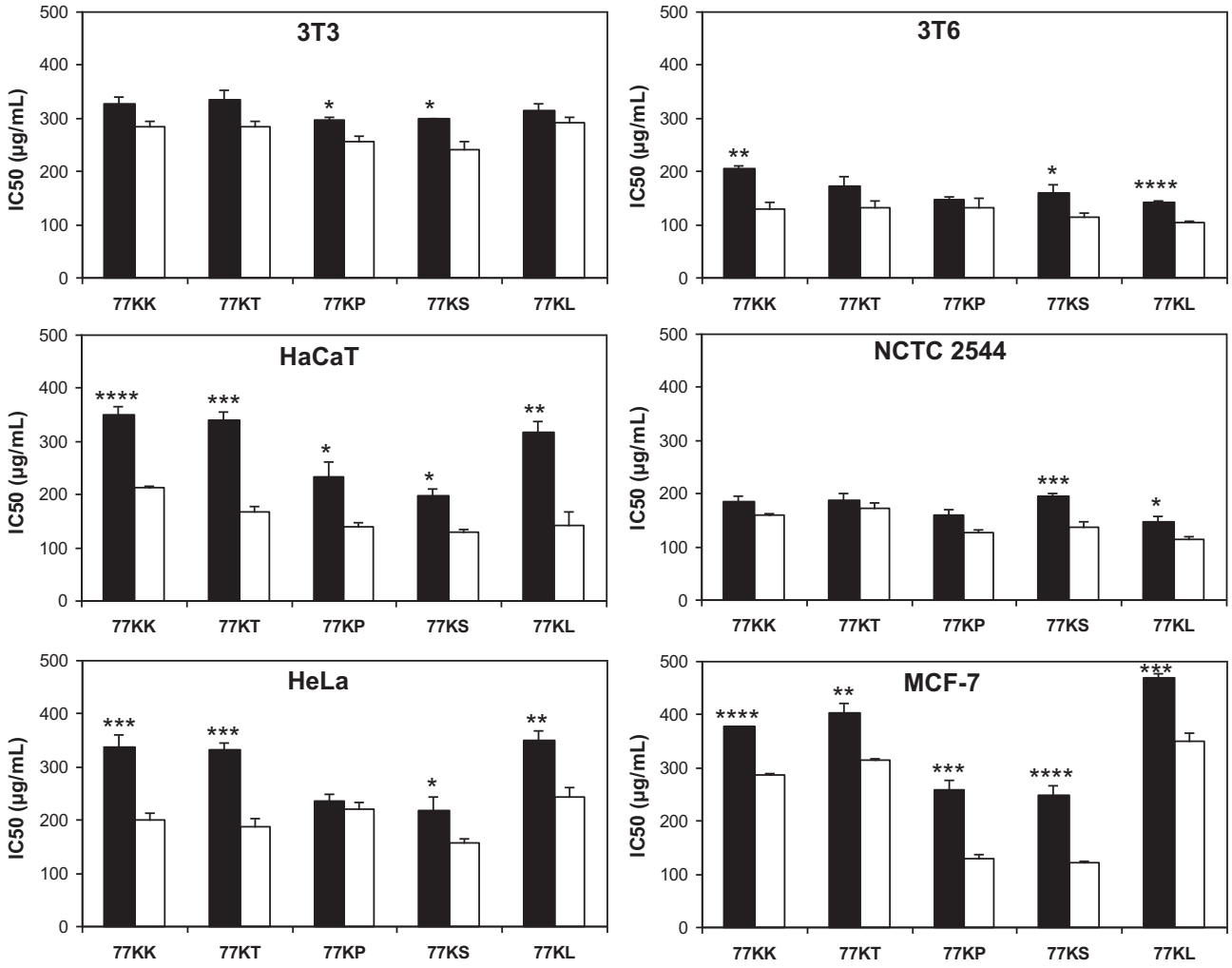


**Fig. 4.** Cytotoxicity of the lysine-based surfactants expressed as $IC_{50}$-values (μg/ml) on 3T3, 3T6, HaCaT, NCTC 2544, HeLa and MCF-7 cell lines, and measured by NRU (dark bars) and MTT (white bars) assays. Data are expressed as mean ± S.E.M. of three independent experiments, performed in triplicate. NRU and MTT assays were compared by the Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ denote significant differences.

### *3.2. Comparative analyses between cell lines and cytotoxicity assays*

The $IC_{50}$-values of the six cell lines obtained from the MTT and NRU assays were analyzed by one-way ANOVA followed by Tukey's *post hoc* test to identify significant differences. The overall results showed that the cell lines differed in their sensitivity to the five surfactants tested. Of note the response of 3T3 cells to the five surfactants differed significantly ($p < 0.005$) from almost all the other cell lines, as determined by the MTT assay. In contrast, the sensitivity of the tumor cells HeLa and MCF-7 was similar to 3T3 fibroblasts in response to 77KL and 77KP; and 77KK, 77KT and 77KL, respectively. The NRU and MTT assays showed that the sensitivity of 3T6 and NCTC 2544 cells to the surfactants did not differ significantly from each other, but showed significant differences ($p < 0.05$) from all the other cell lines by the NRU assay, except in response to 77KS, as this surfactant produced fewer differences between the cell lines. Also worth mentioning is the HeLa and HaCaT cell lines that showed similar sensitivity, except in response to 77KL ($p < 0.005$) and 77KP ($p < 0.005$) as determined by the MTT assay.

Correlation analyses were performed to examine the relationship between the cell types. In general, poor correlations were obtained between the cell lines, even when the comparisons were performed only with the $IC_{50}$-values derived from the same cytotoxicity assay. Nevertheless, significant correlations were obtained for some combinations of cells and endpoints (Table 1).

Furthermore, correlation analyses between the MTT and NRU assays ($IC_{50}$-values) for each surfactant in the six cell lines was performed by mean square root linear regression analysis. The

**Table 1**
Significant correlations of $IC_{50}$-values between the cell types studied by using NRU and MTT cytotoxicity assays.

| Cell line – cytotoxicity assay | Pearson's correlation coeficient | *p* value |
|---|---|---|
| 3T3 NRU vs HaCaT NRU | 0.930 | $p < 0.05$ |
| 3T3 MTT vs HaCaT NRU | 0.899 | $p < 0.05$ |
| 3T3 MTT vs MCF-7 NRU | 0.983 | $p < 0.01$ |
| 3T3 MTT vs MCF-7 MTT | 0.994 | $p < 0.01$ |
| HeLa NRU vs HaCaT NRU | 0.959 | $p < 0.05$ |
| HeLa NRU vs MCF-7 NRU | 0.961 | $p < 0.01$ |
| HeLa NRU vs MCF-7 MTT | 0.986 | $p < 0.01$ |
| HeLa NRU vs 3T3 MTT | 0.982 | $p < 0.01$ |
| HeLa MTT vs NCTC 2544 NRU | 0.953 | $p < 0.05$ |
| HaCaT MTT vs 3T6 NRU | 0.918 | $p < 0.05$ |
| MCF-7 MTT vs HaCaT NRU | 0.910 | $p < 0.05$ |
| MCF-7 MTT vs MCF-7 NRU | 0.989 | $p < 0.01$ |

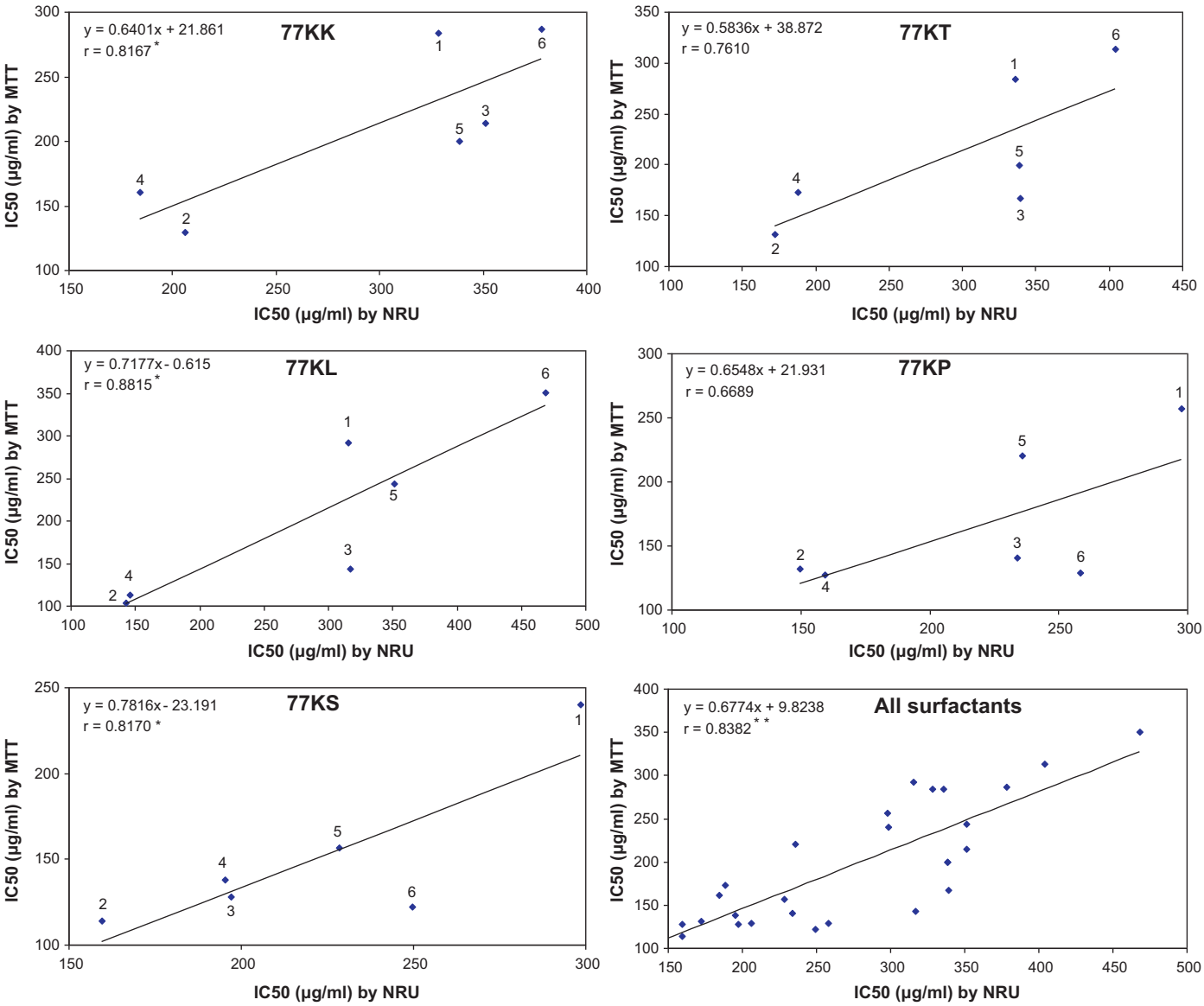


**Fig. 5.** Correlation of $IC_{50}$-values (µg/ml) between the NRU and MTT assays for each individual surfactant and for all surfactants. Each point corresponds to one cell line: (1) 3T3, (2) 3T6, (3) HaCaT, (4) NCTC 2544, (5) HeLa and (6) MCF-7. $r$ = Pearson's correlation coefficient. $^{*}p < 0.05$, $^{**}p < 0.01$ denote significant correlation.

relationship between the assays was expressed by their equations of linear regression and by Pearson's correlation coefficient ($r$). Significant correlations were obtained for 77KK ($p < 0.05$), 77KL ($p < 0.05$) and 77KS ($p < 0.05$), and also when the responses for all five surfactants were correlated simultaneously ($p < 0.01$). These observations indicate that, despite the individual characteristics of each cell line and compound tested, in general relatively good agreement was obtained between the two cytotoxicity assays (Fig. 5).

## 4. Discussion

*In vitro* cytotoxicity assays with established cell lines are useful tools for the general screening of chemicals in toxicological studies (Crespi, 1995). Surfactants are among the most versatile and widely used excipients in pharmaceutical products, and thus research on their toxic effects are required before they are considered for potential applications in topical drugs, cosmetic products (Martinez et al., 2006; Sanchez et al., 2004, 2006a), and drug delivery systems (Nogueira et al., 2011; Wang et al., 2007). It has been reported that the type of (co) surfactant in topical pharmaceuticals and nanoparticulate devices has great impact in their potential cytotoxic effects (Schöler et al., 2001; Weyenberg et al., 2007).

Here we studied the sensitivity of several tumor and non-tumor cell lines to the toxic effects of five anionic lysine-based surfactants that differ in the nature of their counterions. For this purpose, we compared the performance of the MTT and NRU assays, two widely used and relatively simple *in vitro* bioassay methods that provide information on cell metabolic activity and membrane integrity (particularly in the lysosomal compartment), respectively. A comparative study based on a range of different cell lines and *in vitro* endpoints are highly suitable during toxicity screening of chemicals with potential interest in the pharmaceutical industry. The information derived from a more complete study can raise the knowledge concerning to the safety profile of bioactive compounds. Data on HaCaT, 3T6 and NCTC 2544 cell lines have been previously reported by our group (Sanchez et al., 2004, 2006a); however, in this work we used the raw data to recalculate the $IC_{50}$-values using a properly fitted regression model.

The cytotoxicity assays indicated significant differences between the cell lines in their sensitivity to the five compounds tested. Our observations are consistent with earlier studies that reported significant differences in the cytotoxic effects of chemicals depending on the cell type tested (Backorová et al., 2011; Burlando et al., 2008; Lestari et al., 2005; Schröterová et al., 2009; Tan et al., 2008; Wang et al., 2002). Although the effect of the surfactants was concentration-dependent, the cellular attributes of each particular tumor or non-tumor cell line also contributed to the overall outcome. The greater resistance of the tumor cell lines could be partly explained by the fact that healthy and cancer cells show differences in their structure and function, such as differences in metabolic activity and molecular composition (Frey et al., 2007). The particular characteristics of each cell type could lead to varied mechanisms of defense, and consequently distinct sensitivity to the toxic effect of a chemical compound. The overall variability in cell sensitivity was supported by the general poor or moderate correlation between cell lines ($r < 0.85$, correlation not significant). Nevertheless, some significant correlations were obtained. The two tumor cell lines (HeLa and MCF-7) presented a relatively good relationship with each other and also with the 3T3 non-tumor cell line. HeLa and MCF-7 cells have similar phenotypic characteristics (Leporatti et al., 2009), which could explain their similar sensitivity to surfactants. In contrast, no significant correlations were found between the fibroblasts (3T3 and 3T6) or keratinocytes (HaCaT and NCTC 2544). The increased sensitivity of 3T6 and NCTC 2544 cells to the surfactants may explain this lack of correlation. Hayat and Friedberg (1986) reported stronger cytotoxicity in transformed 3T6 cells compared to their untransformed counterparts 3T3 cells, which is consistent with our findings. The high resistance of 3T3 cells could be partly explained by their sensitivity to growth contact inhibition in contrast to 3T6 cells (Todaro and Green, 1963). The contact inhibition property could favor a more differentiated phenotype for the 3T3 cells, in which the cytotoxic effect of the surfactant may be less potent. Moreover, cytotoxicity and phototoxicity studies have shown that HaCaT keratinocytes are more resistant than NCTC 2544 (Burlando et al., 2008; Leccia et al., 1998). The lower sensitivity of HaCaT cells could be attributed to their high degree of differentiation, which involves more developed keratinization, whereas NCTC 2544 cells are scarcely differentiated (Boukamp et al., 1988). Based on the variability of cell responses, we recommended a combination of cell lines of different origins for a reliable primary screening for the potential toxicity of chemical compounds with potential biomedical applications. Cell-culture systems with established banked cell lines are reproducible and high throughput approaches, and thus can provide a forecast of some adverse effects prior to human exposure.

Our results show that the MTT assay was more sensitive in detecting cell damage than NRU assay, regardless of the cell line or compound assessed. MTT assay revealed loss of viability at concentrations at which no significant cytotoxic effect was observed with the NRU assay. These results were not unexpected, since it has been previously reported that responses vary depending on the cytotoxicity assay used (Burlando et al., 2008; Fotakis and Timbrell, 2006; Jondeau et al., 2006; Schröterová et al., 2009; Weyermann et al., 2005). Originally, it was assumed that the reduction of MTT occurred exclusively in the mitochondrial compartment; however, later studies showed that MTT is also reduced by oxido-reductase-type enzymes in microsomal and cytosolic fractions (Berridge et al., 2005; Liu et al., 1997). The NRU assay is based on the capacity of viable cells with intact plasma membranes to incorporate and bind the supravital NR dye in lysosomes. This process is dependent on the capacity of the cell to maintain pH gradients through the production of ATP (Repetto et al., 2008). The NRU endpoint is assumed to be universal among cell types, independently of their nature, while tetrazolium-based assays are more specific, as they measure the activity of intracellular enzymes whose expression, localization and activity depend on the cell type (Schröterová et al., 2009). Therefore, differences in cytotoxic responses could be related to specific toxicological mechanism of the surfactants tested as well as the characteristics of the cell lines. Moreover, the finding that the NRU assay detected lower cytotoxicity than the MTT assay suggests that the mechanism of toxicity exerted by these surfactants involves an early effect on the metabolic activity of the cells, while plasma membrane and lysosomal compartments could be affected at a later stage. Although significant differences were found between the $IC_{50}$-values in some cell lines, significant correlations were obtained between the MTT and NRU assays for the surfactants 77KK, 77KL, 77KS, and also when all compounds were correlated simultaneously. This is generally good relationship between the two endpoints even when individual differences were observed within experimental data is in agreement with previous studies (Borenfreund et al., 1988). On the basis of our results, we recommend that the inter-correlation data from these two assays be carefully analyzed before considering similar tools for the overall evaluation of cytotoxicity. The combination of several endpoints might be recommendable in order to distinguish between the effects on specific organelles or general cytotoxicity.

It is worth mentioning that compounds containing counterions differ in their capacity to interact with biological membranes, and the type of counterion is one of the factors that determine the efficiency of this interaction (Kleszczynska and Sarapuk, 1998; Nogueira et al., 2011). Therefore, given that the class of lysine-based surfactants studied here had the same chemical structure, differing only in the type of counterion, the characteristics of the counterion may be crucial to the cytotoxic effect. Surfactants bound to heavy counterions, lysine (77KK) and Tris (77KT), showed low toxicity. On the basis of this observation, we suggest that there is a relationship between the size of the counterion and the cytotoxic properties of these compounds: the heavier the counterion they are bound to, the lower the cytotoxicity they induce. This finding could be explained by the influence of the volume of surfactant polar head on cell membrane penetration. The larger the volume and radius of the polar head, the lower the penetration, which would explain why high concentrations of these surfactants were required to membrane penetration (Maugras et al., 2001; Sarapuk et al., 1997; Selve et al., 1991). In contrast, surfactant 77KL was the least cytotoxic to tumor cells, indicating a specific interaction of the inorganic lithium counterion with these cells. Moreover, surfactant 77KS was one of the most cytotoxic compounds regardless of the assay or cell line used. This observation suggests non-specific toxicity of the inorganic sodium counterion in the assays and cell lines assessed.

## 5. Conclusions

Our results show significant differences in the cytotoxicity of the surfactants. These differences are attributed to differences in the sensitivity of model cell lines to these compounds and the characteristics of the two assays used. The two tumor cells and 3T3 fibroblasts were more resistant to the surfactants, while 3T6 and NCTC 2544 cells were the most sensitive. The MTT assay was more sensitive in detecting cell damage, regardless of the cell line or compound tested. We conclude that the type of counterion in these compounds determines the degree of surfactant interaction with the cell: in general the compounds with organic counterions are the least cytotoxic. The outcome of this study enhances knowledge about the potential toxic effects of novel biocompatible lysine-based surfactants prior to preformulation trials of pharmaceutical devices. Altogether, our findings highlight the relevance of an appropriate choice and combination of endpoints and cell-culture

systems in toxicity studies, which may raise the information output related to all major toxic effects of bioactive compounds. A complete and detailed toxicological evaluation *in vitro* may increase the reliability of results, and also prevent overestimation or underestimation of cytotoxicity. Furthermore, this comparative study, performed with six different cell types and two endpoints, may also serve as a basis for further toxicological screenings of different chemical compounds.

## Conflict of interest statement

The authors have declared no conflict of interest.

## Acknowledgements

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## Artículo 5

**NEW CATIONIC NANOVESICULAR SYSTEMS CONTAINING LYSINE-BASED SURFACTANTS FOR TOPICAL ADMINISTRATION: TOXICITY ASSESSMENT USING REPRESENTATIVE SKIN CELL LINES**

**(Nuevos sistemas nanovesiculares catiónicos conteniendo tensioactivos derivados de lisina para administración tópica: evaluación de la toxicidad usando líneas celulares representativas de la piel)**

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# Resumen

*Objetivos*

Desarrollar y caracterizar diferentes formulaciones de nanovesículas catiónicas basadas en tensioactivos derivados de lisina como propuesta de nuevos vehículos para la administración tópica de biomoléculas. Además, se propuso estudiar su potencial toxicidad *in vitro* utilizando líneas celulares representativas de la piel.

*Material y métodos*

Tensioactivos catiónicos derivados de lisina del tipo sales de hidrocloruro de $N^{\alpha}$ o $N^{\varepsilon}$-acil lisina metil éster, que difieren en cuanto a la longitud de la cadena hidrocarbonada y a la posición de la carga catiónica, se incorporaron en sistemas de nanovesículas. La matriz lipídica básica de las nanovesículas estaba compuesta por el fosfolípido DMPC (dimiristoilfosfatidilcolina) con o sin la adición de colesterol. Estos sistemas se caracterizaron en cuanto a su tamaño, morfología, índice de polidispersidad, potencial zeta y estado de agregación en medio de cultivo, utilizando técnicas como espectroscopia de correlación de fotones y microscopía electrónica de transmisión. Además, también se valoró la cantidad de tensioactivo incorporado en cada sistema nanovesicular por cromatografía líquida. La citotoxicidad de estas formulaciones se valoró en líneas celulares representativas de la piel (3T3, HaCaT y THP-1), utilizando los ensayos de viabilidad MTT, NRU y LDH. Además, se estudió el potencial fototóxico de las nanovesículas en las líneas de fibroblastos 3T3 y queratinocitos HaCaT, y se valoró la producción de las citoquinas IL-1α e IL-8 como marcadores del potencial inflamatorio.

*Resultados*

Las nanovesículas mostraron tamaño hidrodinámico entre 90 y 255 nm, dependiendo de la composición y del tensioactivo incorporado. Se observó una aglomeración significativa de las formulaciones conteniendo los tensioactivos con la carga positiva en el grupo α-amino de la lisina después de 24 h de incubación en medio de cultivo. Las repuestas de citotoxicidad variaron dependiendo del tensioactivo, línea celular y ensayo de viabilidad. El MTT fue el método más sensible en detectar los efectos tóxicos de las formulaciones con los tensioactivos que tienen la carga en el grupo α-amino de la lisina, mientras que las nanovesículas con el tensioactivo con la carga en el grupo ε-amino presentaron citotoxicidad similar con los tres ensayos de viabilidad. Estos resultados mostraron que las características estructurales de los tensioactivos influyen directamente en la toxicidad del sistema nanovesicular resultante. Además, los diferentes grados de citotoxicidad medidos por los diferentes ensayos de viabilidad, permitieron identificar algunos probables mecanismos de interacción de las nanovesículas con las células. Ninguna de las formulaciones demostró potencial fototoxicidad en los modelos celulares de fibroblastos y queratinocitos, mientras que indujeron solo una respuesta inflamatoria leve en las células HaCaT y THP-1, que se valoraron por la cuantificación de los niveles de IL-1α e IL-8, respectivamente.

*Conclusiones*

La composición de las nanovesículas tiene un papel clave en su toxicidad subyacente, demostrando que el conocimiento de la relación estructura-actividad de los nanomateriales es de extrema importancia cuando estos se desarrollan para aplicaciones biomédicas. Las nanovesículas presentaron, de un modo general, respuestas toxicológicas aceptables en modelos representativos de la piel y, por lo tanto, se propone su utilización como vehículos para la administración tópica de biomoléculas.

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Research paper

# New cationic nanovesicular systems containing lysine-based surfactants for topical administration: Toxicity assessment using representative skin cell lines

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ABSTRACT

Cationic nanovesicles have attracted considerable interest as effective carriers to improve the delivery of biologically active molecules into and through the skin. In this study, lipid-based nanovesicles containing three different cationic lysine-based surfactants were designed for topical administration. We used representative skin cell lines and *in vitro* assays to assess whether the cationic compounds modulate the toxic responses of these nanocarriers. The nanovesicles were characterized in both water and cell culture medium. In general, significant agglomeration occurred after 24 h incubation under cell culture conditions. We found different cytotoxic responses among the formulations, which depended on the surfactant, cell line (3T3, HaCaT, and THP-1) and endpoint assayed (MTT, NRU, and LDH). Moreover, no potential phototoxicity was detected in fibroblast or keratinocyte cells, whereas only a slight inflammatory response was induced, as detected by IL-1α and IL-8 production in HaCaT and THP-1 cell lines, respectively. A key finding of our research was that the cationic charge position and the alkyl chain length of the surfactants determine the nanovesicles resulting toxicity. The charge on the α-amino group of lysine increased the depletion of cell metabolic activity, as determined by the MTT assay, while a higher hydrophobicity tends to enhance the toxic responses of the nanovesicles. The insights provided here using different cell lines and assays offer a comprehensive toxicological evaluation of this group of new nanomaterials.



## 1. Introduction

Cationic lipid-based nanovesicles have been proposed as biocompatible drug delivery devices, with specific properties and able to overcome the barriers imposed by cell membranes [1]. Among various cationic substances, cationic amphiphile molecules have been successfully used in the composition of such carriers [1,2]. Lipid-based devices have a broad spectrum of applications, one of which is the topical delivery of active ingredients, intensively studied in recent years [3,4]. The inclusion of cationic compounds, such as biocompatible cationic amphiphiles, in the basic membrane of liposomes might be a promising approach to increasing the formulation's stability and its specificity for skin drug delivery. The development of new lipid-like molecules, such as cationic amphiphiles derived from lipoaminoacids [5,6], has become a major feature in the search for natural bioactive molecules that could be used in new drug delivery systems [7]. In this context, cationic lysine-based surfactants are a promising group of amino acid-based amphiphiles that can be regarded as an alternative to conventional synthetic amphiphiles due to their multi-functionality and biodegradability, the renewable source of raw materials used during their synthesis and their low cytotoxic potential [5].

The presence of charges at the vesicle surface may influence topical drug delivery. The skin surface bears a net negative charge [8], which, therefore, favors the positively charged vesicles to increase the permeation rate of different model drugs through the skin [9,10]. In contrast, some authors reported contradictory results, in which vesicles with negative charge have higher skin permeation [11,12]. Although extensive studies have focused on the drug release and penetration properties of cationic vesicles, the potential toxic mechanisms of this kind of carrier have not been explained sufficiently. Some authors have studied the effect of structural properties of cationic vesicles on their cytotoxicity [13], while others assessed the mechanisms of cell death induced by catanionic vesicles [14]. The search for reliable conditions to assess nanomaterials' safety is an emerging field that poses many interesting challenges.

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Currently, there are no specific testing requirements for nanotechnology products, and therefore, researchers took liberal approaches to studying toxicity [15,16]. Moreover, it is worth noting that, because of the expense of animal testing in toxicology and pressure from both the general public and government to develop alternatives to *in vivo* testing, *in vitro* cell-based models may be more attractive for preliminary testing of nanomaterials [17]. Here, we developed different formulations of cationic nanovesicles containing biocompatible lysine-based surfactants as surface modification agents and screened *in vitro* toxicological assays both to understand better the potential health hazards of new nanomaterials designed for topical application and to create predictive toxicology approaches for testing nanotechnology-based products. We specifically studied whether the inclusion of cationic lysine-based surfactants, differing in the cationic charge position and in alkyl chain length, may determine the cytotoxic and phototoxic potential of nanovesicular systems and modulate molecular mechanisms such as inflammatory response in representative skin cell lines. Since there is a knowledge gap between the increasing development and use of nanomaterials and the prediction of possible health risks [18], safety evaluation and greater understanding of nanomaterials' impact on human health are essential before any clinical application is explored.

## 2. Experimental

### 2.1. Chemicals and reagents

2,5-Diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), cholesterol and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbeccós Modified Eaglés Medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin–EDTA solution (170,000 U/l trypsin and 0.2 g/l EDTA), and penicillin–streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) were purchased from Lonza (Verviers, Belgium). The 75 $cm^2$ flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland). All other reagents were of analytical grade.

### 2.2. Surfactants included in the nanovesicular systems

Three new biocompatible amino acid-based surfactants derived from $N^{\varepsilon}$ or $N^{\alpha}$-acyl lysine methyl ester salts with one lysine as the cationic polar head (one cationic charge) and one alkyl chain were used as surface modification agents to prepare the cationic vesicular systems reported in this study: $N^{\varepsilon}$-myristoyl lysine methyl ester (MKM) with one alkyl chain of 14 carbon atoms and one positive charge on the α-amino group of the lysine, $N^{\varepsilon}$-palmitoyl lysine methyl ester (PKM) with one alkyl chain of 16 carbon atoms and one positive charge on the α-amino group of the lysine and $N^{\alpha}$-myristoyl lysine methyl ester (MLM) with one alkyl chain of 14 carbon atoms and one positive charge on the ε-amino group of the lysine. MKM and PKM have a hydrophobic chain attached to the ε-amino group of the lysine, while MLM has its hydrophobic chain attached to the α-amino group of the lysine. These lysine-based surfactants were synthesized in our laboratory, as described elsewhere [5,6].

### 2.3. Preparation of cationic nanovesicular formulations

The mixed cationic vesicles were prepared by the film hydration method. Briefly, DMPC only or DMPC and cholesterol (CHOL) were mixed with MKM, PKM, or MLM in the designed molar ratios and dissolved in the mixed chloroform:methanol (1:1, v/v) solvent in a round-bottom flask. The formulations of DMPC:surfactant were prepared at 80:20 M ratio, while the molar composition of the vesicular systems composed of DMPC:CHOL:surfactant was 56:24:20 (30% cholesterol of the total lipid in the formulation). The organic solvent was removed under reduced pressure using a R-210 Rotary Evaporator (Buchi, Switzerland) at 50 °C for 60 min to form a homogeneous thin film. To remove residual traces of the organic solvents, the mixed film was freeze-dried (Christ Alpha 2–4 LD freeze-drying system, Martin Christ, Germany) overnight. Ten milliliters of ultrapurified water was added to hydrate the film, and the resulting suspension was sonicated for 20 min at 60 °C in an Ultrasons-H ultrasonic bath (J.P. Selecta, Spain) to promote the formation of uniform vesicles. The total final concentration of each mixed cationic nanovesicle was fixed at 2 mM.

Nanovesicle dispersions were purified by filtration using Vivaspin 2 centrifugal concentrator (PES membrane, 3000 Da MWCO, Sartorius Stedim Biotech, Goettingen, Germany). The substance filtrated was used to determine the extent of incorporation of the cationic surfactants into the vesicles. The amount of unincorporated surfactant was assessed by high-performance liquid chromatography (HPLC), following the analytical method previously described [5].

### 2.4. Nanovesicle characterization

The mean hydrodynamic size and the polydispersity index (PDI) of the cationic nanovesicles were determined at 25 °C by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK). The measurements were performed in ultrapurified water immediately after preparation ($t$ = 0 h) and in cell culture medium with 5% FBS at $t$ = 0 h and after a 24 h incubation at 37 °C ($t$ = 24 h). Each measurement was performed using at least three sets of at least ten runs.

The zeta potential (ZP) values of the nanovesicle formulations were assessed by determining the electrophoretic mobility of a particle with the Malvern Zetasizer ZS equipment. The measurements were performed in ultrapurified water and cell culture medium at 25 °C using at least three sets of at least 20 runs.

The morphology and size analysis of the vesicular systems were analyzed by transmission electron microscopy (TEM), and the images were obtained with a Jeol JEM-1010 electron microscope (Jeol Ltd., Tokyo, Japan) operating at an acceleration voltage of 80 kV. A droplet (5 μl) of the vesicles dispersed in ultrapure water was placed on a carbon-coated copper grid, forming a thin liquid film. The negative staining of samples was obtained with a 2% (w/v) solution of phosphotungstate acid (pH 6.5, with KOH). The excess solution was removed by a filter paper and was followed by thorough air-drying.

### 2.5. Cell cultures

The murine Swiss albino fibroblasts, 3T3, and the spontaneously immortalized human keratinocyte, HaCaT, cell lines were grown in DMEM medium (4.5 g/l glucose) supplemented by 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% $CO_2$. The 3T3 and HaCaT cells were routinely cultured in 75 $cm^2$ culture flasks and were trypsinized using trypsin–EDTA when the cells reached approximately 80% confluence. The human monocytic leukemia cell line THP-1 was grown in RPMI 1640 medium supplemented by 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol at 37 °C, 5% $CO_2$. The three cell lines were obtained from Eucellbank (Universitat de Barcelona, Spain).

### 2.6. Cytocompatibility assays

The 3T3 ($1 \times 10^5$ cell/ml) and HaCaT ($7.5 \times 10^4$ cell/ml) cells were seeded into 96-well cell culture plates in 100 μl of complete culture medium. Cells were incubated for 24 h under 5% $CO_2$ at 37 °C and the medium was then replaced with 100 μl of fresh medium supplemented by 5% FBS containing the vesicular system dispersions in the 0.5–100 μM concentration range. THP-1 cells were seeded into 24-well plates at a density of $1 \times 10^6$ cell/ml and then treated with vesicle dispersions in the 1–100 μM concentration range. The final volume in each well was 500 μl and the medium used contained 5% FBS. Each concentration was tested in triplicate and control cells were exposed to medium with 5% FBS only. The cell lines were exposed for 24 h to each nanovesicle treatment.

To determine whether the NVs interact with the viability assays, UV–visible absorbance measurements were carried out [19,20]. NVs at 100 μM were suspended in DMEM medium (without FBS and phenol red) containing MTT (0.5 mg/ml) or NR (50 μg/ml) dyes. After 3 h incubation under cell culture conditions, the NVs were pelleted by ultracentrifugation, rinsed and extracted with DMSO or a solution containing 50% ethanol absolute and 1% acetic acid in distilled water for MTT and NR dyes, respectively. The extracted solution was transferred to a quartz cuvette and the absorbance read at 550 nm and at intervals from 300 to 700 nm on a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan).

#### 2.6.1. MTT assay

The MTT assay is based on the protocol first described by Mossmann [21]. In this assay, living cells reduce the yellow tetrazolium salt MTT to insoluble purple formazan crystals. After 24 h exposure of 3T3 and HaCaT cells to the various vesicular systems, the treatment-containing medium was removed, and 100 μl of MTT in PBS (5 mg/ml) diluted 1:10 in FBS-free medium without phenol red was then added. Plates were further incubated for 3 h, after which time the medium was removed. The purple formazan product was then dissolved by adding 100 μl of DMSO to each well. Plates were then placed in a microtitre-plate shaker for 10 min at room temperature, and the absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader.

After 24 h incubation of THP-1 cells with the treatments, the plates were centrifuged and supernatants were collected and kept at −80 °C till subsequent analysis (see section 1.9). Then, 300 μl of a MTT solution of 0.75 mg/ml was added to each well. Cells were incubated for 3 h at 37 °C, plates were then centrifuged, medium discarded, and cells lysed in 250 μl/well of a mixture of HCl/isopropanol. 100 μl of the resulting solutions was transferred to a 96-well plate and the absorbance was read as described above. Cell viability was calculated as the percentage of tetrazolium salt reduction by viable cells on each sample against the untreated cell control (cells with medium only).

#### 2.6.2. NRU assay

Based on the protocol described by Borenfreund and Puerner [22], the NRU assay was performed following exposure to the nanovesicles. 3T3 and HaCaT cells were incubated for 3 h with NR dye solution (50 μg/ml) dissolved in medium without FBS and phenol red. Cells were then washed with PBS, followed by the addition of 100 μl of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water to extract the dye. Plates were gently shaken for 10 min to ensure complete dissolution. We then measured the absorbance of the extracted solution at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of uptake of NR dye by lysosomes against the untreated cell control (cells with medium only).

#### 2.6.3. LDH assay

LDH leakage was determined in the conditioned medium 24 h after cationic vesicles' treatment in 3T3 and HaCaT cell lines, using a commercially available kit (Takara Bio Inc., Otsu, Japan), in line with the instructions provided by the manufacturer. This assay quantifies cytotoxicity based on the measurement of LDH activity released from dead or plasma membrane-damaged cells into the supernatant. Results are expressed as percentage of control, with 1% Triton X used as positive control.

### 2.7. Cell morphology analysis

The 3T3 cells at a density of $1 \times 10^5$ cells/ml were grown on sterile cover glass in 24-well plates and then treated without (control) or in the presence of the $IC_{50}$ concentrations (determined by the MTT assay) of the various formulations of cationic vesicles for 24 h. After incubation, cell morphology was analyzed by a phase contrast microscope (Olympus BX41, Olympus, Japan). Images were digitised by an Olympus XC50 camera connected to Olympus cell^B computer software.

### 2.8. Phototoxicity assay

Cell lines 3T3 and HaCaT were used as *in vitro* models to predict cutaneous phototoxicity. Two plates were seeded with cells: one for irradiation (+UVA) and the other wrapped in foil and therefore non-irradiated (−UVA). The cells were treated with the various vesicle formulations, as described in Section 2.6. After treatment application, the plates were pre-incubated for 1 h at 37 °C in a humidified 5% $CO_2$ and then irradiated with a dose of 2.5 J/cm$^2$ UVA light. Following irradiation, the plates were incubated again under the same conditions to complete 24 h exposure. MTT and NRU endpoint assays were used to assess the phototoxic potential of the formulations. The phototoxic effect was evaluated by the OECD phototoxic validation test [23], with some modifications. A photoirritation-factor (PIF) was calculated, using the following formula:

$$PIF = IC_{50}(-UVA)/IC_{50}(+UVA) \quad (1)$$

Based on the results, a test substance with a PIF < 2 predicts "no phototoxicity"; a PIF > 2 and <5 predicts "probable phototoxicity" and a PIF > 5 predicts "phototoxicity".

### 2.9. Cytokine production

IL-1α and IL-8 concentrations, as markers of induced inflammatory stimuli, were measured by commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (Diaclone Research, France and ImmunoTools, Friesoythe, Germany, respectively). The results are expressed in pg/ml.

To assess whether there was an inflammatory reaction measured by the presence of IL-1α, HaCaT cells at a density of $1 \times 10^5$ - cells/ml were grown in 24-well plates and then exposed to a range of concentrations from 1 to 100 μM of the various vesicle formulations diluted in medium with 1% FBS. After 24 h exposure, conditioned medium was recovered, centrifuged, and used for the determination of extracellular IL-1α (IL-1α release). Monolayers were washed with PBS, then lysed in 300 μl of PBS containing 0.5% of Triton X-100 and used for the determination of intracellular IL-1α (cell-associated IL-1α). The protein content of the cell lysate determined by a commercial kit (Bio-Rad, Hercules, CA, USA) based on the dye-binding procedure of Bradford [24]. Cell viability was determined by the LDH leakage assay as described previously.

IL-8 release was assessed in cell-free supernatants after incubation of the various cationic vesicle formulations with THP-1 cells.

The supernatants were collected directly from the cytotoxicity assay plates, centrifuged, and stored at −80 °C until analysis.

### 2.10. Statistical analysis

All *in vitro* experiments were performed at least three times, using three replicate samples for each formulation concentration tested. Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses used the Student's *t*-test or one-way analysis of variance (ANOVA) to determine the differences between the datasets, followed by Dunnett's post hoc test for multiple comparisons using SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ and $p < 0.005$ were considered significant.

## 3. Results and discussion

Cationic lipid-based vesicles have attracted considerable interest because of their use as effective drug delivery systems [25,26]. More specifically, these kinds of carriers have been studied to improve the delivery of biologically active molecules into and through the skin [10,12]. Among a range of applications, cationic nanovesicles could be used in cosmetic formulations and epicutaneous drug release, because the addition of polar amphiphiles increases the likelihood of skin penetration [27]. In the present study, we provided new cationic nanocarriers as possible vehicles for dermal and transdermal applications. The cationic amphiphiles used as surface modification agents have biocompatible properties and can be considered much more suitable for practical applications than current commercial surfactant systems [28]. Furthermore, we showed previously that amphiphiles with the positive charge on the α-amino group of lysine (MKM and PKM) have pH-dependent membrane lytic activity [29,30]. Some researchers have reported that the use of pH-sensitive lipid vesicles for skin delivery of biologically active molecules resulted in enhanced effects [31].

Here, experiments were performed to investigate the physicochemical parameters of the nanovesicles and, more particularly, to see how their composition affects their interaction with cells that are representative of the skin and play a key role in irritant, inflammatory and immunological reactions. The *in vitro* methods proposed here to assess the safety of new nanomaterials are adapted to nanoscale colloids. They are a direct extension of methods known for other macroscopic biomaterials or soluble drug toxicology assessments. However, since there are no defined protocols for assessing nanotoxicity [15,16], the use of the current assays to evaluate the toxic potential and the mechanisms involved is of great importance in determining structure/function relationships between nanomaterials and toxicity.

### 3.1. Characterization of cationic nanovesicles

Nanomaterial size and zeta potential are very important parameters in drug delivery applications. As keeping nanomaterials in solution for longer periods results in aggregation [32], we prepared fresh formulations for each subsequent study to guarantee nanosized vesicles. Table 1 shows the results for each formulation. Average nanovesicle hydrodynamic diameter is between 90 and 255 nm, as determined by DLS analysis. The addition of cholesterol decreased the mean nanovesicle diameter, with the exception of the formulations containing MKM. When the nanovesicles containing MKM and PKM were dispersed in cell culture medium (DMEM with 5% FBS), the size increase was slight by 0 h, but significant agglomeration to micron-sized structures occurred after 24 h incubation under cell culture conditions. The easy aggregation in cell culture medium is probably attributed to the high ionic nature of the solution, resulting in the formation of the secondary particles

**Table 1**
Characterization parameters of the different cationic nanovesicles.

| | DMPC:MKM (80:20) | DMPC:CHOL:MKM (56:24:20) | DMPC:PKM (80:20) | DMPC:CHOL:PKM (56:24:20) | DMPC:MLM (80:20) | DMPC:CHOL:MLM (56:24:20) |
|---|---|---|---|---|---|---|
| *Size (nm) ± SEM*[a] | | | | | | |
| *t* = 0 h water | 94.16 ± 2.05 | 107.33 ± 0.94 | 253.07 ± 26.05 | 184.77 ± 6.64 | 174.40 ± 7.16 | 127.50 ± 1.96 |
| *t* = 0 h DMEM 5% FBS | 94.42 ± 6.50 | 159.77 ± 5.71 | 229.37 ± 12.64 | 197.73 ± 6.57 | 229.63 ± 16.33 | 170.27 ± 9.49 |
| *t* = 24 h DMEM 5% FBS[b] | 1781.67 ± 45.72/110.3 ± 5.17 | 2028.67 ± 21.23/154.33 ± 11.95 | 1059.50 ± 10.61/118.93 ± 2.09 | 1488 ± 19.59/125.67 ± 11.94 | 193.47 ± 7.75 | 151.77 ± 4.44 |
| *PDI ± SEM*[a] | | | | | | |
| *t* = 0 h water | 0.231 ± 0.004 | 0.278 ± 0.019 | 0.427 ± 0.017 | 0.331 ± 0.020 | 0.394 ± 0.003 | 0.256 ± 0.006 |
| *t* = 0 h DMEM 5% FBS | 0.385 ± 0.015 | 0.236 ± 0.001 | 0.522 ± 0.001 | 0.288 ± 0.001 | 0.445 ± 0.007 | 0.319 ± 0.029 |
| *t* = 24 h DMEM 5% FBS[b] | 0.903 ± 0.075 | 1.00 ± 0.000 | 0.605 ± 0.002 | 0.973 ± 0.027 | 0.294 ± 0.001 | 0.352 ± 0.007 |
| *Zeta potential (mV) ± SEM*[a] | | | | | | |
| *t* = 0 h water | 42.7 ± 0.90 | 41.2 ± 1.66 | 52.8 ± 1.15 | 55.17 ± 0.67 | 78.7 ± 2.56 | 44.9 ± 0.40 |
| *t* = 0 h DMEM 5% FBS | 1.23 ± 1.64 | -3.13 ± 0.81 | 6.78 ± 0.94 | 0.86 ± 0.11 | 13.00 ± 0.49 | 8.61 ± 0.16 |
| *% Incorporation of surfactant into nanovesicles ± SEM*[a] | | | | | | |
| | 88.56 ± 0.009 | 75.96 ± 0.054 | 99.10 ± 0.007 | 98.96 ± 0.006 | 90.85 ± 0.053 | 79.05 ± 0.038 |

[a] Mean of three experiments ± standard error of the mean (SEM).
[b] Incubated under cell culture conditions: 37 °C, 5% $CO_2$.

[33]. In contrast, the nanovesicles containing MLM did not suffer agglomeration in cell culture medium, which can be attributed to their higher charge density (p$K_a$ MLM = 8.1) [29], corroborated by the ZP values determined in cell culture medium. The ZP values of all formulations are highly positive (>40 mV) and did not differ significantly from each other. In contrast, in cell culture medium almost neutral values were obtained. The high positive ZP values in water indicate the stability of the prepared formulations and reflect the net charge on the surface of the vesicles. This is also of great importance in preventing fusion or aggregation of nanovesicles [13]. It has been reported that a physically stable formulation would have a minimum ±30 mV ZP as a borderline value of colloidal stability [34]. The PDI values reported in Table 1 range from 0.23 to 0.42, indicating a relatively homogenous vesicular population. A PDI value lower than 0.3 indicates a homogenous and monodisperse population [34].

The TEM morphological analysis showed that all the cationic vesicle formulations displayed clear negative staining images with a roughly spherical shape (Fig. 1). The nanovesicles containing the surfactants with the positive charge on the α-amino group of lysine (PKM and MKM) were in general much smaller (∼20 to 50 nm) than those obtained by DLS. These differences were especially significant for the nanovesicles with MKM (Fig. 1a and b), while the formulations with PKM showed more heterogeneous size distribution (Fig. 1e and f). The mean hydrodynamic particle size measured by DLS did not capture the real population distribution of the nanovesicles observed by TEM. The latter's aggregate population was undetected even as a peak by DLS analysis. This disparity between DLS and TEM analysis, previously reported [32,35,36], might be a result of the resolution limitations of DLS. DLS provides a scattered intensity-based size of a colloidal particle, and thus, the mean diameter is biased toward larger vesicles, even though they may occupy a much smaller fraction of the vesicle population [35]. Moreover, these observed differences might be attributed to aggregation [32,37] or to the swelling of the nanovesicles in the presence of water [36]. In contrast, the nanovesicles containing the surfactant MLM, which have the positive charge on the ε-amino group of lysine, did not show the same significant disparity between DLS and TEM analysis (Fig. 1c and d). In general, the TEM images corroborated the mean size obtained by DLS. Moreover, TEM images also revealed the formation of a multilayered membrane in the vesicles containing the surfactant MLM (Fig. 1c and d), while those containing the surfactants MKM and PKM showed unilamellar membranes in both presence and absence of cholesterol in the basic membrane (Fig. 1a, b, e, and f).

The HPLC analysis of the filtrated samples (obtained from the purification process performed to remove the unincorporated amount of surfactant) revealed that the cationic surfactants were highly incorporated into the formulations (from 75% to 99% incorporation). (Table 1). It is noteworthy that the vesicles containing cholesterol have a lower degree of surfactant incorporation than those with DMPC only. Moreover, the formulations with the surfactant PKM have almost total incorporation of this compound into the nanovesicle. This observation was fully expected since the longer the alkyl chain of a surfactant, the greater its tendency to incorporate into lipid-based vesicles due to its hydrophobicity [38].

### 3.2. Cytocompatibility studies

The cytotoxic potential of new nanovesicles designed for biomedical application was evaluated on representative skin cells using established *in vitro* methods. HaCaT keratinocyte and 3T3

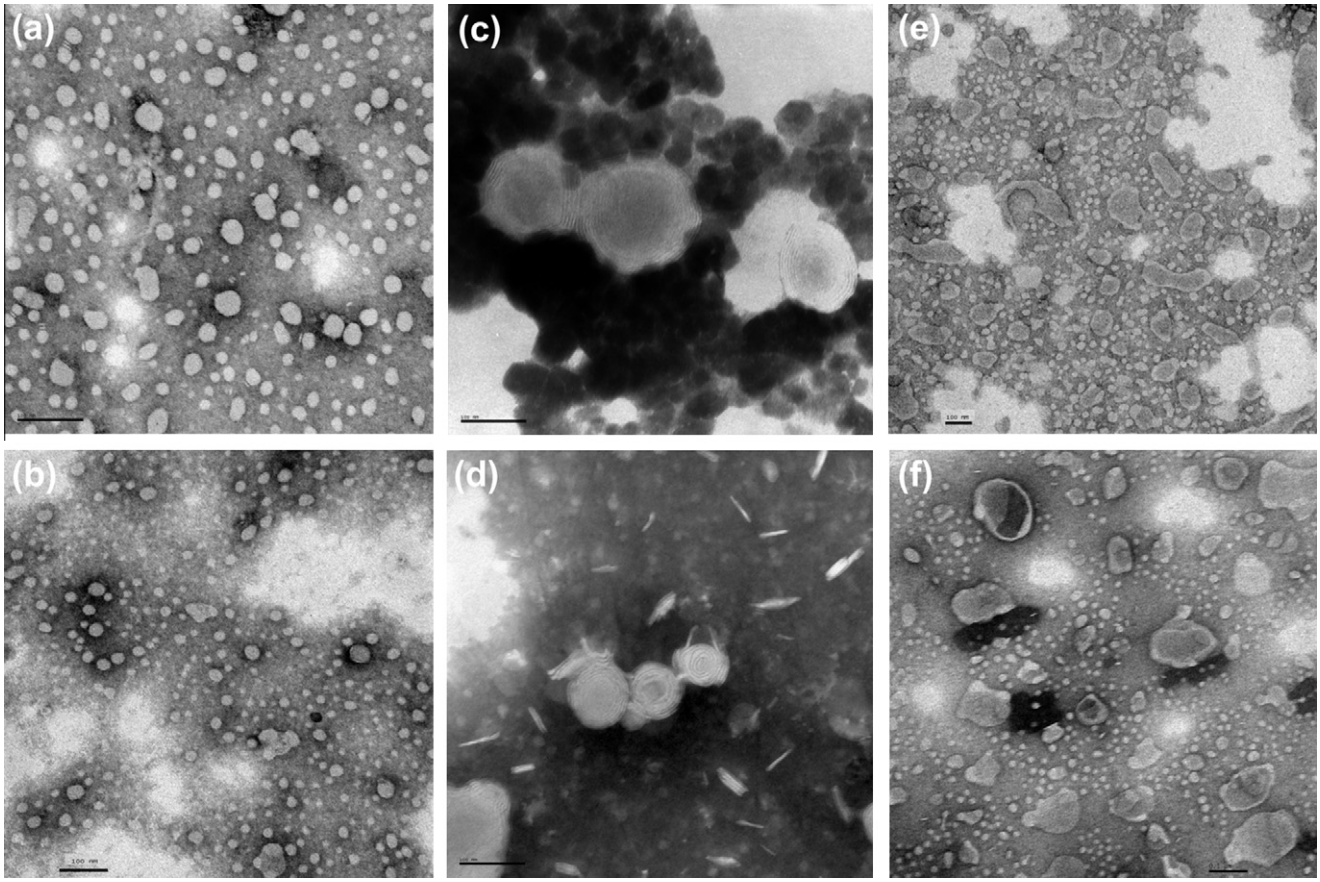



**Fig. 1.** TEM images of cationic nanovesicles (a) DMPC:MKM (80:20, molar ratio), (b) DMPC:CHOL:MKM (56:24:20, molar ratio), (c) DMPC:MLM (80:20, molar ratio), (d) DMPC:CHOL:MLM (56:24:20, molar ratio), (e) DMPC:PKM (80:20, molar ratio), (f) DMPC:CHOL:PKM (56:24:20, molar ratio). Scale bars correspond to 100 nm.

fibroblast cultures gave an appropriate *in vitro* model for skin irritation [39], while the human monocytic leukemia cell line THP-1 is considered surrogate of cutaneous dendritic cells in *in vitro* skin sensitization studies [40]. The combination of different cell lines and cytotoxicity assays gives information concerning general and specific toxicological mechanisms [41] that could help in understanding the toxic response of nanomaterials.

All the formulations containing the surfactants showed, as expected, higher cytotoxicity than the vesicles formulated without surfactant (data not shown). The cationic charge of the vesicles is probably responsible for the initial binding to the surface membrane by ionic interaction with the negatively charged cell membrane [42], which might enhance the toxic effects of these formulations. Indeed, the cytotoxic effects observed showed many disparities between formulations that, in fact, depend on the surfactant, cell line, and endpoint assay. The vesicles containing MKM and PKM (positive charge on the α-amino group of lysine) have a clear and dose-dependent decrease in MTT activity in the three cell lines studied after 24 h, while the NRU and LDH assays showed a significant decline in cell viability only at doses >50 μM (Fig. 2). In contrast, the vesicular systems containing MLM (positive charge on the ε-amino group of lysine) showed similar cytotoxic responses by the three endpoint assays. These differences are reflected by comparing the $IC_{50}$ values of the vesicles, as shown in Table 2.

In studies of MTT and NR dye interactions with nanovesicles, we observed minimal interference with each dye. These data were proved by the UV–vis measurements (data not shown). Only the formulations containing PKM induced a slight increase in the MTT absorbance values at 550 nm. This might be due to a false positive reaction in which MTT was converted to formazan in the absence of cells. However, these small interferences did not result in further increases in cell viability with increasing concentration (false viability), which is in contrast to previous reported data for other types of nanomaterials [19,20]. These data prove that these viability endpoints are suitable for the intended purpose.

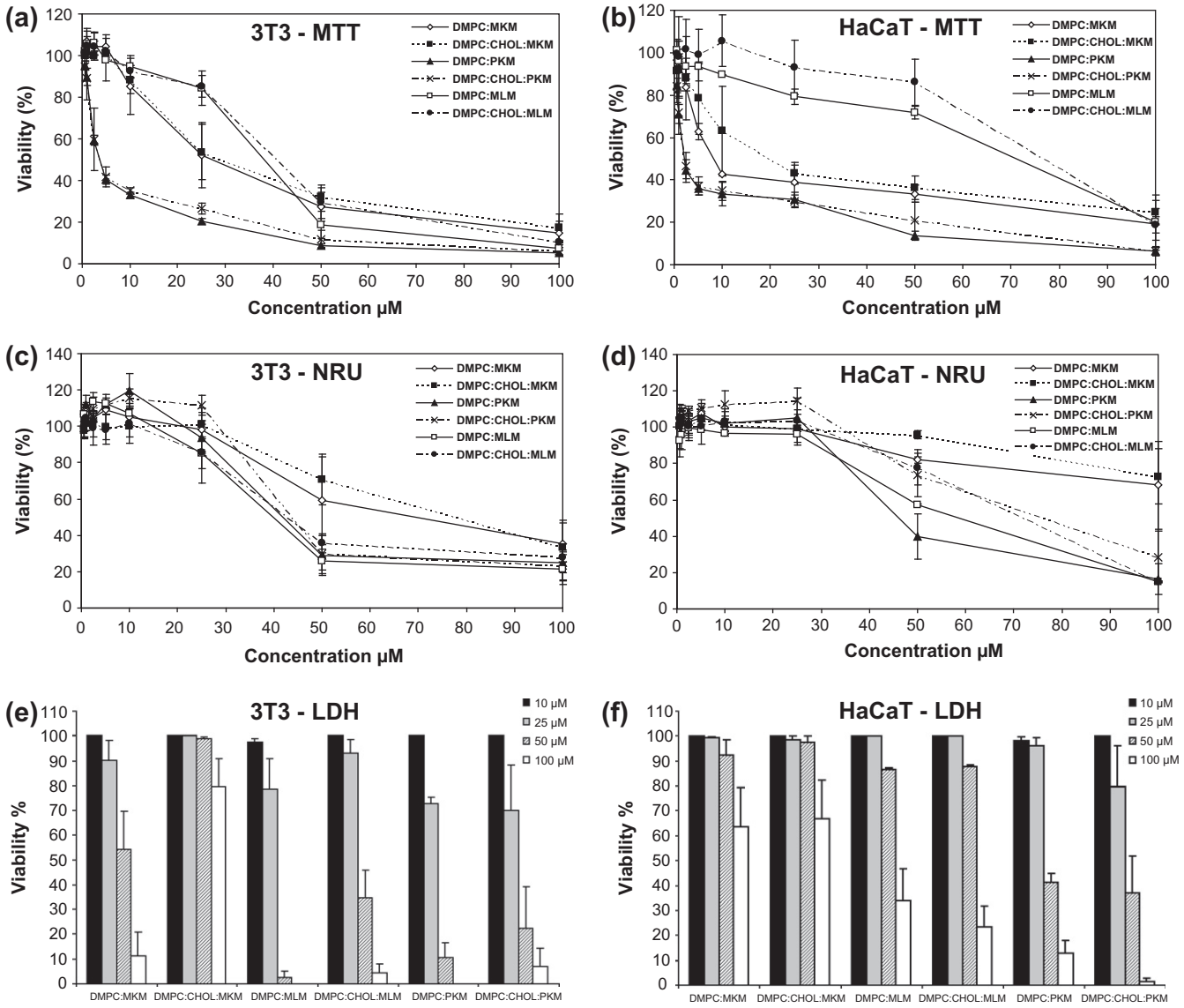
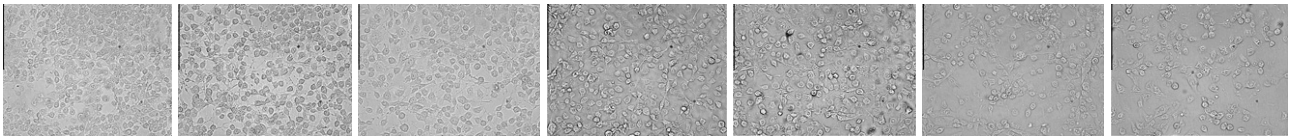



**Fig. 2.** Cell viability measured by the (a and b) MTT, (c and d) NRU, and (e and f) LDH assays on (a, c, and e) 3T3 and (b, d, and f) HaCaT cell lines. Each cell line was exposed to increasing concentrations of the nanovesicle formulations, ranging from 0.5 to 100 μM. Results are given as a percentage of untreated control cells. The nanovesicles did not induce loss of cell viability when concentrations were lower than 10 μM, as determined by the LDH assay (data not shown). Results are expressed as mean ± SEM of three independent experiments, performed in triplicate.

**Table 2**
Cytotoxicity of the nanovesicle formulations expressed as $IC_{50}$ values (μM) on 3T3, HaCaT and THP-1 cell lines.

| Nanovesicle formulation | $IC_{50}$ (μM)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3T3 | | | HaCaT | | | THP-1 |
| | MTT | NRU | LDH | MTT | NRU | LDH | MTT |
| DMPC:MKM | 28.16 | 71.14 | 60.01 | 8.03 | 162.22 | 129.43 | 13.92 |
| DMPC:CHOL:MKM | 31.05 | 72.59 | 79.82 | 19.51 | 191.32 | 141.35 | 21.18 |
| DMPC:PKM | 3.76 | 41.62 | 33.24 | 2.12 | 47.68 | 46.94 | 12.31 |
| DMPC:CHOL:PKM | 3.86 | 43.88 | 35.56 | 2.26 | 70.31 | 46.34 | 11.73 |
| DMPC:MLM | 36.41 | 39.23 | 32.23 | 65.66 | 64.32 | 84.42 | 89.41 |
| DMPC:CHOL:MLM | 39.11 | 42.96 | 36.14 | 73.25 | 71.41 | 77.53 | 102.48 |

*Cell morphology – 3T3*[b]




[a] Mean of three independent experiments.
[b] Induced by treatment with the $IC_{50}$ determined by MTT assay. Morphological changes were analyzed under a phase contrast microscope.

A key finding of our research was that the structural characteristic of the surfactants included in the nanovesicular systems directly affect the toxicological effects of such nanomaterials. Firstly, the position of the cationic charge in the amphiphile molecule was critical in determining the sensitivity of the endpoint used to assess the formulation's cytotoxicity. On the one hand, the nanovesicles containing the compounds with the positive charge on the α-amino group of lysine (MKM and PKM) have greater cytotoxicity detected by MTT than by NRU and LDH endpoints. On the other hand, the nanovesicles containing MLM (with the positive charge on the ε-amino group of lysine) displayed in general the same level of cytotoxicity with the three endpoints. These effects might be due to a different interaction mechanism of the vesicular systems within cell as a function of the cationic charge position on the amphiphile included in them. The MTT assay is a measurement of cell metabolic activity within the mitochondrial compartment, while the NRU and LDH assays measure membrane integrity. NR dye diffuses through intact cell membranes to accumulate within lysosomes, while the LDH endpoint detects cells in the last stages of cell death [43]. Based on the mechanisms of cell

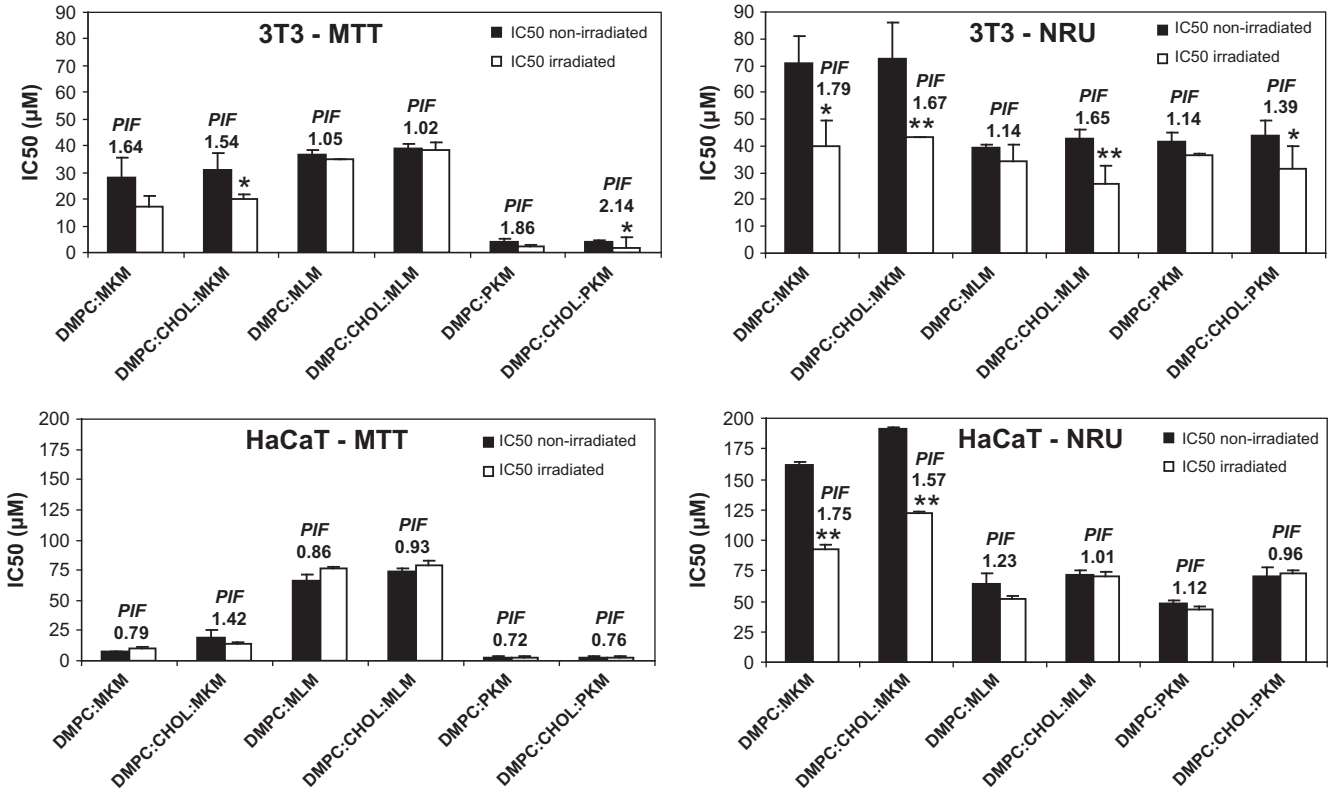



**Fig. 3.** Cytotoxicity and phototoxicity induced by the nanovesicle formulations expressed as $IC_{50}$ values on 3T3 and HaCaT cell lines. Values were obtained from MTT and NRU assays. Black bars = non-irradiated cells and white bars = UVA-irradiated cells (2.5 J/cm$^2$). The photoirritation-factor (PIF) was calculated as described in Section 2.8. Results are expressed as mean ± SEM of three independent experiments, performed in triplicate. Statistical analyses were performed using the Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.005$ denote significant differences.

damage detected by each cytotoxicity assay, our overall results suggest that the vesicles containing MKM and PKM interacted early with the mitochondrial compartment, first affecting cell metabolic activity, while the plasma membrane is affected in an lesser extent. The early interaction of these nanovesicles with the mitochondria might be due to their cell internalization before any damage to the cell membrane. The ability of these nanomaterials to be cell internalized was corroborated using the fluorophores nile red and calcein as drug models (unpublished results). Moreover, the pH-sensitive activity of MKM and PKM [29,30] could favor this increased mitochondrial damage: the nanovesicles containing these compounds might have the ability to lysis the endosome membrane after cell internalization, enhancing, thus, their potential toxicity in the cell cytoplasm (especially to the mitochondria). Finally, it is also worth noting that the length of the surfactant alkyl chain also affected the nanovesicles' cytotoxicity. Regardless of the cell line and endpoint used, the vesicular systems containing the amphiphile PKM were the most cytotoxic. Therefore, we can conclude that the longer the alkyl chain of the surfactant, the greater the cytotoxicity of the resulting vesicular system. In line with these findings, our previous studies revealed that the amphiphiles also displayed significant differences in their phospholipid bilayer–perturbing properties [29,30], corroborating that the interaction processes with cell membrane are directly dependent on surfactant structure.

Together with the disparities observed with different cytotoxic assays, we also found that each cell line used showed different sensitivity to the cationic nanovesicles. Unfortunately, no clear conclusion was achieved about which cell was the most sensitive. Although we have no explanation for these differences at present, these data showed cell-specific differences in cationic vesicle processing and toxicity. The results given are in line with previous studies [42,44], in which was reported that cytotoxic effects of particulate carrier systems differ, depending on the cell lines used, due to the innate nature, metabolic abilities (e.g. enzymes present) and capabilities of these cells. All in all, our results showed that the surfactants have a strong effect on the interaction of nanovesicles with the cells and, thus, on their cytotoxicity. These findings corroborated previous studies that found that the type and concentration of surfactant strongly affected the toxic responses of solid lipid nanoparticles [44] and also have a slight effect on the cytotoxicity of lipoplex formulations [1,2].

### 3.3. Morphological analysis

Light microscopy analysis was used to view the effects of nanovesicles on the morphology of 3T3 cells after 24 h of incubation. Untreated control cells showed a well-spread and flattened morphology (Table 2). In contrast, 3T3 cells treated with the $IC_{50}$ concentrations of the nanovesicles displayed prominent morphological changes, including rounding, reduced spreading, and shrunken cells. These morphological changes corroborate and are directly attributed to the cytotoxic effects of each vesicular system.

### 3.4. Phototoxicity assessment

Keratinocytes and fibroblasts are considered biologically relevant targets for skin irritants and photoirritants [45]. We therefore chose HaCaT and 3T3 cells as model cell systems to study the cutaneous phototoxic potential of nanovesicles. No potential phototoxic effects were observed, with PIF values lower than 2 (non-phototoxic) in all cases (Fig. 3). In general, the $IC_{50}$ values in

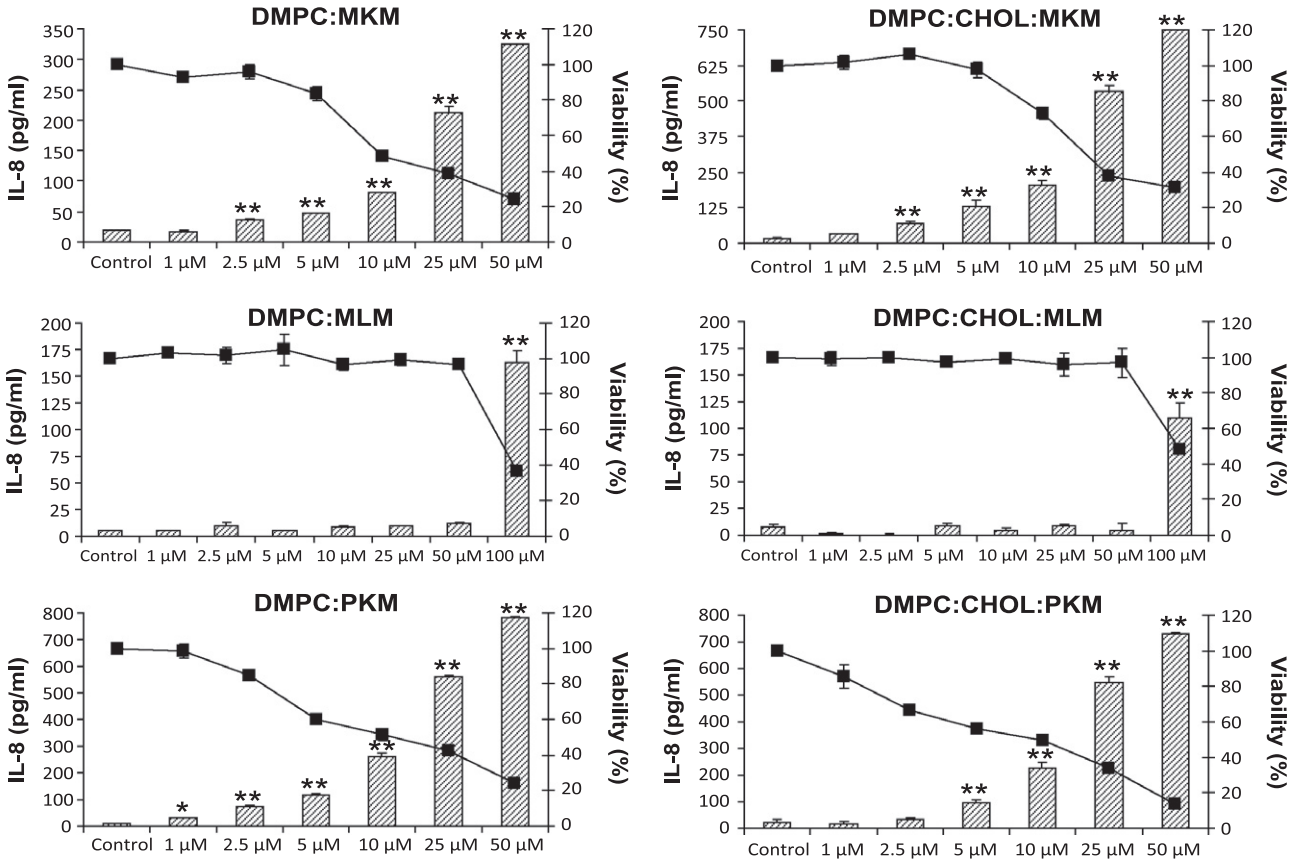



**Fig. 4.** IL-8 release (bars) induced by THP-1 cells with increasing concentrations of each nanovesicle formulation. Cell viability (line) was determined by MTT assay and is expressed as percentage of control. Results are expressed as mean ± SEM of three independent experiments, performed in triplicate. Statistical analyses were performed using ANOVA followed by Dunnett's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.005$ denote significant differences. Different scale bars were used to express the results better.

irradiated (+UVA) and non-irradiated (−UVA) plates were similar to the HaCaT cells, and only the formulations containing the amphiphile MKM showed a significant increase ($p < 0.005$) in the toxic response after irradiation, as determined by the NRU assay. In contrast, more significant differences between (−UVA) and (+UVA) plates were observed with 3T3 cells, especially with the NRU assay. Although the MTT assay gave lower $IC_{50}$ values (for the formulations containing MKM and PKM) for the (+UVA) plates than the NRU assay did (as also found in the cytotoxicity assays, see Section 3.2), the latter assay was more sensitive in detecting the UVA irradiation effects in both cell lines studied. These results suggest that the nanovesicle treatment followed by UVA irradiation has a greater effect on the plasma membrane of the cells than on their metabolic activity. Furthermore, the results of nanovesicle phototoxicity revealed the different sensitivity of the two cell lines regardless of the endpoint used, with HaCaT cells being, in general, less sensitive to the phototoxic effects. This variability in the effects of photoirritants on human keratinocytes and 3T3 cells was previously demonstrated in the original phototoxicity validation study [46] and in the studies of other amino acid-based surfactants [47]. All these data indicate the importance of assessing specific effects with different endpoints in a variety of different cell types.

As also discussed above for the general cytotoxicity assays, it is worth mentioning that the structural features of the amphiphiles also affected the phototoxicity of the nanovesicles. Vesicles containing MKM (positive charge on the α-amino group of lysine) had a significant phototoxic effect with both cell lines and endpoint assays, while those containing MLM (positive charge on the ε-amino group of lysine) had practically no phototoxicity in either cell line. Moreover, the length of the amphiphile alkyl chain also affected the phototoxicity of the formulations. Although the vesicles containing PKM (with 16 carbon atoms) were the most cytotoxic formulations, they showed lower phototoxic effects than those containing MKM (with 14 carbon atoms). Altogether, the results obtained showed that even though the formulations with MKM were the least cytotoxic in almost all conditions tested (see Section 2.2), they displayed the highest potential phototoxicity of the nanovesicular systems.

### 3.5. Inflammatory response

To gain an insight into possible inflammatory reactions attributable to the nanovesicle formulations, secretion of cytokines (IL-1α and IL-8) by two different cell lines (HaCaT and THP-1, respectively) was examined by ELISA. THP-1 cells were stimulated with 5 ng/ml of lipopolysaccharide (LPS) from Escherichia coli O55:B5 (Sigma, St. Louis, MO), in order to examine the capacity of this cell model to up-regulate cytokine (IL-8) production [48]. We found significantly higher IL-8 concentrations (652.14 ± 13.86 pg/ml, $p < 0.005$), while cell viability was 87.5% as detected by the MTT assay. THP-1 cells were chosen because they were proposed as an *in vitro* model able to produce and release the potent pro-inflammatory cytokine IL-8 [48,49]. Therefore, this *in vitro* model is a promising tool for the screening of new nanomaterials with possible inflammatory response. IL-8 (Fig. 4) release was induced in THP-1 cells in a dose-dependent manner by the formulations containing the amphiphiles MKM and PKM. The cationic vesicles containing MKM showed significant cytokine release at concentrations higher than 2.5 μM ($p < 0.005$), while those containing PKM induced significant IL-8 release at concentrations higher than 1 μM and 5 μM, for the formulations with DMPC only or DMPC and cholesterol as lipid matrix, respectively. Interestingly, the surfactant MLM, which differs in the position of the cationic charge, did not show a dose–response release of IL-8, but only induced a significant increase in the level of this cytokine at the highest concentration. Indeed, since the significant release at the highest concentrations might be related to cell death, it is reasonable to consider cytokine release responses given by concentrations that displayed viability higher than 75% as significant [40,49]. Therefore, we can reasonably consider as significant IL-8 releases those induced at concentrations equal to or lower than 2.5 μM and 5 μM for the formulations containing PKM and MKM, respectively, while no significant response can be attributed to the formulations containing MLM, as even a 10-fold higher concentration (50 μM, viability >75%) did not induce IL-8 release. The IL-8 release induced by nanomaterials has also been studied in different cell lines and, in line with our findings, only slight or negligible inflammatory response due to increased levels of IL-8 was found [16,50].

Keratinocytes participate actively in inflammatory and immunological skin reactions [51]. The human keratinocyte HaCaT cell line was stimulated with SDS 20 μg/ml, as positive control, to confirm IL-1α up-regulation [52]. Under these conditions, we found significantly higher cell-associated IL-1α concentrations (239.12 ± 9.87 pg/mg protein, $p < 0.005$) with cell viability of 85.7% as detected by the LDH assay. Therefore, the combination of keratinocyte and cytokine production (IL-1α as a pro-inflammatory cytokine) offers a simplified *in vitro* model to evaluate the potential toxicity of new nanomaterial-based formulations with cutaneous applications. In this study, IL-1α production (both cell-associated and that released into extracellular media) was investigated in human keratinocytes following their exposure to nanovesicle formulations. The intracellular amount of IL-1α was standardized with the total cell protein content, and the LDH assay was used as an indicator of cell viability. Fig. 5 shows our results for cell-associated IL-1α (pg/mg protein) and IL-1α release

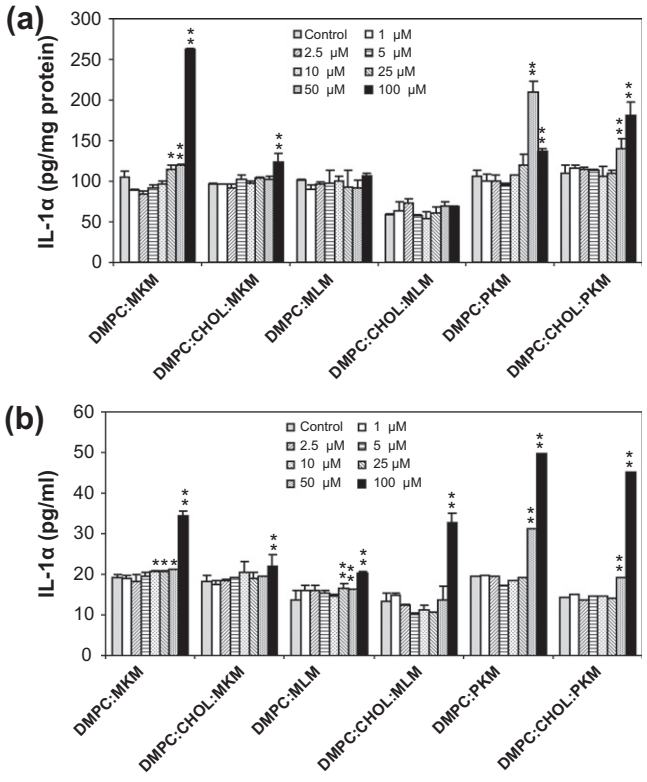



**Fig. 5.** (a) Cell-associated IL-1α (pg/mg protein) and (b) IL-1α release (pg/ml) to the culture medium by HaCaT cells with increasing concentrations of each nanovesicle formulation. The concentrations tested were 1 μM (white bars), 2.5, 5, 10, 25, 50 and 100 μM (black bars). Results are expressed as mean ± SEM of three independent experiments, performed in triplicate. Statistical analyses were performed using ANOVA followed by Dunnett's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.005$ denote significant differences.

(pg/ml) after cationic vesicle treatment. Neosynthesis or release of IL-1α only achieved significant levels above the basal values (negative control) at the highest exposure conditions (25–100 μM) in almost all cases, but the levels obtained were below those of the positive control SDS. For some formulations, there was a down-regulatory effect in cell-associated IL-1α and an increase in IL-1α release at the highest concentrations, which can be directly attributable to the loss of cell membrane integrity (as seen in the LDH assay results, see Fig. 2e and f). The formulations containing cholesterol in the lipid matrix induced, in general, lower IL-1α production. Moreover, the nanovesicles containing MLM were those that induced lower production of IL-1α, as also observed for IL-8 release. This means that the inflammatory response of the nanovesicles might also be directly related, as reported for the cytotoxic and phototoxic studies, to the position of the cationic charge of the amphiphile: the formulations containing the surfactant with the positive charge on the ε-amino group of the lysine have the least inflammatory potential. In the same way as described for the IL-8 and since the significant release at the highest concentrations might be related to cell death, the neosynthesis or release of IL-1α was not significant. All in all, and despite some significant responses found for IL-8 and IL-1α release in comparison with control cells, our results suggest that only a very slight inflammatory and allergenic effect was induced by nanovesicle formulations.

## 4. Conclusions

We demonstrated that the cytotoxicity of cationic nanovesicles differed between the three representative skin cell lines as well as when the *in vitro* endpoint varied, showing that the selected cell type and assay can affect the final outcome. Moreover, no potential phototoxic effect was induced by all nanovesicular systems, while only a slight inflammatory response occurred due to cytokine release. The formulations with MLM showed the lowest tendency to induce phototoxicity and inflammation. Overall, our findings showed that nanovesicle composition plays a primary role in the underlying toxicity. The cytotoxic responses of the nanovesicles varied especially as a function of the cationic charge position on the amphiphile included in them. Furthermore, the surfactant with the highest hydrophobicity tends to enhance the toxic potential of the formulations. All these findings suggest that differential toxicity according to vesicle composition could be an important concept when developing new nanomaterials for biomedical applications. In conclusion, the combination of all assays used in the present study offers an in-depth and comprehensive evaluation of the potentially toxic effects of nanomaterials. Due to their generally low toxicity, the nanovesicles containing MKM and MLM are especially recommended for topical administration.

## Acknowledgments

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## Artículo 6

**LYSINE-BASED SURFACTANTS IN NANOVESICLE FORMULATIONS: THE ROLE OF CATIONIC CHARGE POSITION AND HYDROPHOBICITY IN *IN VITRO* CYTOTOXICITY AND INTRACELLULAR DELIVERY**

**(Tensioactivos derivados de lisina en formulaciones de nanovesículas: efecto de la posición de la carga catiónica y hidrofobicidad en la citotoxicidad *in vitro* y liberación intracelular)**

**Daniele Rubert Nogueira, M. Carmen Morán, Montserrat Mitjans, Lourdes Pérez, David Ramos, Joaquín de Lapuente, M. Pilar Vinardell**

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# Resumen

*Objetivos*

Desarrollar y caracterizar sistemas nanovesiculares conteniendo tensioactivos catiónicos derivados de lisina, estudiar como estos compuestos modulan la toxicidad *in vitro* de las nanovesículas y valorar si aumentan su probabilidad de captación celular o bien su habilidad en promover una liberación intracelular eficiente.

*Material y métodos*

Se desarrollaron nanovesículas con matriz lipídica básica formada por el fosfolípido DMPC (dimiristoilfosfatidilcolina), con o sin la adición de colesterol, incluyendo en su composición tensioactivos catiónicos derivados de lisina del tipo sales de hidrocloruro de $N^{\alpha}$ o $N^{\varepsilon}$-acil lisina metil éster que difieren en cuanto a sus características estructurales. Estos sistemas fueron caracterizados en cuanto a sus características fisicoquímicas utilizando espectroscopia de correlación de fotones, microscopía electrónica de transmisión y cromatografía líquida. La citotoxicidad se valoró en las líneas celulares 3T3 y HeLa utilizando los ensayos de viabilidad MTT, NRU y LDH. Los mecanismos involucrados en la citotoxicidad se investigaron a través de estudios de apoptosis, ciclo celular, peroxidación lipídica y genotoxicidad. Se valoró su compatibilidad con los componentes de la sangre, utilizando el ensayo de hemólisis y electroforesis en gel de proliacrilamida para identificar adsorción de proteínas plasmáticas. Por último, se estudió la captación celular y el comportamiento intracelular de las nanovesículas utilizando la línea HeLa como modelo celular y marcadores fluorescentes como la calceína y el rojo nilo.

*Resultados*

La caracterización de las nanovesículas mostró agregación significativa después de 24 h de incubación en medio de cultivo, a excepción de los sistemas que tienen el tensioactivo con la carga positiva en el grupo ε-amino de la lisina. Las repuestas citotóxicas de las nanovesículas variaron dependiendo del tensioactivo, línea celular y ensayo de viabilidad. El MTT fue el método más sensible en detectar los efectos tóxicos de las formulaciones con los tensioactivos que tienen la carga en el grupo α-amino, indicando la presencia de mecanismos diferentes involucrados en su citotoxicidad. La inducción de disfunción mitocondrial, estrés oxidativo y apoptosis, en conjunto con lisis de la membrana celular a concentraciones altas, fueron los mecanismos encontrados como responsables de la toxicidad de las nanovesículas. El conjunto de resultados de este trabajo mostró que las características estructurales de los tensioactivos directamente afectan la toxicidad del sistema nanovesicular resultante. Los estudios por microscopía de fluorescencia utilizando el marcador rojo nilo demostraron que las nanovesículas fueron internalizadas por las células, mientras que la utilización del marcador de endocitosis calceína mostró que las formulaciones conteniendo los tensioactivos con la carga en el grupo α-amino liberaron de modo significativo el material endocitado en el citoplasma celular. Por último, también se demostró compatibilidad aceptable de las nanovesículas con los componentes de la sangre.

*Conclusiones*

Las características estructurales de los tensioactivos incluidos en las nanovesículas determinaron los mecanismos de interacción de estos sistemas con las células y, por lo tanto, su toxicidad y su comportamiento intracelular. La combinación del amplio rango de ensayos *in vitro* utilizado en este trabajo constituye un análisis completo y fidedigno de los potenciales efectos tóxicos de los nanomateriales.

# Lysine-based surfactants in nanovesicle formulations: the role of cationic charge position and hydrophobicity in *in vitro* cytotoxicity and intracellular delivery

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## ABSTRACT

Understanding nanomaterial interactions within cells is of increasing importance for assessing their toxicity and cellular transport. Here, we developed nanovesicles containing bioactive cationic lysine-based amphiphiles, and assessed whether these cationic compounds increase the likelihood of intracellular delivery and modulate toxicity. We found different cytotoxic responses among the formulations, depending on surfactant, cell line and endpoint assayed. The induction of mitochondrial dysfunction, oxidative stress and apoptosis were the general mechanisms underlying cytotoxicity. Fluorescence microscopy analysis demonstrated that nanovesicles were internalized by HeLa cells, and evidenced that their ability to release endocytosed materials into cell cytoplasm depends on the structural parameters of amphiphiles. The cationic charge position and hydrophobicity of surfactants determine the nanovesicle interactions within the cell and, thus, the resulting toxicity and intracellular behavior after cell uptake of the nanomaterial. The insights into some toxicity mechanisms of these new nanomaterials contribute to reducing the uncertainty surrounding their potential health hazards.

# Introduction

Nanomaterials (NMs) are classically defined as substances that have one or more external dimensions on a sub-100 nm scale (Horie et al. 2012), although some authors classify them as all submicronic particles up to 200 nm (Paillard et al. 2010). At this size, NMs might be easily taken up by cells and interact in a unique fashion with biological systems, which opens up a wide range of interesting applications, including the development of drug and gene delivery systems (Robbens et al. 2010).

Lipid bilayer vesicles are the most prominent colloidal drug carriers, as they can transport drug molecules into the interior of the vesicle, solubilize drugs in the lipid bilayer, or adsorb drugs at the lipid-water interface (Cevc 2012; Liang and Chou 2009). To date, all pharmaceutically used lipid-based vesicles consist of phospholipids (mainly phosphatidylcholine) supplemented with cholesterol, due to the widespread, but not always justified, belief that this is a prerequisite for bilayer stability (Cevc 2012). The inclusion of additives in lipid-based vesicles might help to fulfill the unmet, or partially unmet, goals of these lipid delivery systems. For example, it may increase absorption, improve controlled release and target specificity (Bombelli et al. 2005), and facilitate intracellular delivery (Chen et al. 2004). Additives with non-viral properties, including surfactants, lipids, peptides and polymers, are being intensively studied (Chen et al. 2004; Liang and Chou 2009; Zhang et al. 2004). In this line of research, current directions and the reported advantageous features of the amino acid-based surfactants (Colomer et al. 2012; Nogueira et al. 2011a, 2012a; Pérez et al. 2009) make them a promising group of novel additives for this kind of drug carriers (Lundberg et al. 2011; Morán et al. 2010). The amino acid-based amphiphiles have a structural relationship with endogenous substances and their synthesis can largely be based on non-toxic building blocks, which potentiallly gives them good biocompatibility (Infante et al. 2010; Lundberg et al. 2011).

Advances in strategies for treating a wide variety of diseases require the efficient delivery of active compounds into the cytosol of target cells (Hu et al. 2007). One common strategy for the intracellular delivery of encapsulated and/or intercalated material *via* lipid-based vesicles exploits intracellular pH gradients (Pollock et al. 2010). Therefore, an approach for cytosolic drug delivery is the development of pH-sensitive lipid-based vesicles (Di Marzio et al. 2011; Simões et al. 2004), which contain pH-responsive components that are stable at physiological pH (7.4), but undergo destabilization under the acidic environments encountered during endocytosis. Consequently, their contents are released at intracellular level (Di Marzio et al. 2011). In our previous studies on amino acid-based surfactants, we showed that new cationic lysine-based amphiphiles (hydrochloride salts of $N^{\varepsilon}$-acyl lysine methyl ester) have pH-responsive membrane lytic activity (Nogueira et al. 2012a, 2012b). Thus, they are highly suitable for incorporation in carriers designed for intracellular drug delivery. The potential advantages of this class of cationic lysine-based surfactants as additives in devices designed for drug delivery motivated us to develop new nanovesicles (NVs) containing these amphiphiles as surface modification agents. We study whether these cationic compounds increase the likelihood of intracellular delivery, establish the physicochemical properties of such carriers and

modulate their cytotoxic potential. In line with the likely advantages of cationic delivery systems, cationic lipid-based carriers have attracted considerable interest because of their use as effective drug and gene delivery systems (Dakwar et al. 2012; Lundberg et al. 2011; Ramezani et al. 2009).

In the present study, we assessed the impact of including three cationic lysine-based surfactants in lipid-based NVs. These amphiphiles differed in the cationic charge position and the alkyl chain length. Consequently, we investigated specifically whether these structural parameter changes play a key role in the general outcome of the NVs. The potential topical application of these formulations were explored in our previous study, in which we showed no phototoxicity, slight inflammatory potential, and acceptable toxic responses in representative skin cell lines (Nogueira et al. 2013). Here, we tested the feasibility of these NVs as efficient drug delivery devices for intravenous administration and intracellular delivery of biomolecules. With this objective, we focused on the performance of a broad *in vitro* nanotoxicity study of these new nanocarriers, together with an assessment of their uptake by cells and ability to deliver contents intracellularly, using nile red and calcein as fluorescent markers, respectively. We aimed to increase understanding of the various steps in nanocarrier cytotoxicity, as little is known about the mechanisms underlying this effect. The potential toxic mechanisms of the increasing number of nanocarriers have not been explained sufficiently. Additionally, relationships between the cytotoxic responses and NM composition are not well understood. Thus, the search for reliable conditions to assess NM safety is an emerging field that poses many interesting challenges (Marquis et al. 2009).

## Materials and Methods

### Chemicals and reagents

Acridine orange (AO), ethidium bromide (EB), propidium iodide, ribonuclease A (RNAse A), nile red (NR), calcein, 2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), cholesterol (CHOL) and dimethylsulphoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin-EDTA solution (170,000 U/l trypsin and 0.2 g/l EDTA) and penicillin-streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) were purchased from Lonza (Verviers, Belgium). The 75 $cm^2$ flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland). All other reagents were of analytical grade.

### Surfactants included in the nanovesicular systems

Three new biocompatible amino acid-based surfactants with one lysine as the cationic polar head (one cationic charge) and one alkyl chain were used as surface modification agents to prepare the cationic nanovesicular systems reported in this study. The surfactants were: $N^{\varepsilon}$-myristoyl lysine methyl ester (MKM) with one alkyl chain of 14 carbon atoms

and one positive charge on the α-amino group of the lysine; $N^{\varepsilon}$-palmitoyl lysine methyl ester (PKM) with one alkyl chain of 16 carbon atoms and one positive charge on the α-amino group of the lysine; and $N^{\alpha}$-myristoyl lysine methyl ester (MLM) with one alkyl chain of 14 carbon atoms and one positive charge on the ε-amino group of the lysine. MKM and PKM have a hydrophobic chain attached to the ε-amino group of the lysine, while MLM has its hydrophobic chain attached to the α-amino group. These lysine-based surfactants were synthesized in our laboratory, as described elsewhere (Colomer et al. 2012; Pérez et al. 2009).

**Preparation of cationic nanovesicular formulations**

The mixed cationic NVs were prepared by the film hydration method, as previously described (Nogueira et al. 2013). DMPC and cholesterol (CHOL) were selected as basic lipid membrane components and were mixed with MKM, PKM or MLM in the designed molar ratios, as described in the Table 1. The total final concentration of each mixed cationic NV was fixed at 2 mM. NV dispersions were purified by filtration using Vivaspin 2 centrifugal concentrator (PES membrane, 3,000 MWCO, Sartorius Stedim Biotech, Goettingen, Germany). The filtered substance was used to determine the extent of incorporation of the cationic surfactants into the vesicles and the amount of unincorporated surfactant was assessed by high-performance liquid chromatography, following the previously described analytical method (Pérez et al. 2009).

NVs physically encapsulating Nile red as a fluorescent marker (NR-NVs) were also prepared. The formulation DMPC:MKM (80:20, w/w) was used as a model in this study. Nile red 1 mol% (of the final concentration of the formulation), was dissolved with the phospholipid and the surfactant prior to obtaining the film. Then, the nanovesicle preparation followed the same procedure as that described above. The NR-NVs were further purified to eliminate the non-encapsulated dye by ultracentrifugation with multiple washing with ultrapure water.

**Table 1.** Scheme of the nanovesicles' composition.

| | Molar composition (%) | | |
|---|---|---|---|
| Nanovesicles | DMPC | COLESTEROL (CHOL) | Surfactant (MKM, PKM o MLM) |
| DMPC:surfactant | 80 | - | 20 |
| DMPC:CHOL:surfactant | 56 | 24 | 20 |

**Nanovesicle characterization**

The mean hydrodynamic diameter and the polydispersity index (PDI) of the cationic NVs were determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK). Before measurement, the NVs were appropriately diluted in ultrapure water or cell culture medium with 5% (v/v) FBS. Readings were taken at 25ºC immediately after preparation (t = 0 h) and after a 24 h incubation at 37ºC (t = 24 h). Each measurement was performed using at least three sets of a minimum of ten runs. The zeta potential (ZP) values of the NVs were assessed by determining electrophoretic

mobility with the Malvern Zetasizer ZS equipment. The measurements were also performed in ultrapure water and cell culture medium with 5% (v/v) FBS (t = 0 h, 25ºC), using at least three sets of a minimum of 20 runs. These experiments were performed as previously described (Nogueira et al. 2013), but additional analyses were carried out to assess the NV stability in ultrapure water after 24 h and 1 week incubation at 37ºC and 4ºC, respectively.

The morphology and size of the NVs were analyzed by transmission electron microscopy (TEM), as previously described (Nogueira et al. 2013). The images were obtained with a Jeol JEM-1010 electron microscope (Jeol Ltd., Tokyo, Japan) operating at an acceleration voltage of 80 kV.

**Cell cultures**

The 3T3 (murine Swiss albino fibroblasts) and the HeLa (human epithelial cervical cancer) cell lines were grown in DMEM medium (4.5 g/l glucose) supplemented by 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37ºC, 5% $CO_2$. These cells were routinely cultured in 75 $cm^2$ culture flasks and were trypsinized using trypsin-EDTA when the cells reached approximately 80% confluence. The cell lines were obtained from Eucellbank (Universitat de Barcelona, Spain). We selected these cell lines as model systems because the use of cells from different species and with different embryonic origins is an important approach to understand the cell specific responses induced by NMs (Fröhlich et al. 2012).

**Cytotoxicity assays: MTT, NRU and LDH**

The 3T3 (1 x $10^5$ cells/ml) and HeLa (5 x $10^4$ cells/ml) cells were seeded into the 60 central wells of 96-well cell culture plates in 100 µl of complete culture medium. Cells were incubated for 24 h under 5% $CO_2$ at 37ºC. The medium was then replaced with 100 µl of fresh medium supplemented by 5% (v/v) FBS containing the NV dispersions in the 0.5 – 100 µM concentration range. The surfactants only and NVs without any surfactant (DMPC only and DMPC:CHOL 70:30) were also assessed in the same concentration range. Each concentration was tested in triplicate and control cells were exposed to the medium with 5% (v/v) FBS only. The cell lines were incubated for 24 h with each treatment.

The MTT assay is a measurement of cell metabolic activity in the mitochondria of viable cells and was performed as previously described (Nogueira et al. 2013). Cell viability was calculated as the percentage of tetrazolium salt reduction by viable cells in each sample and the values were normalized by the untreated cell control (cells with medium only).

The NRU assay determines the accumulation of NR dye in the lysosomes of cells and reflects the functionality of the lysosomes and plasma membrane (Fröhlich et al. 2012). The assay was performed following the previously described methodology (Nogueira et al. 2013). The effect of each treatment was calculated as the percentage of uptake of NR dye by lysosomes against the untreated cell control (cells with medium only).

LDH leakage was determined in the conditioned medium using a commercially available kit (Takara Bio Inc, Otsu, Japan), according to the instructions provided by the

manufacturer. This assay is an indicator of plasma membrane integrity and quantifies cytotoxicity by measuring LDH released from dead or plasma membrane-damaged cells into the supernatant (Yang et al. 2009). Results are expressed as a percentage of the control, with 1% (v/v) Triton-X used as a positive control.

One of the possible limitations of the cytotoxicity study of NMs is their interference with the dyes used in the viability assays. Therefore, we determined whether the NVs interact with the viability assays using UV-visible absorbance measurements (Monteiro-Riviere et al. 2009, 2010). NVs at 100 μM were suspended in DMEM medium (without FBS and phenol red) containing MTT (0.5 mg/ml) or NR (50 μg/ml) dyes, and the occurrence of dye interference was assessed following the procedure previously described (Nogueira et al. 2013).

**Apoptosis**

NV-induced apoptosis in 3T3 cells was quantified using acridine orange/ethidium bromide (AO/EB) double staining, according to standard procedure (Squier and Cohen 2001). Samples were examined under a fluorescence microscope. Briefly, cells were seeded (1 x $10^5$ cells/ml) in 24-well plates and treated with $IC_{50}$ concentration (calculated by MTT assay). After 24 h incubation, the cells were trypsinized and centrifuged at 1200 rpm for 5 min. Then, the fluorescent dyes AO (0.5 μg/ml) and BE (10 μg/ml) were added to the cellular pellet. The freshly stained cell suspension was dropped on a glass slide and covered by a cover slip. Slides were observed under a fluorescent microscope (Olympus BX41 microscope equipped with a UV-mercury lamp, 100W Ushio Olympus, and a filter set type MNIBA3 470-495 nm excitation and 505 nm dichromatic mirror) and the percentage of viable, apoptotic, late apoptotic and necrotic cells was determined in at least 100 cells.

**Cell cycle analysis by flow cytometry**

The 3T3 fibroblasts were cultured in 60 mm petridishes for 24 h at a density of 1 x $10^5$ cells/ml and then treated with $IC_{20}$ and $IC_{50}$ concentrations of each NV formulation. After 24 h treatment, the cells were harvested by trypsinization, washed in cold PBS, fixed in ice-cold 70% ethanol and kept at -20ºC. Fixed cells were centrifuged, resuspended in DNA extraction buffer (0.2 M $Na_2PO_4$ and 0.1 M citric acid, pH 7.8) and incubated for 30 min at 37ºC. Then, the cells were centrifuged and stained with staining solution (20 μg/ml propidium iodide, 200 μg/ml RNAse A and Triton X-100 in PBS). The samples were kept in dark conditions for 1 h and measured with the Beckman Coulter ADC Epics XL flow cytometer (Beckman Coulter, FL, USA). The amount of propidium iodide intercalating to DNA was used as the parameter to determine the cell cycle distribution phases. Aggregates were excluded gating single cells by their area vs. peak fluorescence signal. DNA analysis on single fluorescence histograms was done using Multicycle software (Phoenix Flow Systems, CA, USA).

**Genotoxicity**

DNA damage in the form of unrepaired single- and double-strand DNA breaks was detected using an alkaline single cell gel electrophoresis/comet assay, according to the

method described by Singh et al. (1988), with some modifications by Di Guglielmo et al. (2012). The 3T3 cells were treated with the $IC_{10}$, $IC_{20}$ and $IC_{30}$ concentrations (calculated by MTT assay) of each NV formulation. After 24 h incubation, the cells from 2 wells of each treatment were trypsinized and transferred to eppendorfs. Slides containing the samples were prepared. The cells were lysated and then incubated in alkaline electrophoresis buffer for DNA unwinding and conversion of alkali-labile sites to single-strand breaks. Electrophoresis was performed in the same buffer for 30 minutes at 25 V and 300 mA. After that, 20 µL of 5 µg/ml DAPI solution was added to each slide for the fluorescence microscopy analysis. The migration of nuclear DNA from the cells was measured using the COMET ASSAY IV® Program (Perspective Instruments) for 50 randomly selected cell images, and the mean percentage of DNA in the tail (% Tail DNA) was calculated in each trial. Methyl methanesulfonate (MMS) at a concentration of 400 µM was used as the positive control.

**Oxidative damage**

Oxidative stress can be determined by biomarker stress products from lipid peroxidation (Marquis et al. 2009). We employed the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to indicate the oxidative damage caused by NVs. MDA is the end product of the peroxidation of polyunsaturated fatty acids, and has been extensively used as an index for lipid peroxidation. The 3T3 fibroblasts were cultured in 60 mm petri dishes for 24 h at a density of 1 x $10^5$ cells/ml and then treated with $IC_{50}$ concentrations of each NV formulation. After 24 h treatment, the cells were trypsinized, centrifuged and resuspended in PBS. For the lipid extraction, cells were incubated with 3.2% SDS and 30% acetic acid, and 0.8% TBA was added. The reaction was carried out at 95ºC for 1 h and the absorbance was then determined at 532 nm using a microplate reader. The MDA concentration in each sample was obtained from an MDA calibration curve (0 - 15 nM/ml). The values were normalized against the total cell protein content and the results expressed as nM MDA/mg protein. The protein content of the cell lysate was determined by a commercial kit (Bio-Rad, CA, USA) based on the Bradford dye-binding procedure (Bradford 1976). Hydrogen peroxide ($H_2O_2$) at a concentration of 100 µM was included in the assay as a positive control.

**Blood compatibility studies**

**Hemolysis and agglutination assays**

Erythrocytes were isolated from rat blood, which was obtained from anesthetized animals by cardiac puncture and drawn into tubes containing EDTA. The procedure was approved by the institutional ethics committee on animal experimentation. The hemolysis assay was performed following the previously described procedure (Nogueira et al. 2011a). Aliquots of 25-µl of erythrocyte suspension were exposed to NV concentrations of 20, 100 and 200 µM, and dissolved in PBS buffer in a total volume of 1 ml. Two controls were prepared by resuspending erythrocyte suspension either in buffer alone (negative control) or in distilled water (positive control). The samples were incubated at room temperature for 10 minutes or 1 h and then centrifuged at 10,000 rpm for 5 min. Absorbance of the hemoglobin release

in supernatants was measured at 540 nm using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan) and the percentages of hemolysis were determined by comparison with the positive control samples completely hemolyzed with distilled water. For the erythrocyte agglutination studies, 10 µl of each sample that had been subjected to a hemolysis assay (100 µM for 1 h) were placed on a glass slide, covered by a cover slip and analyzed by a phase contrast microscope (Olympus BX41, Olympus, Japan).

**Human plasma protein adsorption by SDS-PAGE and total protein assay**

Blood was drawn from the authors by venipuncture into tubes containing EDTA. It was then centrifuged at 3000 rpm to obtain fresh plasma. NVs (200 µM) were suspended in plasma diluted with PBS to 10% of normal strength and incubated for 1 h at 37ºC with constant shaking. The NVs were separated from the plasma by multiple cycles of ultracentrifugation and washing steps with PBS. Firstly, an aliquot of the supernatant of each sample was taken and mixed with electrophoresis sample buffer. Then, the proteins adsorbed to the NVs were desorbed by sonication in electrophoresis sample buffer for 20 min. Both samples were incubated at 95ºC for 5 min. Thereafter, samples were applied onto pre-cast polyacrylamide gel (7.5% resolving gel and a 5% stacking gel). Electrophoresis was carried out for 10 min at 60 V followed by 35 min at 200 V. Protein bands were viewed by staining with Coomassie Brilliant Blue R-250 for an hour under gentle shaking. Samples were then destained with a mixture of 7.5% methanol and 10% acetic acid. The molecular weight of the membrane proteins was estimated from the molecular size marker (Bio-Rad Precision Plus Unstained Standard), and ranged from 10 to 250 kDa. Finally, portions of the supernatant and the fluid desorbed from NVs were also taken for total protein assay using the Bio-Rad kit (Bio-Rad, CA, USA), which is based on the Bradford dye-binding procedure (Bradford 1976), using bovine serum albumin (BSA) as a protein standard.

**Cell uptake studies**

**Intracellular localization of Nile red-labeled nanovesicles**

To study the cell uptake, DMPC:MKM (80:20, w/w) NVs that physically encapsulated Nile red (NR-NVs) were prepared. HeLa cells were plated in 24-well plates at a density of 5 x $10^4$ cells/ml on round cover glasses (Marlenfeld GmbH & Co.KG, Lauda-Könlgshofen, Germany) and incubated overnight at 37ºC under 5% $CO_2$. After that, the culture medium was replaced with fresh medium containing NR-NVs at a final concentration of 50 µM (0.5 µM of NR) and incubated for 2 h and 24 h. Following incubation, the test samples were aspirated and the cells were washed three times with PBS and fixed with 4% (v/v) formaldehyde in PBS (pH 7.4) for 15 min at room temperature. The individual cover glasses were then mounted on clean glass slides with Prolong® Gold antifade reagent (Invitrogen, OR, USA) for subsequent fluorescence microscopy analysis (Olympus BX41 microscope equipped with a UV-mercury lamp, 100W Ushio Olympus, and a filter set type MNIBA3 470-495 nm excitation and 505 nm dichromatic mirror). Images were digitized on a computer through a video camera (Olympus digital camera XC50) using an image processor (Olympus cell^B Image Acquisition Software). The images were then analyzed

with ImageJ software (v. 1.46, National Institutes of Health, MD, USA) (Collins 2007; Rasband 1997) to calculate the mean fluorescence value of the cells, which corresponds to the cell internalization of NVs. For each condition, ~20 individual cells from different fields and images were analyzed and their total fluorescence intensity was quantified.

**Intracellular release of calcein**

Calcein (a membrane-impermeable fluorophore) was used as a tracer molecule to monitor the effect of the NVs on endosomes after cell internalization. HeLa cells were plated (5 x $10^4$ cells/ml) in 24-well plates on round cover glasses (Marlenfeld GmbH & Co.KG, Lauda-Könlgshofen, Germany) and incubated overnight at 37ºC under 5% $CO_2$. Then, calcein (1 mg/ml) was added to the cells with or without (control cells) 50 μM of each NV formulation in DMEM medium without FBS and phenol red. After 1 h incubation at 37ºC, the cells were washed four times with PBS and incubated in complete medium for 3 h to allow intracellular trafficking. The cells were then washed four times with PBS and fixed with 4% (v/v) formaldehyde in PBS (pH 7.4) for 15 min at room temperature. Each individual cover glasses was mounted on a clean glass slide with Prolong® Gold antifade reagent (Invitrogen, OR, USA) and analyzed on a Olympus BX41 fluorescence microscope equipped with a UV-mercury lamp (100W Ushio Olympus) and a filter set type MNIBA3 (470-495 nm excitation, 510-550 nm emission and 505 nm dichromatic mirror). Images were digitized on a computer through a video camera (Olympus digital camera XC50) using an image processor (Olympus cell^B Image Acquisition Software). Thereafter, ImageJ software was used to calculate the average pixel intensity of calcein fluorescence within regions of interest (ROI) drawn on to collected images. Images of ~20 individual cells were analyzed for each treatment. This was done by drawing three ROI inside the cell (excluding any calcein-containing vesicles and, thus, representing the cytoplasm only) and the results were obtained in arbitrary fluorescence units (Jones et al. 2003).

**Statistical analysis**

The results of all *in vitro* assays are expressed as mean ± standard error of the mean (SEM) of three independent experiments, which were performed using three replicate samples for each concentration tested. The cytotoxicity of each formulation was expressed in terms of its $IC_{50}$ (the concentration causing 50% death of the cell population), calculated from concentration-response curves. Statistical analyses used the Student's *t* test or the one-way analysis of variance (ANOVA) to determine the differences between the datasets, followed by Dunnett's *post-hoc* test for multiple comparisons using SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ and $p < 0.005$ were considered significant.

## Results

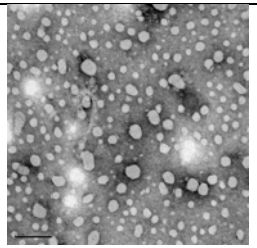
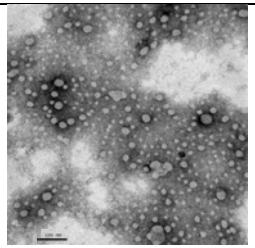
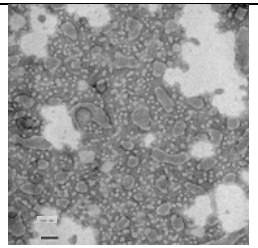
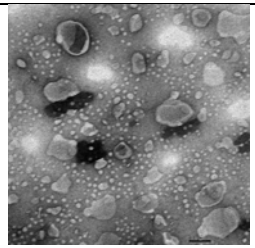
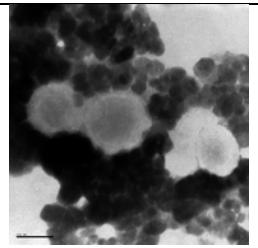
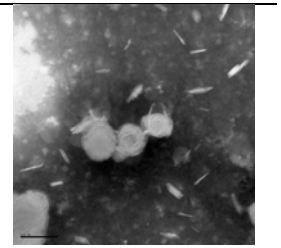
**Characterization of cationic nanovesicles**

As was previously reported (Nogueira et al. 2013), we assessed the effects of the dispersion medium (ultrapure water or cell culture medium) on NV hydrodynamic size (Table 2). Here, we have extended the NV characterization and also evaluated the effects of temperature and exposure time on the stability of the formulations in ultrapure water.

DLS measurements showed that after 24 h incubation (37ºC) in ultrapure water, the NVs showed slightly lower diameters or did not consistently increase in comparison to fresh prepared NVs (t = 0), with the exception of the NVs containing PKM, which almost doubled in size. When the NVs were dispersed in cell culture medium (DMEM with 5% [v/v] FBS) the size increase was slight by 0 h, but significant agglomeration to micron-sized structures occurred after 24 h incubation under cell culture conditions (37ºC), with the exception of the NVs containing MLM. As observed from the PDI values, the increase in NV polydispersity was slight to moderate after 24 h incubation at 37ºC in water. However, in general, substantially higher PDI values were obtained after incubation in cell culture medium. The ZP values of all formulations dispersed in water were highly positive (> 40 mV), whereas almost neutral values were obtained in cell culture medium. The NVs containing MLM showed the most positive ZP values in DMEM medium (~10 mV). The purification process with a Vivaspin column resulted in no significant changes in NV size, PDI, or ZP (data not shown). Preservation of the mean hydrodynamic size and zeta potential was observed for at least one week at 4ºC, with the exception of the formulation DMPC:CHOL:PKM, proving the generally good physical stability of these colloidal formulations. Finally, the HPLC measurements of the filtered samples (obtained from the purification process performed to remove the unincorporated amount of surfactant) revealed that the cationic surfactants were highly incorporated into the NVs (from 75 to 99% incorporation).

The TEM analysis (Table 2) showed that the cationic NVs containing MKM and PKM were predominantly much smaller (~ 20 – 50 nm) than the hydrodynamic size determined by DLS (~ 100 or 200 nm, respectively). These differences were especially significant for the NVs with MKM, while the formulations with PKM showed a more heterogeneous size distribution (2 predominant populations: 20 – 50 nm and 100 – 150 nm). In contrast, TEM images corroborated the mean hydrodynamic size obtained by DLS for the NVs containing MLM. Moreover, the TEM images revealed the formation of a multilayered membrane in the NVs containing MLM, while those containing MKM and PKM showed unilamellar membranes in both the presence and absence of cholesterol in the basic membrane.

**Table 2.** Characterization properties of the cationic nanovesicles. Values are reported as mean of three independent experiments ± SEM.

| | DMPC:MKM (80:20) | DMPC:CHOL:MKM (56:24:20) | DMPC:PKM (80:20) | DMPC:CHOL:PKM (56:24:20) | DMPC:MLM (80:20) | DMPC:CHOL:MLM (56:24:20) |
|---|---|---|---|---|---|---|
| | | | *Size (nm) ± SEM* | | | |
| t = 0 h water | 94.16 ± 2.05 | 107.33 ± 0.94 | 253.07 ± 26.05 | 184.77 ± 6.64 | 174.40 ± 7.16 | 127.50 ± 1.96 |
| t = 0 h DMEM 5% FBS | 94.42 ± 6.50 | 159.77 ± 5.71 | 229.37 ± 12.64 | 197.73 ± 6.57 | 229.63 ± 16.33 | 170.27 ± 9.49 |
| t = 24 h water [a] | 89.39 ± 3.42 | 82.59 ± 5.58 | 355.6 ± 31.49/ 68.71 ± 7.44 [c] | 368.47 ± 19.56/ 77.94 ± 4.26 [c] | 189.53 ± 5.38 | 119.57 ± 4.16 |
| t = 24 h DMEM 5% FBS [a] | 1781.67 ± 45.72/ 110.3 ± 5.17 [c] | 2028.67 ± 21.23/ 154.33 ± 11.95 [c] | 1059.50 ± 10.61/ 118.93 ± 2.09 [c] | 1488 ± 19.59/ 125.67 ± 11.94 [c] | 193.47 ± 7.75 | 151.77 ± 4.44 |
| t = 1 week water [b] | 78.41 ± 4.76 | 102.86 ± 4.19 | 277.9 ± 22.16 | 349.23 ± 18.45/ 78.64 ± 4.10 [c] | 173.27 ± 8.19 | 130.70 ± 3.20 |
| | | | *PDI ± SEM* | | | |
| t = 0 h water | 0.231 ± 0.004 | 0.278 ± 0.019 | 0.427 ± 0.017 | 0.331 ± 0.020 | 0.394 ± 0.003 | 0.256 ± 0.006 |
| t = 0 h DMEM 5% FBS | 0.385 ± 0.015 | 0.236 ± 0.001 | 0.522 ± 0.001 | 0.288 ± 0.001 | 0.445 ± 0.007 | 0.319 ± 0.029 |
| t = 24 h water [a] | 0.371 ±0.025 | 0.378 ± 0.024 | 0.508 ± 0.003 | 0.523 ± 0.044 | 0.399 ± 0.003 | 0.239 ± 0.005 |
| t = 24 h DMEM 5% FBS [a] | 0.903 ± 0.075 | 1.00 ± 0.000 | 0.605 ± 0.002 | 0.973 ± 0.027 | 0.294 ± 0.001 | 0.352 ± 0.007 |
| t = 1 week water [b] | 0.323 ± 0.024 | 0.372 ± 0.025 | 0.598 ± 0.040 | 0.629 ± 0.011 | 0.363 ± 0.024 | 0.241 ± 0.002 |
| | | | *Zeta potential (mV) ± SEM* | | | |
| t = 0 h water | 42.7 ± 0.90 | 41.2 ± 1.66 | 52.8 ± 1.15 | 55.17 ± 0.67 | 78.7 ± 2.56 | 44.9 ± 0.40 |
| t = 0 h DMEM 5% FBS | 1.23 ± 1.64 | -3.13 ± 0.81 | 6.78 ± 0.94 | 0.86 ± 0.11 | 13.00 ± 0.49 | 8.61 ± 0.16 |
| t = 1 week water [b] | 46.67 ± 1.13 | 53.97 ± 1.85 | 52.23 ± 0.87 | 32.15 ± 2.90 | 52.53 ± 0.79 | 45.90 ± 1.00 |
| | | | *% incorporation of surfactant into NVs ± SEM* | | | |
| | 88.56 ± 0.009 | 75.96 ± 0.054 | 99.10 ± 0.007 | 98.96 ± 0.006 | 90.85 ± 0.053 | 79.05 ± 0.038 |
| | | | *TEM diameter (nm)* [d] | | | |
| t = 0 h water | 20 - 50 | 20 - 50 | 20 – 50/ 100 - 150 | 20 – 50/ 100 - 150 | 150 - 200 | 90 - 110 |
| *TEM images* [d] |  |  |  |  |  |  |

[a] Incubated under cell culture conditions: 37ºC, 5% $CO_2$; [b] 4ºC; [c] predominanting size is indicated first; [d] predominant population; [e] Scale bars = 100 nm

## Cytotoxicity assays

### Cell viability studies

The cytotoxicity of each surfactant in its free form was firstly assessed in 3T3 cell line (Figure 1a), in which it was observed a greater reduction of the cell viability in comparison to the correspond formulations. Thereafter, the NV effects on the viability of 3T3 and HeLa cells were evaluated by three different endpoints (MTT, NRU and LDH). Indeed, the cytotoxic effects of NVs showed many disparities between formulations that, in fact, depended on the surfactant, cell line and endpoint assayed. These disparities can be seen in the dose-response curves illustrated in Figure 1. The NVs containing MKM and PKM induced a clear dose-dependent decrease in MTT activity (Figures 1b,c). In contrast, the NRU assay showed markedly low cytotoxicity with a significant decline in cell viability only at the highest doses assessed (> 50 μM) (Figures 1d,e). In the same way, the LDH release results only showed significant cytotoxicity at 50 and 100 μM in 3T3 cells, and to a lesser extent in HeLa cells (Figures 1 f,g). NVs containing MLM showed relatively similar cytotoxic responses in the three endpoint assays. The NVs that formed agglomerates after 24 h incubation in cell culture medium (those containing MKM and PKM) induced more marked cytotoxic effects to the mitochondria (as shown by the MTT assay) than well-dispersed NVs (those containing MLM). NVs without any surfactant were also tested as a control of each formulation type and, as expected, it was observed that they induced negligible cytotoxic effects (Figures 1b-g). Finally, the NVs containing cholesterol were less cytotoxic to both cell lines, which might be related to the low surfactant incorporation into these formulations (Table 2). Finally, in studies of MTT and NR dye interactions with NVs, we observed only minimal interference of the NVs with the MTT dye and no interaction with the NR dye. The formulations induced only a slight increase in the MTT absorbance values at 550 nm. These data were proved by the UV-vis measurements (data not shown).

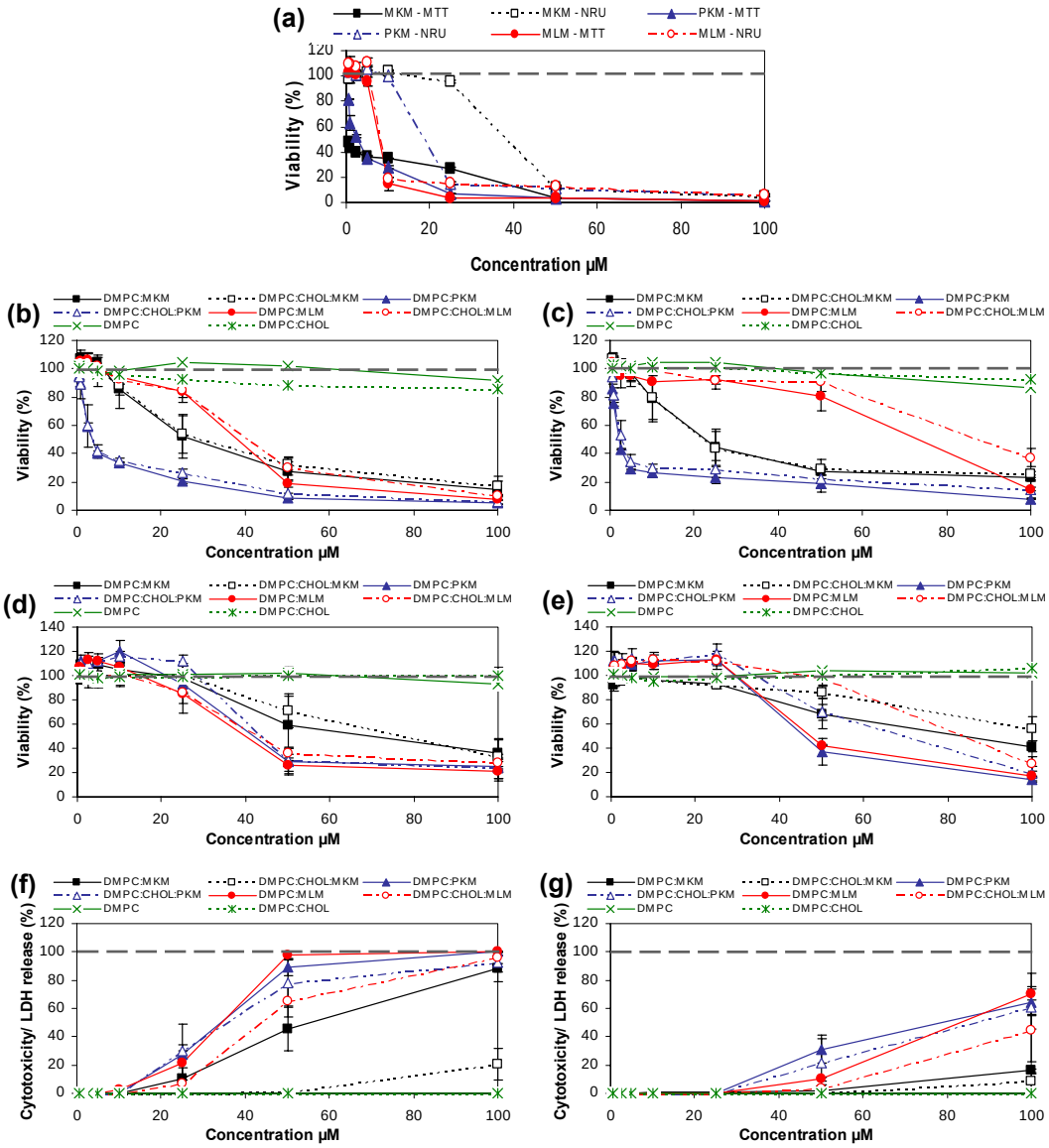



**Figure 1.** Cell viability measured by the MTT (a-c) and NRU (d,e) assays, and cytotoxicity expressed by LDH release (f,g) on 3T3 (a,b,d,f) and HeLa (c,e,g) cell lines. The cells were exposed to increasing concentrations of the surfactants only (a) or of the NV formulations (b-g), ranging from 0.5 to 100 µM. Results of MTT and NRU assays are given as a percentage of untreated control cells, whereas the cytotoxicity by LDH release was calculated in relation to the positive control set as 100% LDH release. The discontinuous straight line in each graph corresponds to untreated control cells set as 100% cell viability (MTT and NRU) or to positive control cells set as 100% cell death (LDH). Results are expressed as mean ± SEM of three independent experiments, performed in triplicate.

## Apoptosis

To assess the extent and mode of cell death, AO/EB staining was carried out and the samples analyzed under a fluorescence microscope. The data from this experiment revealed that the NV treatment ($IC_{50}$ concentrations) increased the number of cells undergoing apoptosis and necrosis (Figure 2a). The most significant effects were observed with the NVs containing PKM, which were also the most cytotoxic in all the viability assays. The untreated cells were observed with a green intact nuclear structure (Figure 2b),

while after NV treatment we observed cells showing chromatin condensation (Figure 2c), blebbing and nuclear margination (early and moderate apoptosis) (Figures 2d,e), together with cells showing apoptotic body separation and a reddish-orange color (late apoptosis) (Figure 2e) and orange nuclei with normal chromatin distribution (necrosis) (Figure 2c).

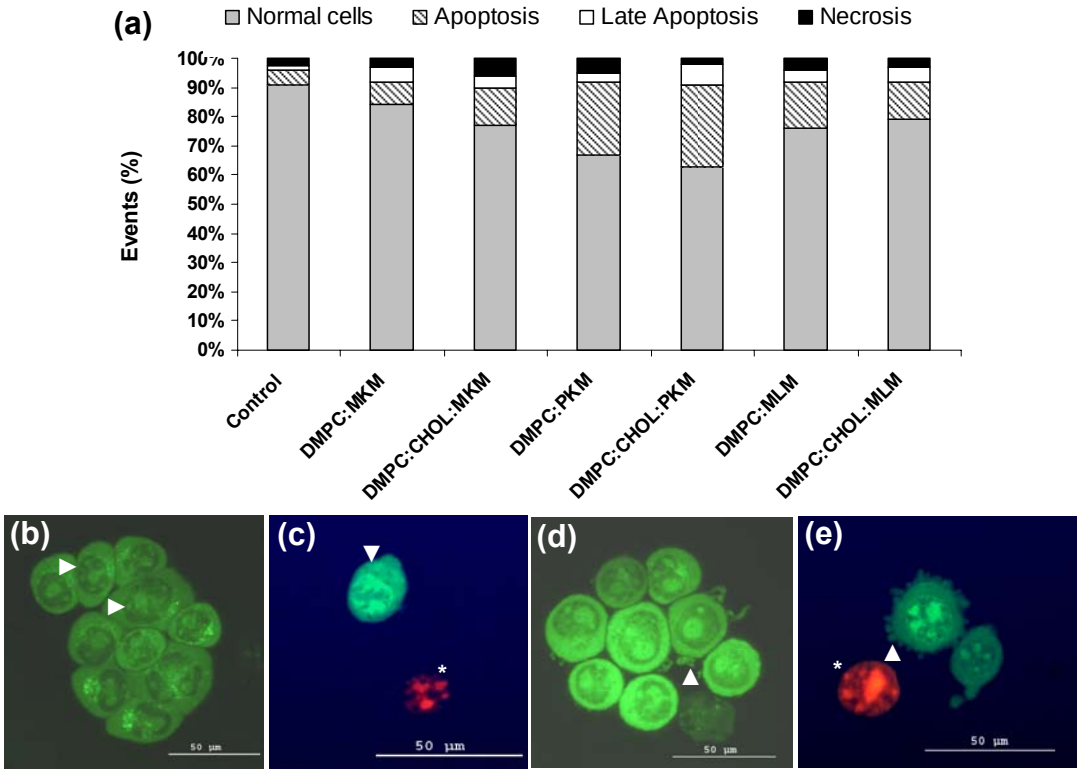



**Figure 2.** Effect of various cationic NVs on apoptosis of 3T3 cells determined by fluorescence microscopy after AO and BE staining. (a) Per cent of viable, apoptotic and necrotic cells after 24 h treatment with the $IC_{50}$ concentrations (calculated by MTT assay) of each NV formulation. Fluorescent micrographs of (b) untreated control cells, (c) DMPC:MKM, (d) DMPC:CHOL:MKM and (e) DMPC:PKM. Legends: (►) typical live nuclei, (▼) chromatin condensation (early apoptosis), (▲) blebbing and nuclear margination (early to moderate apoptosis), (*) necrosis, (**) late apoptosis. Scale bar: 50 µm.

**Cell cycle analysis**

In addition to the cell viability studies, flow cytometric analysis was performed to clarify the influence of each NV formulation on the cell-cycle distribution. The treatment of 3T3 cells with the $IC_{20}$ and $IC_{50}$ concentrations of NVs revealed no significant induction of cell cycle arrest, and only small significant changes were observed in the percentages of cells in each cell phase (Figure 3). Even the most cytotoxic NVs (those containing PKM) showed only a maximal increase in the percentage of cells in the G2/M phase from 15.15% (control) to 20.95%, and a decrease in G1 and S phases from 50.19 and 32.31% (control) to 46.71 and 27.60%, respectively. Moreover, no significant cell population was observed in the sub-G1 phase, which could be attributed to the low proportion of apoptotic cells in relation to the total cell count.

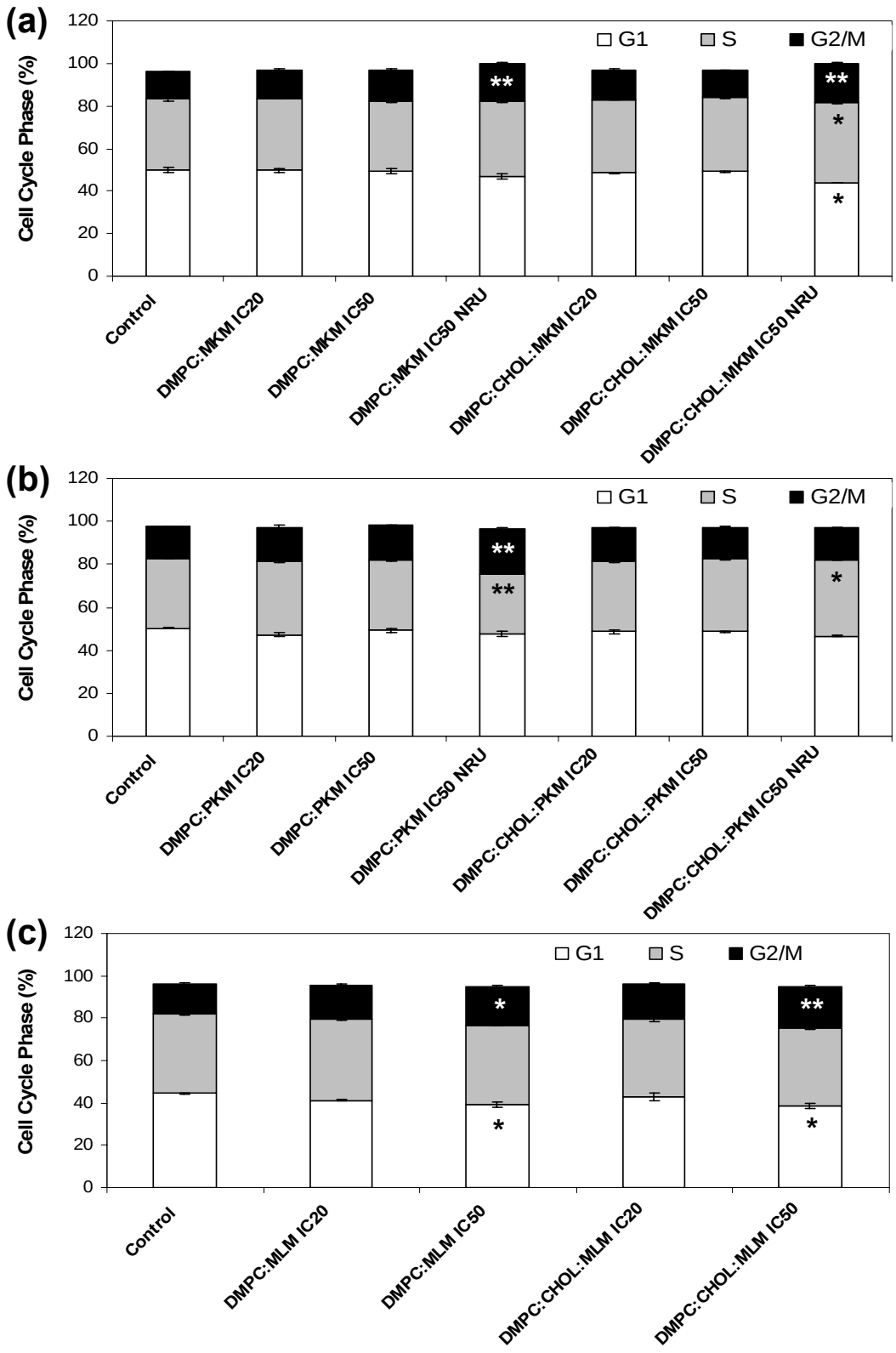



**Figure 3.** Cell-cycle analysis of 3T3 cells following 24 h treatment with $IC_{20}$ and $IC_{50}$ concentrations of each NV formulation. NVs containing (a) MKM, (b) PKM and (c) MLM. Results are expressed as mean ± SEM of three independent experiments, performed in duplicate. Statistical analyses were performed using ANOVA followed by Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.005$ denote significant differences.

**Genotoxicity**

DNA damage was further studied using a comet assay, by measuring the % Tail DNA in the control and treated cells. A comet-like tail implies the presence of a damaged DNA strand, and the length of the tail increases with the extent of the DNA damage (Figures 4a-c). The tested concentrations ($IC_{10}$, $IC_{20}$ and $IC_{30}$) of the NVs containing PKM and MLM induced significantly ($p < 0.005$) higher DNA damage than the negative control (Figure 4d). However, these significant responses were ~ 4- to 20-fold lower than that induced by the positive MMS control. No clear dose-dependent response was observed among the three concentrations assessed.

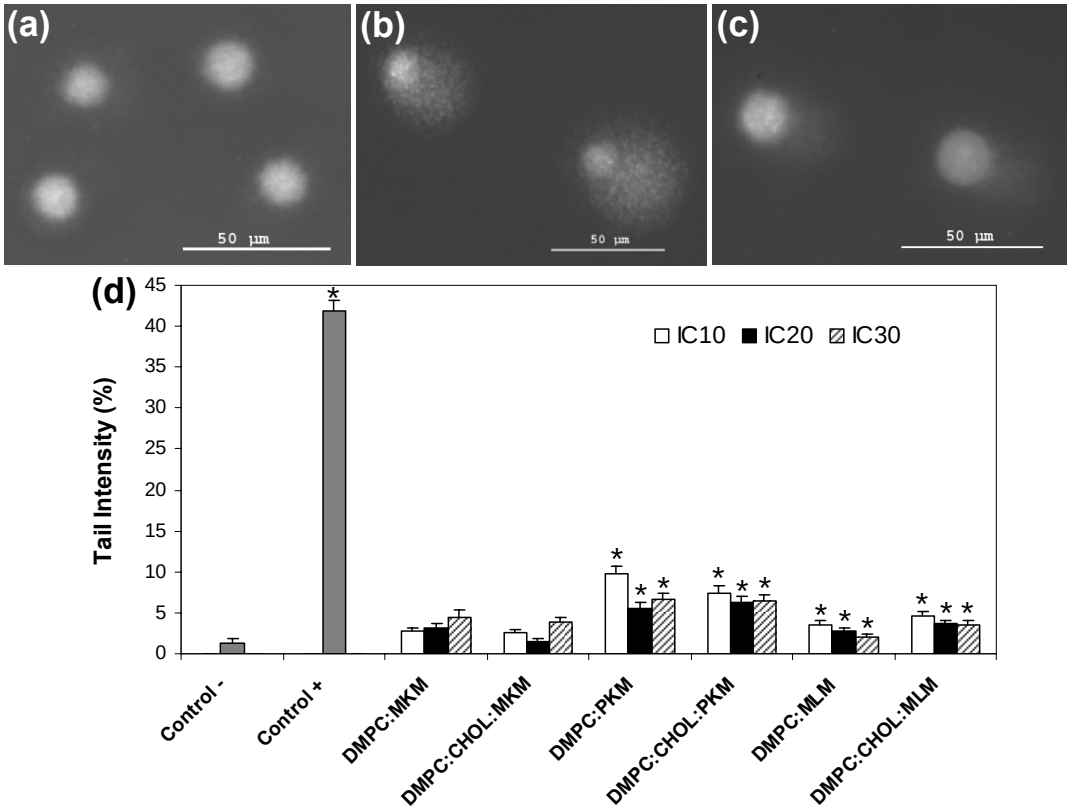



**Figure 4.** DNA damage in 3T3 cells determined by a comet assay. The cells were treated for 24 h with the $IC_{10}$, $IC_{20}$ and $IC_{30}$ concentrations (calculated by MTT assay) of each NV formulation. Fluorescent micrographs of (a) untreated control cells, (b) cells treated with 400 µM of MMS (positive control) and (c) cells treated with $IC_{20}$ of DMPC:CHOL:PKM. Scale bar: 50 µm. (d) % Tail DNA. Values shown are the mean of 50 randomly selected comet images of each sample ± SEM. Statistical analyses were performed using ANOVA followed by Dunnett's multiple comparison test. * $p < 0.005$ denotes significant differences.

**Oxidative damage**

MDA concentrations were measured to elucidate the lipid peroxidation induced by NVs. The NVs containing the amphiphiles MKM and PKM statistically ($p < 0.05$) elevated the intracellular MDA level after 24 h exposure, while no significant ($p > 0.05$) response was induced by the formulations containing MLM (Figure 5). The inclusion of cholesterol in the NV formulations resulted in lower MDA generation. Enhanced responses were

observed as the concentration increased (from $IC_{50}$ MTT to $IC_{50}$ NRU), with the exception of the formulation DMPC:CHOL:PKM. This failure in dose-dependent response might be directly attributed to the cell death. The NVs DMPC:MKM ($IC_{50}$ NRU) and DMPC:PKM ($IC_{50}$ MTT and NRU) showed the most prominent responses, as they elevated intracellular MDA to concentrations higher than that induced by the positive $H_2O_2$ control.

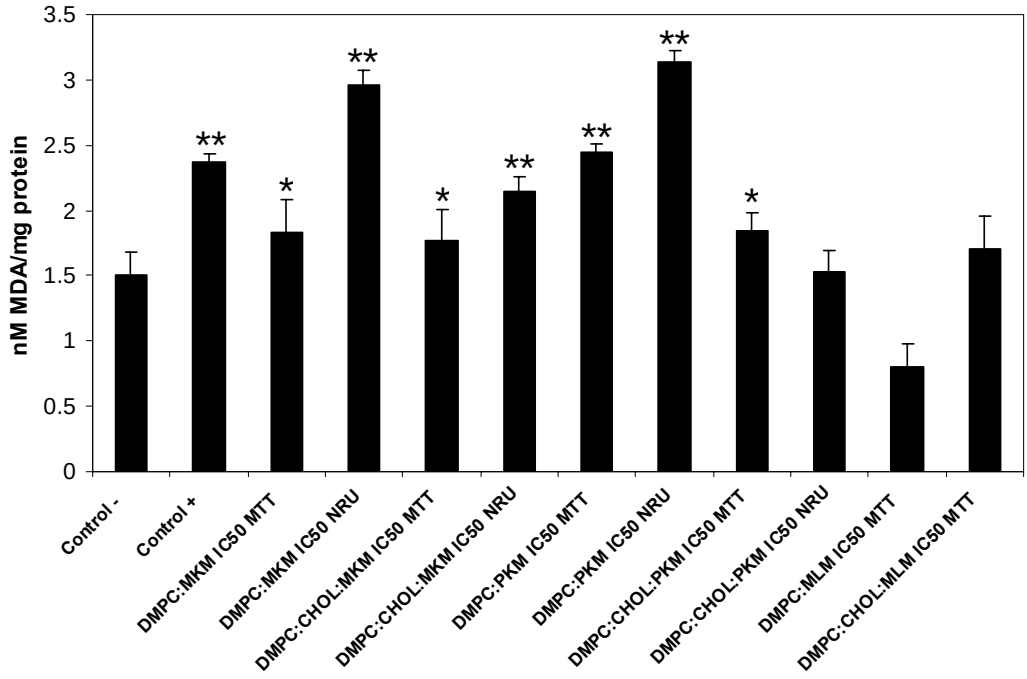



**Figure 5.** Quantitative analysis of MDA concentrations after exposure of 3T3 cells for 24 h to the $IC_{50}$ concentrations of each NV formulation. MDA is a marker of lipid peroxidation and, thus, of cellular oxidative stress. Results are expressed as mean ± SEM of three independent experiments, performed in duplicate. Statistical analyses were performed using ANOVA followed by Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.005$ denote significant differences.

**Blood compatibility studies**

The hemocompatibility of the cationic NVs was studied by hemolysis experiments. The release of hemoglobin was used to quantify the erythrocyte-damaging properties of the NVs. The hemolytic activity of the NVs, with concentrations up to 200 μM, was negligible (less than 5%) after 10 min incubation (data not shown). When the hemolysis assay was performed for 1 h (Figure 6a), the formulations containing MKM showed increased hemolysis at 100 μM, while the NVs containing PKM only prompted enhanced hemolysis at 200 μM. NVs containing MLM did not increase the hemolytic activity at any concentration assayed. Moreover, the NVs containing MKM and MLM did not induce agglutination of erythrocytes after 1 h of treatment (Figures 6b-h), whereas a slight tendency to agglutinate was noticed with the NVs containing PKM (Figures 6e,f).

The plasma proteins adsorbed to the NVs were firstly evaluated by SDS-PAGE. In brief, we analyzed the plasma protein present in the supernatant after incubation with NVs. Figure 6i shows the protein banding patterns that appeared after electrophoresis. No qualitative differences were observed in any protein band in comparison to the control

plasma sample, which indicates that a negligible or very small amount of plasma protein was adsorbed onto the NVs following incubation under *in vitro* conditions. To further examine the plasma protein adsorption, we assessed the proteins desorbed from the NVs surface, obtained from washed particle pellets. The gel of the proteins adsorbed to NVs from 10% plasma is shown in Figure 6j. The lane corresponding to the final wash fluid shows essentially no protein, which confirms that the wash protocol reduced unbound protein to an insignificant level. No substantial amount of protein was associated with the NVs after plasma contact, which indicates the lack of nonspecific adsorption. We only observed quite a faint band in the 50 – 75 kDa range of the gel, which corresponds to albumin (67 kDa), the most abundant plasma protein. Moreover, it seems that the albumin adsorption was higher in the formulations containing cholesterol (Figures 6j, lanes 2, 4 and 6). Finally, quantitative analyses of total protein adsorption were performed using the BioRad assay. In agreement with the qualitative data obtained from the SDS-PAGE gels, no significant differences were observed in the amount of protein in the control plasma and NV-treated plasma (data not shown).

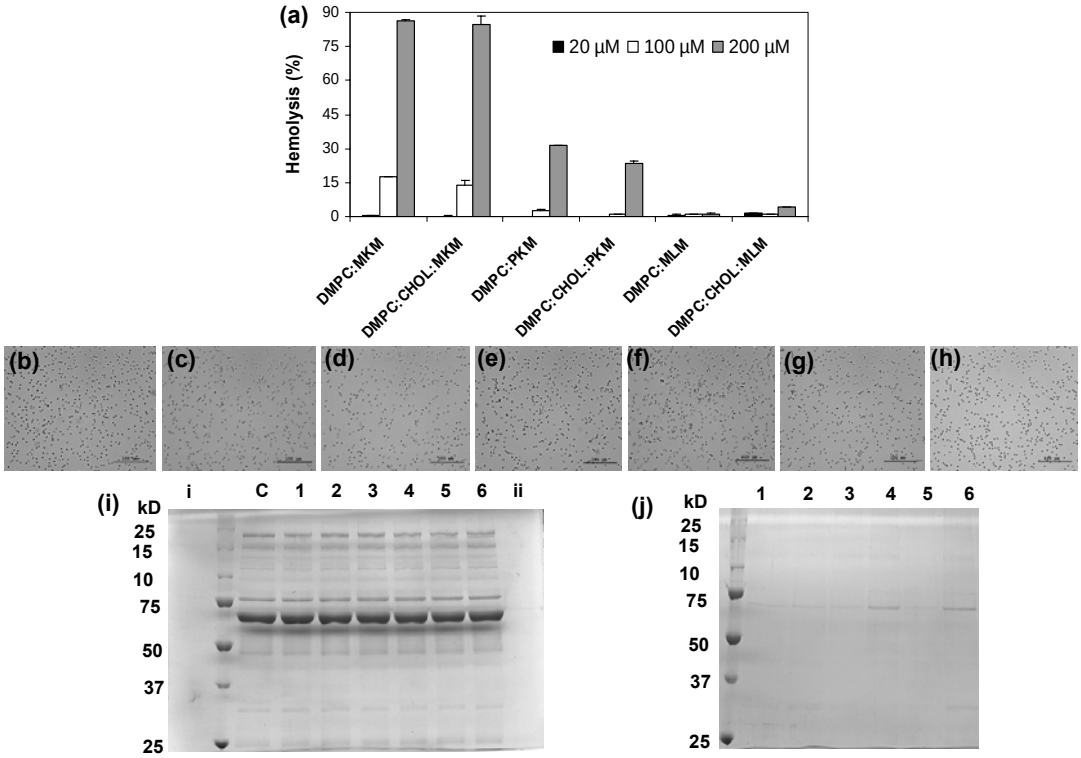



**Figure 6.** Blood compatibility of NVs. (a) Percentage of hemolysis caused by NVs after 1 h of incubation with rat erythrocytes. Each value represents the mean ± SEM of three experiments. Agglutination of rat erythrocytes observed by phase microscopy after 1h of incubation with 100 µM of each NV formulation: (b) control, (c) DMPC:MKM, (d) DMPC:CHOL:MKM, (e) DMPC:PKM, (f) DMPC:CHOL:PKM, (g) DMPC:MLM, (h) DMPC:CHOL:MLM. Scale bar: 100 µm. Plasma protein adsorption assessed by SDS-PAGE: (i) protein present in the supernatant and (j) protein adsorbed to the NVs after incubation of 10% plasma with 100 µM of each NV formulation for 1 h at 37ºC. 1 = DMPC:PKM, 2 = DMPC:CHOL:PKM, 3 = DMPC:MKM, 4 = DMPC:CHOL:MKM, 5 = DMPC:MLM, 6 = DMPC:CHOL:MLM, C = control 10% plasma, i and ii = wash fluids from DMPC:PKM and DMPC:MKM samples, respectively.

**Cell uptake studies and pH-dependent membrane-lytic activity of NVs**

The cell uptake of fluorescent-labelled NR-NVs by the HeLa cell line was visualized by a fluorescence microscope after 2 h and 24 h incubation (Figure 7A). Fluorescent punctate spots were observed mainly in the cell cytosol, which indicates that the NVs were taken up by the cells. Moreover, a smaller number of fluorescent spots were detected along the cell membrane, but not in the nucleus. Interestingly, 24 h of incubation resulted in a more intensive dotted pattern of fluorescent NVs inside the cell, together with some diffuse fluorescence in the cytosol. The quantitative analysis of the images corroborated the greater localization of the NVs in the intracellular compartments after 24 h of incubation (Figure 7C). The cell uptake was ~ 2-fold higher than after 2 h of incubation ($p < 0.005$).

The ability of NVs to release endocytosed materials into cell cytoplasm was examined by fluorescence microscopy following uptake of calcein and NVs into HeLa cells (Figure 7B). Cells treated with calcein alone (control cells) showed a punctuate distribution of fluorescence, which is consistent with constitutive endocytosis of the external medium. Moreover, the co-incubation of calcein and NVs containing MLM only induced a low release of calcein from endosomal compartments. Almost all of the membrane-impermeable fluorophore was restricted within intracellular vesicles appearing as bright punctate structures in a similar level of the control cells. In contrast, when the cells were co-incubated with calcein and NVs containing MKM or PKM, green diffuse fluorescence staining was observed in the cytoplasm, which indicates that calcein had been released from the endosomes. Stronger diffuse staining in the cytoplasm was observed after cell treatment with the NVs containing MKM , whilst a larger number of bright intact vesicles still existed after cell treatment with the NVs containing PKM. Figure 7D shows the quantitative analyses of the cytosolic calcein distribution., which corroborate that the NVs containing MKM or PKM induced significant endosomal destabilization and calcein release ($p < 0.005$), whereas those containing MLM prompted low or negligible membrane-lytic activity at the endosomal compartments ($p < 0.05$ for DMPC:MLM and $p > 0.05$ for DMPC:CHOL:MLM). The presence of cholesterol confers to each formulation a low ability to lysis the endosomal membrane, which can be attributed to the low incorporation of the surfactants into the NV lipid-based structure.

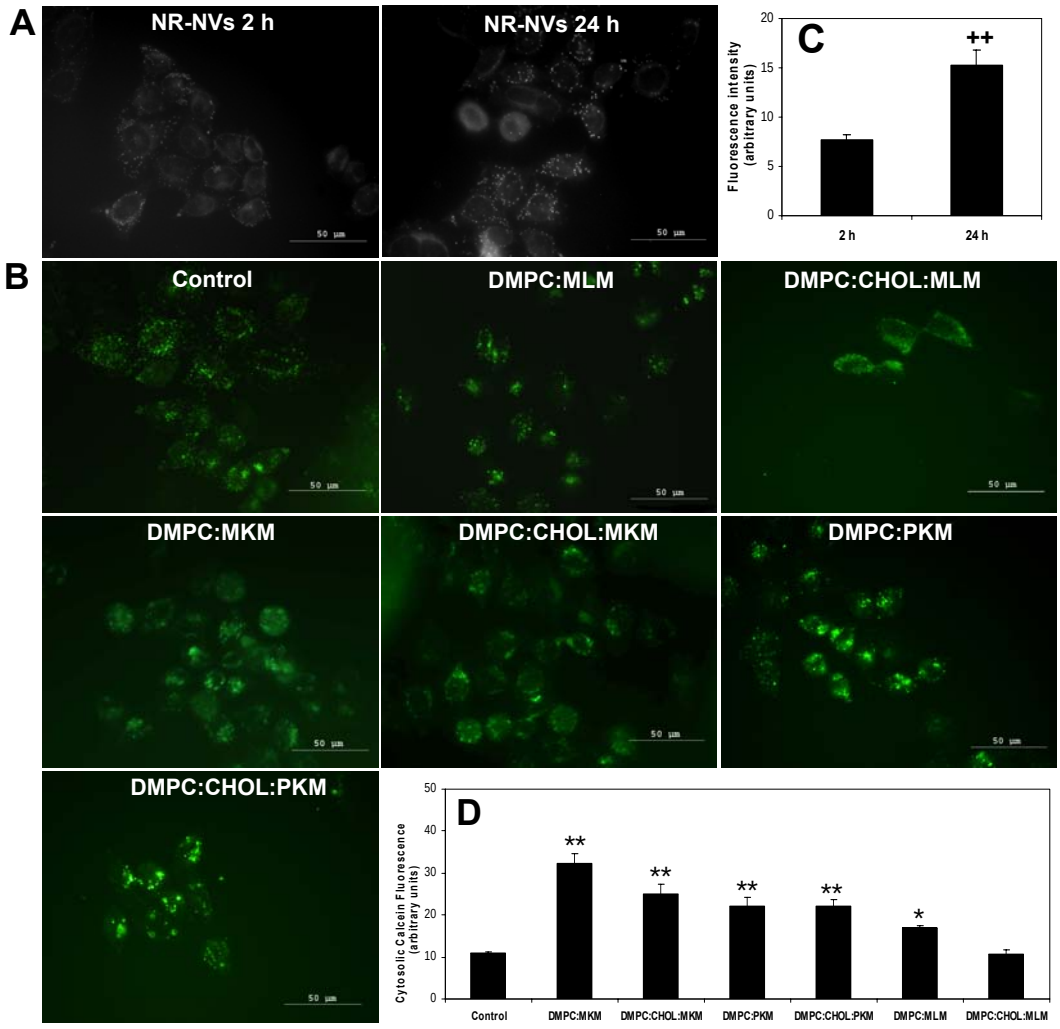



**Figure 7.** (A) Localization of NR-NVs (DMPC:MKM) by HeLa cells after 2h and 24 h of incubation at 37ºC. Cell uptake is visualized using fluorescence microscopy. (B) Fluorescence microscopy images of HeLa cells showing the subcellular distribution of calcein fluorescence. The cells were treated with 1 mg/ml calcein (control), and both 1 mg/ml calcein and 50 μM of each NV formulation. Images were acquired at 3 h after 1 h of uptake. Scale bar: 50 μm. (C) Quantitative fluorescence analysis of images like those in 'A' and (D) of images like those in 'B'. The results represent the mean of values determined for ~20 cells ± SEM. See the Materials and Methods section for details of the analysis performed. Statistical analyses were performed using Student's *t* test ($^{++}$ $p < 0.005$) or ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$ and ** $p < 0.005$).

## Discussion

Chemical composition of the NMs is one of the most important factors influencing cellular interaction. Therefore, the role of including cationic amphiphiles in lipid-based NVs was investigated. We hypothesised that these compounds influences not only the cytotoxic effects, but also the physicochemical properties and intracellular behavior after cell uptake. In order to corroborate this hypothesis, we prepared six different NVs formulations containing three lysine-based surfactants that differ in the cationic charge position and hydrophobicity.

NV hydrodynamic size characterization in cell culture medium suggested significant agglomeration, especially for formulations containing MKM and PKM (positive charge on the α-amino group of lysine). These amphiphiles have pKa values of 5.3 and 4.5 (Nogueira et al. 2012a), respectively, which means that at physiological conditions they predominantly exist as unprotonated species and, therefore, the resulting NVs tend to aggregate. The tendency of NMs to agglomerate in cell culture medium during an *in vitro* toxicity assessment has been previously reported (Horie et al. 2012; Monteiro-Riviere et al. 2009, 2010). The easy aggregation or agglomeration in cell culture medium is probably attributed to the high ionic nature of the solution and the electrostatic/van der Waals interaction between protein and NMs, which result in the formation of secondary particles (Horie et al. 2012). In contrast, NVs containing MLM (positive charge on the ε-amino group of lysine) were not agglomerated in the cell culture medium. This may be due to their higher charge density at physiological pH (pKa MLM = 8.1), corroborated by the ZP values determined in the cell culture medium. A high charge density increases the physical stability of colloidal dispersions (Liang et al. 2009). These overall findings showed that the cationic charge position in the amphiphile determines the aggregation state of NVs. The high positive ZP values of the NVs in water indicated the stability of the prepared formulations by preventing fusion or aggregation of NVs (Liang et al. 2009). A physically stable formulation would have a minimum ± 30 mV ZP as a borderline value of colloidal stability (Di Marzio et al. 2011; Müller et al. 2011). Interestingly, the mean hydrodynamic diameters measured by DLS did not capture the real size distribution of the NVs observed by TEM. DLS measurements of NVs containing MKM and PKM were larger than those determined from the TEM images of the corresponding samples. Disparity between DLS and TEM might be a result of the resolution limitations of DLS (Coldren et al. 2003; Ojogun et al. 2009), aggregation (Ahmad et al. 2012; Bai et al. 2009; Venkatesan et al. 2011) and swelling of the NVs in the presence of water (Mehrotra et al. 2011), or even to the fact that DLS gives the mean hydrodynamic diameter of the particle core surrounded by the solvation layers, whereas TEM gives the diameter of particles alone in the dry state (Gao et al. 2010).

High throughput cell-based tests have been the initial step in biological screening approaches (Monteiro-Riviere et al. 2009). Moreover, *in vitro* cell culture-based cytotoxicity screening assays, designed to minimize animal use in toxicity testing, have become and are expected to remain the mainstay of modern toxicology testing strategies (National Research Council 2007). The use of model cell lines from different origins and more than one cytotoxicity assay is a reliable approach to systematically investigate the influence of NM properties on the degrees and pathways of cytotoxicity (Bhattacharjee et al. 2013; Fröhlich et al. 2012; Sohaebuddin et al. 2010). 3T3 cell model is considered very sensitive to verify *in vitro* cytotoxic effects of NMs (Uboldi et al. 2012; Mahto et al. 2010), while HeLa tumor cell line is a culture model widely used in cytotoxicity and especially in cell uptake studies of a broad range of particulate nanocarriers (Kelsch et al. 2012; Salado et al. 2012). The tumor HeLa cell line was more resistant to NV cytotoxic effects, which was in agreement with our previous study (Nogueira et al. 2011b). These results are also in line with those reported by several authors (Fröhlich et al. 2012; Müller et al. 1997; Schöler et al. 2001; Sohaebuddin et al. 2010; Xia et al. 2008), who noticed that the

cytotoxic effects of particulate carrier systems differ depending on the cell lines used, due to the innate nature, metabolic abilities (e.g. enzymes present) and capabilities of the cell lines. In an attempt to understand the toxic response of NMs, here we used different cytotoxicity assays. The higher sensitivity of the MTT assay for detecting the cytotoxic effects of the NVs containing MKM and PKM suggests that the cytotoxicity primarily originated at the mitochondrial compartment after cellular internalization of NVs rather than physical damage to both lysosomal (NRU assay) and plasma (LDH assay) membranes (Yang et al. 2009). Noteworthy is that the intracellular reactions of internalized NMs were reported to lead to cellular metabolism/mitochondrial dysfunction (Horie et al. 2012). Concerning to cell membrane integrity, we observed different effects when the cell line was varied, being NRU assay more sensitive in HeLa cells and LDH in 3T3 fibroblasts. These results corroborated the different interaction mechanisms of these NVs within cells from different origins and species. In contrast, NVs formulated with MLM generally displayed the same level of cytotoxicity with the three endpoints. These NVs have higher positive ZP in cell culture medium, which might support their higher binding to the negatively charged lipid bilayer by electrostatic interaction and, thus, their toxic effects on the cell membrane to the same extent as cell metabolism depletion (Venkatesan et al. 2011; Xia et al. 2008). All in all, these results led to a key finding of our research: the structural characteristic of the surfactant included in the NV directly affects its cytotoxic effects. Firstly, the position of the cationic charge in the amphiphile molecule was critical in determining the resulting formulation's cytotoxicity. Secondly, the hydrophobicity of the surfactant was directly related to the NV toxic effects: the longer the alkyl chain of the amphiphile, the higher the NV cytotoxicity. Based on the variability of cell responses, a combination of cell lines and endpoints might be suitable for a reliable *in vitro* evaluation of nanotoxicity. Finally, the small interference of the NVs with the MTT dye did not result in further increase in cell viability with increasing NM concentration, which is in contrast to previous reported data for other types of NMs (Monteiro-Riviere et al. 2009, 2010). These data prove that these small interactions were no significant and, therefore, that the viability endpoints are suitable for the intended purpose.

After showing that NVs exerted cytotoxic effects on the proliferation of fibroblasts in a typically dose-dependent manner, we examined whether the induction of apoptosis was the possible molecular mechanism involved in the cytotoxicity of NVs. Morphological alterations detected after staining with AO/EB demonstrated that the NV treatments could induce apoptosis in cultured fibroblasts. Whereas the apoptotic effects could be attributed to the observed induction of oxidative damage (AshaRani et al. 2009), mitochondrial dysfunction, which can be determined by MTT assay (Fisher et al. 2003), is reported as an intrinsic pathway of apoptosis. Therefore, the apoptotic effects of the NVs containing especially MKM and PKM can be directly related to oxidative stress and early mitochondrial injury. Additionally, plasma membrane integrity modifications that are detected by LDH assay could be related to the late stage of apoptosis (Fisher et al. 2003) and necrosis. Recent reports have identified apoptosis as a major mechanism of cell death in exposure to NMs (Hsin et al. 2008; Pan et al. 2007). However, in our case, although the number of apoptotic cells increased, most cells remained viable after NV treatments. This suggests that other factors also contribute to cell death, aside from the apoptotic pathway.

Toxicity studies were further extended to cell-cycle analysis, which showed that NV treatment did not induce many significant alterations, as the cell cycle distribution was very similar to the control. This suggests that cell cycle arrest is not the mechanism underlying NV cytotoxicity. In this line, genotoxicity did not appear to be one of the main mechanisms involved in NV toxic effects. Although some significant values of % Tail DNA were obtained with respect to the negative control, the overall responses can be considered biologically non-significant, as the % values were close to the negative control values and much lower than those displayed by the positive MMS control. These results are in agreement with the cell cycle analysis, as, if present, DNA damage would be evidenced in cell cycle progression with the accumulation of cells in a determined cycle phase (AshaRani et al. 2009).

NMs might be able to disturb the oxidative balance in a cellular environment. This phenomenon is called oxidative stress and results in abnormally large concentrations of intracellular reactive oxygen species (ROS) (Marquis et al. 2009). In response to oxidative stress, cell surface and organelle membrane lipids may undergo peroxidation (Choi et al. 2007). A marker of lipid peroxidation elicited by ROS is increased MDA production, which was induced by the NVs containing MKM or PKM. The inverse correlation between cell viability and MDA level made the latter a key analytical marker for induced cell damage, which in turn indicated that oxidative stress was probably a key route by which these NVs induce cytotoxicity. ROS-induced membrane lipid peroxidation may occur both at cellular and organelle levels, especially in the membranes of metabolically active mitochondria. This makes mitochondrial injury a marker of elevated intracellular ROS level (Choi et al. 2007; Jones and Grainger 2009). The early compromised cell viability detected by the MTT reduction assay may also result from this insult.

Intravenous administration of a nanocarrier is limited by its hemolytic activity, among other factors. Thus, hemolysis should be assessed to determine the biocompatibility of a material (He et al. 2009). The low hemolytic activity of NVs at concentrations that were also non-cytotoxic (< 100 μM) indicated the hemocompatibility of the formulations. Generally, a percentage of hemolysis of less than 5% was regarded as non-toxic (He et al. 2009). Moreover, the strong positive charge of the NVs did not induce erythrocyte agglutination. This is in contrast to previous reported data for cationic carriers (Eliyahu et al. 2002), but corroborates the hemocompatible properties of the NVs. The NVs were more toxic to 3T3 and HeLa cells than to erythrocytes. This greater sensitivity may reflect differences in intrinsic structural properties of membranes and might be attributed to the fact that erythrocytes cannot internalize foreign particles, which leads to poor insertion into the phospholipid membrane and consequently low hemolytic activity (Yessine et al. 2003). Furthermore, protein adsorption on particulate drug carriers is regarded as a key factor for their *in vivo* fate and might also play an important role in directing their cell uptake and toxicity (Dutta et al. 2007). Minimal differences in protein pattern were observed after plasma incubation with NVs. This indicates that negligible or very small amount of plasma protein was adsorbed onto NVs following incubation under *in vitro* conditions. Overall, our results showed that the NVs positive charge, attributed to the surface modification with cationic lysine-based amphiphiles, did not compromise the desirable blood compatibility of the lipid-based vesicles.

The therapeutic effects of the drug-loaded NMs would firstly depend on internalization by the diseased cells (Mei et al. 2009). Here, the observed punctuate distribution of fluorescence in the cell cytosol indicated the cell uptake of the NR-NVs. The more intense fluorescence at 24 h indicated a higher cell uptake after a longer incubation time, whereas the diffuse fluorescence in the cytosol can be attributed to dye release after endosome rupture. Internalization is probably mediated through an endocytic pathway through non-specific interactions (e.g., adsorptive endocytosis) with cell membrane (Park et al. 2006). Moreover, the results obtained after the concomitant cell incubation with calcein and NV suggest that the NMs entered the cell by endocytosis (see discussion below). Noteworthy is that the formation of secondary vesicles due to aggregation in cell culture medium did not inhibited their cellular uptake. Finally, and in relation to the toxic effects of NVs, the hydrophobicity (Arora et al. 2012) and the electrostatic interactions between the cationic NVs and the anionic cell surface (Venkatesan et al. 2011) might be responsible for the cell uptake.

The high efficiency of amphiphiles with a positive charge on the α-amino group of lysine (MKM and PKM) at disrupting cell membranes within the pH range characteristic of late endosomes (Nogueira et al. 2012a) prompted an evaluation of the ability of NVs containing these compounds to release endocytosed materials into the cytoplasm of cells. One common strategy for the intracellular delivery of encapsulated and/or intercalated material via lipid-based vesicles exploits intracellular pH gradients (Pollock et al. 2010; Torchilin et al. 1993). We used calcein as a tracer molecule, which is internalized by the cell through endocytosis and is used to monitor the stability of endosomes following NM uptake (Hu et al. 2007). In the absence of NVs, endosomal compartmentalization of calcein was observed, indicating that the endosome membranes were not damaged (Chen et al. 2009; Hu et al. 2007). When the MLM was included into the NVs, it was observed a different behavior depending on the composition of the basic lipid membrane. Only the formulations without cholesterol had the ability to release some calcein from endosomes, which can be due to a non specific interaction between these NVs and the endosomal membrane. In contrast, the NVs containing MKM and PKM induced efficient and greater release of endocytosed material into the cytoplasm regardless of the composition of the lipid-based matrix. These results corroborated the pH-responsive membrane-lytic activity of the latter surfactants demonstrated in our previous study (Nogueira et al. 2012a). Since MKM and PKM are more protonated at acidic pH (pka = 5.3 and 4.5, respectively), they might interact with negatively-charged endosomal membranes, induce influx of water and ions, and eventually bring about endosome destabilization and drug release (Park et al. 2006). The stronger diffuse staining observed after cell treatment with the NVs containing MKM may be directly related to the higher membrane-lytic activity of MKM at the pH range characteristic of the endosomal compartments (Nogueira et al. 2012a). The efficient intracellular delivery of therapeutic compounds into the cytosol by destabilizing endosomal membranes under mildly acidic conditions would manipulate or circumvent non-productive trafficking from endosomes to lysosomes, in which degradation may occur (Chen et al. 2009).

All considered, these data on the cytotoxic pathways and intracellular behavior show a structure-activity relationship of the cellular events in response to NVs, which is

summarized in Table 3. The main finding is that the position of the cationic charge in surfactant molecule determines the NV cytotoxic responses, induction of oxidative stress, endosomal release and hemolytic activity. NVs containing the surfactants with cationic charge on the α-amino group of lysine affect firstly the mitochondria, followed by the lysosomal and plasma membrane, as demostrated by the corresponding *in vitro* endpoints. Moreover, the surfactant hydrophobicity correlates directly with the cytotoxic and genotoxic effects. In contrast, the surfactant chemical structure does not affect the NV effects on apoptosis, cell cycle, erythrocyte agglutination and plasma protein adsorption.

Finally, it is appropriate to comment on the importance of these findings for nanotoxicology. Here, we showed that some NVs have the ability to lysis the endosomal membrane and are therefore potential carriers for specific intracellular drug delivery. However, we also demonstrated that they have an effect on normal cell biological functions and that such effect is presented in terms of various toxic mechanisms. We must highlight the *in vitro* approach used in this study as a reliable predictive model to provide an affordable database on the biological activities of new NMs as a function of their chemical composition, which would help understand their risks and opportunities before any *in vivo* application. An initial animal-based screening approach is not feasible with regard to laboratory capacities and costs, and it certainly is not desirable from an animal welfare viewpoint (Hartung 2010). Relevant *in vitro* data may suggest the feasibility of new NMs for further applications. Therefore, only the NMs with such relevance must be addressed for additional *in vivo* studies to corroborate the initial hypothesis demonstrated through *in vitro* screening assays.

**Table 3.** Summary of the structure-activity relationship of the cellular events in response to NVs.

| | DMPC:MKM | DMPC:CHOL:MKM | DMPC:PKM | DMPC:CHOL:PKM | DMPC:MLM | DMPC:CHOL:MLM |
|---|---|---|---|---|---|---|
| Surfactant charge position | α-amino | α-amino | α-amino | α-amino | ε-amino | ε-amino |
| Surfactant alkyl chain length | 14C | 14C | 16C | 16C | 14C | 14C |
| Surfactant pH-sensitive activity [a] | Yes | Yes | Yes | Yes | No | No |
| Cytotoxicity by MTT [b] (mitochondrial dysfunction) | + + | + + | + + + | + + + | + | + |
| Cytotoxicity by NRU [b] | + | + | ++ | + | ++ | + |
| Cytotoxicity by LDH [b] | – | – | + | + | + | + |
| Apoptosis [c] | + | + | + | + | + | + |
| Cell cycle alteration [c] | – | – | – | – | – | – |
| Genotoxicity [c] | – | – | + | + | – | – |
| Oxidative stress [c] | + + | + | + + + | + | – | + |
| Endosomal release [c] | + + + | + + + | + + + | + + + | + + | – |
| Hemolysis [c] | + | + | + | + | – | – |
| Erythrocyte agglutination [c] | – | – | – | – | – | – |
| Plasma protein adsorption [c] | – | – | – | – | – | – |

[a] As previously reported (Nogueira et al. 2012a, 2012b).
The number of plus signs is a semiquantitative measure according to which: [b] + refers to low ($50<IC_{50}<100$ μM), + + to medium ($10<IC_{50}<50$ μM) and + + + to high cytotoxicity ($IC_{50}<10$ μM). Negligible cytotoxicity was considered when the $IC_{50}$ was higher than 100 μM (–); [c] + refers to 5-33% value higher than the response of untreated control cells, + + to 33-66%, and + + + to > 66%. A < 5% value change was considered as equivocal effect (–).

## Conclusions

Here, we used an *in vitro* toxicological approach to elucidate the biological activity and intracellular behavior of new cationic NV formulations containing biocompatible lysine-based amphiphiles. We demonstrated the cell-specific nature of NV toxicity as well as different toxic responses when the *in vitro* endpoint varied. A possible mechanism of toxicity is proposed involving NV cell uptake followed by induction of oxidative stress, which in turn cause mitochondrial dysfunction and ultimately result in apoptosis of cells. This mechanism is especially proposed as the cytotoxicity pathway induced by the NVs containing MKM and PKM, as those with MLM did not prompt significant oxidative stress and mitochondrial injury. The cytotoxicity of the latter NVs seems to be attributed to an initial damage to the cell membranes. NV cytotoxicity as well as their cellular uptake might be attributable to the cationic charge. Our findings suggest that the cationic charge position and hydrophobicity of the amphiphiles determine the NV interaction within the cell and, thus, their resulting toxicity and intracellular behavior after cell uptake. NVs containing MKM and PKM (with a cationic charge on the α-amino group of lysine) are especially recommended as pH-sensitive nanocarriers for intracellular drug delivery. They have the ability to specifically lyse the endosome membrane in mildly acidic conditions, as evidenced here and demonstrated in our preliminary study (Nogueira el al. 2012a). Desirable blood compatibility was substantiated by minimal interaction with erythrocytes and lack of plasma protein adsorption, which also corroborates the potencial application of these NVs as carriers of intravenous biomedical drugs that have their site of action in the intracellular compartments. However, we are aware that further *in vivo* studies must be conducted in this field to prove this hypothesis. Finally, as each NM type shows unique physicochemical properties, the combination of assays used here, together with all the information provided, offers an in-depth and comprehensive evaluation of the toxic effects of novel NMs and contributes to reducing the uncertainty surrounding their potential health hazards.

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### Declaration of interest

The authors state that they have no conflict of interest.

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## Artículo 7

**_IN VITRO_ ANTITUMOR ACTIVITY OF METHOTREXATE VIA pH-SENSITIVE CHITOSAN NANOPARTICLES**

**(Actividad antitumoral _in vitro_ del metotrexato a través de nanopartículas de quitosano pH-sensibles)**

**Daniele Rubert Nogueira, Lorena Tavano, Montserrat Mitjans, Lourdes Pérez, M. Rosa Infante, M. Pilar Vinardell**

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# Resumen

*Objetivos*

Desarrollar nanopartículas poliméricas basadas en el quitosano y conteniendo el tensioactivo aniónico derivado de lisina (77KL) como excipiente bioactivo, encapsular en este vehículo el fármaco antitumoral metotrexato e investigar el efecto de la inclusión del tensioactivo, con propiedad lítica de membrana dependiente del pH, en la actividad antitumoral *in vitro* del fármaco encapsulado.

*Material y métodos*

Las nanopartículas se prepararon utilizando una técnica modificada del método de gelificación iónica, en lo cual se incluyó el tensioactivo aniónico derivado de lisina, 77KL, en el proceso de complejación con el polímero catiónico quitosano. Se caracterizaron las nanopartículas en cuanto a su tamaño, morfología, potencial zeta e índice de polidispersidad. También se valoró la eficiencia de encapsulación del fármaco antitumoral metotrexato y su cinética de liberación de las nanopartículas a diferentes valores de pH. Se cuantificó el metotrexato liberado en el medio por espectrofotometría a 306 nm. La actividad antitumoral de las nanopartículas conteniendo metotrexato se valoró en comparación al fármaco libre en solución, utilizando las células tumorales HeLa y MCF-7. Los queratinocitos HaCaT se utilizaron como control celular no tumoral. Se utilizaron los ensayos de viabilidad MTT, NRU y LDH, y los mecanismos involucrados en la actividad citotóxica se investigaron a través de estudios de apoptosis, ciclo celular e integridad lisosomal. Por fin, se estudió la actividad pH-dependiente de las nanopartículas y su efecto en el comportamiento intracelular de estos vehículos, utilizando eritrocitos y el ensayo de hemólisis como modelo de membrana del endosoma, y la línea HeLa como modelo celular juntamente con la calceína como marcador fluorescente de endocitosis.

*Resultados*

Se obtuvieron nanopartículas con tamaño hidrodinámico de ~300 nm y potencial zeta de ~30 mV. Sin embargo, los estudios de microscopía electrónica mostraron nanopartículas con tamaño muy inferior, en el rango de 20 a 140 nm. El metotrexato tuvo un alto índice de encapsulación (~63%) y se demostró una liberación más rápida del fármaco a medida que se bajó el pH del medio (6,5 y 5,4). La actividad antitumoral del metotrexato encapsulado en las nanopartículas se demostró significativamente mayor en comparación con el fármaco libre. Además, estas nanopartículas demostraron toxicidad mayor en el pH característico del tejido tumoral (pH~6,6). La toxicidad tanto del fármaco libre como encapsulado fue considerablemente inferior en las células no tumorales HaCaT. Los estudios del mecanismo de la actividad antitumoral demostraron, de un modo general, que el fármaco encapsulado indujo efectos apoptóticos más significativos y una inhibición mayor del progreso del ciclo celular. Por último, los estudios de hemólisis a diferentes pH y los análisis de microscopía de fluorescencia mostraron la actividad pH-dependiente de las nanopartículas y su capacidad de liberar de modo significativo el material endocitado en el citoplasma celular.

*Conclusiones*

La combinación de las propiedades fisicoquímicas y pH-dependientes de las nanopartículas aumentó de modo significativo la actividad antitumoral *in vitro* del metotrexato encapsulado en comparación con su forma libre. Por lo tanto, se propone estas nanopartículas como potenciales vehículos para la liberación de fármacos antitumorales en el tejido tumoral y también en los compartimientos intracelulares.

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# *In vitro* antitumor activity of methotrexate via pH-sensitive chitosan nanoparticles

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ABSTRACT

Nanoparticles with pH-sensitive behavior may enhance the success of chemotherapy in many cancers by efficient intracellular drug delivery. Here, we investigated the effect of a bioactive surfactant with pH-sensitive properties on the antitumor activity and intracellular behavior of methotrexate-loaded chitosan nanoparticles (MTX–CS–NPs). NPs were prepared using a modified ionotropic complexation process, in which was included the surfactant derived from $N^{\alpha}$,$N^{\varepsilon}$-dioctanoyl lysine with an inorganic lithium counterion. The pH-sensitive behavior of NPs allowed accelerated release of MTX in an acidic medium, as well as membrane-lytic pH-dependent activity, which facilitated the cytosolic delivery of endocytosed materials. Moreover, our results clearly proved that MTX–CS–NPs were more active against the tumor HeLa and MCF-7 cell lines than the free drug. The feasibilty of using NPs to target acidic tumor extracellular pH was also shown, as cytotoxicity against cancer cells was greater in a mildly acidic environment. Finally, the combined physicochemical and pH-sensitive properties of NPs generally allowed the entrapped drug to induce greater cell cycle arrest and apoptotic effects. Therefore, our overall results suggest that pH-sensitive MTX–CS–NPs could be potentially useful as a carrier system for tumor and intracellular drug delivery in cancer therapy.



## 1. Introduction

Chitosan (CS) is a naturally occurring polymer that has been attracting increasing attention in pharmaceutical and biomedical applications because of its biocompatibility, biodegradability, non-toxicity, cationic properties and bioadhesive characteristics [1–3]. CS nanoparticles (NPs) have been investigated as a promising colloidal drug carrier for targeted delivery to specific sites, as well as for gene and vaccine delivery, and cancer therapy [3–5]. The ionotropic gelation technique is one of the most widely used of a variety of methods to prepare CS-NPs [1,2,6,7]. This procedure is based on reversible crosslinking by electrostatic interaction (between protonized $-NH_3^+$ and an anion such as tripolyphosphate), instead of chemical crosslinking. It avoids the potential toxicity of reagents and the possibility of damaging the drugs, especially with biological agents [2,8].

Methotrexate (MTX) acts as an antagonist of folic acid, which is necessary for DNA synthesis, and has a therapeutic effect on many types of cancer cells that overexpress folate receptors on their surfaces [9]. MTX is currently widely used as a major chemotherapeutic agent for human malignancies such as acute lymphoblastic leukemia, malignant lymphoma, osteosarcoma, breast cancer and head and neck cancer [10]. However, its clinical efficacy is often compromised by the acquisition of resistance in cancer cells, due to cellular efflux of the molecule [11]. In this context, the encapsulation of antitumor drugs in nanoparticulated systems like polymeric NPs, which retain a higher drug concentration within the cell, might overcome the shortcomings associated with conventional drug delivery strategies [12]. MTX-loaded CS-based NPs have been developed using especially modified forms of chitosan, to improve controlled drug delivery to tumors [5,13]. A delivery

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system based on covalently conjugated CS-MTX has also been described, but has the drawback of using a cross-linked agent [14]. On the basis of the above studies and the need for an efficient drug carrier for cancer therapy, we decided to investigate the effect of a bioactive excipient with pH-sensitive properties on the antitumor activity and intracellular behavior of MTX-loaded CS-NPs.

Lysosomal degradation of the chemotherapeutic agent is another major hurdle for successful cancer therapy. For nanocarriers to act efficiently, they must overcome intracellular barriers, such as endosomes, and release the drugs into the cytosol before they are ultimately trafficked to lysosomes [15]. Therefore, delivery devices with bioactive excipients have been developed, which selectively release drugs or genes into the cytoplasm by sensing low pH in endosomes [16]. Bioactive compounds that could be used in nanocarriers include amino acid-based surfactants that have shown pH-responsive membrane-lytic activity [17–19]. The anionic amphiphile derived from $N^{\alpha}$,$N^{\varepsilon}$- dioctanoyl lysine with a lithium counterion (77 KL) in particular showed pH-sensitive membrane-lytic behavior and low cytotoxicity [17]. This suggests that it may have a specific ability to destabilize the endosomal membrane in a mildly acidic environment without considerable toxic effects to the cell. Therefore, it was chosen as a bioactive excipient to be included in the pH-sensitive CS-NPs designed here as a promising carrier for the intracellular delivery of cancer drugs. In this context, CS-TPP-NPs modified by the cationic amphiphile cetyltrimethylammonium bromide are noteworthy, as they showed improved physicochemical properties, attributed to the inclusion of the surfactant [1]. The green fluorescent dye calcein and fluorescence microscopy analysis was used to monitor the capacity of the NPs to destabilize the endosomal membrane. MTX, a drug widely used in the treatment of cancer, was incorporated into CS-NPs, and the cytotoxic activity of the latter against tumor and non-tumor cell lines was assessed using *in vitro* toxicological assays.

## 2. Experimental

### 2.1. Chemicals and reagents

Chitosan (CS) of medium molecular weight (deacetylation degree, 75–85%; viscosity, 200–800 cP according to the manufacturer's data sheet), pentasodium tripolyphosphate (TPP), methotrexate (MTX), acridine orange (AO), ethidium bromide (EB), propidium iodide, ribonuclease A (RNAse A), calcein, 2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin-EDTA solution (170,000 U/l trypsin and 0.2 g/l EDTA) and penicillin-streptomycin solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) were purchased from Lonza (Verviers, Belgium). The 75 $cm^2$ flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland). All other reagents were of analytical grade.

### 2.2. Surfactant included in the nanoparticles

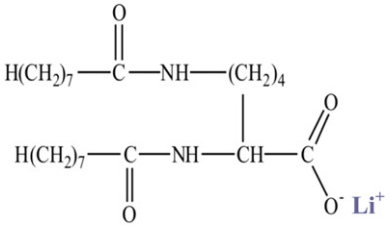
An anionic amino acid-based surfactant derived from $N^{\alpha}$, $N^{\varepsilon}$-dioctanoyl lysine and with an inorganic lithium counterion (77 KL) was included in the NP formulation (Table 1). This surfactant was synthesized by our group as previously described [20]. The choice of 77 KL as a bioactive excipient in the NP formulation was based on our previous studies, which showed its pH-sensitive membrane-disruptive activity, together with improved kinetics in the endosomal pH range and low cytotoxic potential [17,21].

### 2.3. Preparation of MTX nanoparticles

CS-NPs were prepared according to a modified version of the ionic gelation technique [6], which is based on the ionotropic complexation of CS with TPP anions. The surfactant 77 KL was included in the complexation process and the NPs were prepared with a selective CS:TPP:77 KL ratio of 5:1:0.5 (w/w/w).

Unloaded CS NPs (unloaded–CS–NPs) were prepared by dropwise addition of a premixed TPP and 77 KL solution (both at 0.1%, w/v, with a TPP:77 KL ratio equal to 1:0.5, w/w) to CS (0.1%, w/v) previously dissolved in acetic acid solution (1%, v/v).

**Table 1**
Physicochemical properties of the anionic lysine-based surfactant 77 KL.

| Chemical structure | | |
|---|---|---|
|  | Molecular weight | 405.6 g/mol |
| | Critical micellar concentration (CMC) | $2.9 \times 10^3$ μg/ml |
| | p*Ka* | 6.2 |
| | Number of alkyl chains | 2 |
| | Length of alkyl chain | C8 |

The pH of the CS final solution was adjusted to 5.5 with 1 M NaOH [7]. The NPs were formed spontaneously and the gelation process was carried out under constant magnetic stirring (500 rpm) for 30 min at room temperature.

MTX-loaded CS NPs (MTX–CS–NPs) were prepared as follows: to a premixed TPP and 77 KL solution (both at 0.1%, w/v, with a TPP:77 KL ratio equal to 1:0.5, w/w), MTX was added to provide a final concentration of 0.07% (w/v) of the antitumor drug. MTX-NPs were prepared by dropwise addition of this premixed solution (TPP:77 KL:MTX, with a final ratio of 1:0.5:1, w/w/w) to the previously prepared CS solution (0.1%, w/v). The NPs were formed at room temperature and dark conditions, under constant magnetic stirring (500 rpm) for 30 min. The purification of the resulting NPs was carried out by exhaustive dialysis for 4 h, using a Spectra/Por® dialysis bag of 3500 MWCO (Spectrum Medical Industries, CA, USA).

### 2.4. Nanoparticle characterization

The mean hydrodynamic diameter and the polydispersity index (PDI) of the NPs were determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK). Before measurement, the NPs were appropriately diluted in both distilled water and cell culture medium with 5% FBS, and the readings were taken at 25 °C immediately after preparation ($t = 0$ h) and after 24 h incubation at 37 °C ($t = 24$ h). Each measurement was performed using at least three sets of ten runs.

The zeta potential (ZP) values of the NPs were assessed by determining electrophoretic mobility with the Malvern Zetasizer ZS equipment. The measurements were also performed in both ultrapure water and cell culture medium with 5% FBS at 25 °C using at least three sets of 20 runs. ZP is a measurement of the electric charge at the surface of the particles and indicates the physical stability of colloidal systems.

The morphology and size of the NPs were analyzed by transmission electron microscopy (TEM). A droplet (5 μl) of the NPs dispersed in distilled water was placed on a carbon-coated copper grid to form a thin liquid film, and the negative staining of samples was obtained with a 2% (w/v) solution of uranyl acetate. The images were obtained with a Jeol JEM-1010 electron microscope (Jeol Ltd., Tokyo, Japan) operating at an acceleration voltage of 80 kV.

### 2.5. Drug entrapment efficiency

The drug association efficiency was determined using the dialysis technique for separating the non-encapsulated drug from the NPs. Five milliliters of drug-loaded NPs was dropped into a dialysis bag (Spectra/Por®, 3500 MWCO, Spectrum Medical Industries, CA, USA), the free drug was dialyzed for 60 min each time and the dialysis was completed when no drug was detectable in the recipient solution. The percentage of encapsulation efficiency (E%) of MTX in the NPs was measured using UV spectrophotometry (306 nm, UV-160A spectrophotometer, Shimadzu, Kyoto, Japan) and calculated as follows [22]:

$$E\% = 100 \times (ND - D)/ND \quad (1)$$

where ND and D are the drug concentrations before and after the dialysis, respectively. The E% value was the mean of three NP batches.

### 2.6. In vitro release study

In vitro release assessments from MTX-loaded CS NPs were carried out for 24 h in phosphate buffered saline (PBS) at pH 7.4, 6.5 and 5.4. An aliquot of NPs (100 μg/ml) was placed in a dialysis bag (Spectra/Por®, 3500 MWCO, Spectrum Medical Industries, CA, USA) and suspended in 15 ml of PBS at 37 °C under gentle magnetic stirring (100 rpm). At scheduled times, 2 ml of medium was withdrawn and replaced with an equal volume of fresh medium. The amount of MTX released was estimated by UV spectrophotometry (306 nm, UV-160A spectrophotometer, Shimadzu, Kyoto, Japan). All of the experimental procedure was performed in triplicate. The release of

the free drug was also investigated in the same way. The cumulative release percentage (CR%) of MTX at each time point was determined using the following equation:

$$CR\% = (Mt/Mi) \times 100 \quad (2)$$

where M*i* and M*t* are the initial amount of drug encapsulated in the NPs and the amount of drug released at the time *t*, respectively.

### 2.7. Cell cultures and treatments

The tumor cell lines HeLa (human epithelial cervical cancer) and MCF-7 (human breast cancer), and the non-tumor cell line HaCaT (spontaneously immortalized human keratinocytes) were grown in DMEM medium (4.5 g/l glucose) supplemented by 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C, 5% $CO_2$. These cells were routinely cultured in 75 $cm^2$ culture flasks and were trypsinized using trypsin-EDTA when the cells reached approximately 80% confluence. All cell lines were obtained from Eucellbank (Universitat de Barcelona, Spain).

HeLa ($5 \times 10^4$ cells/ml), MCF-7 ($1 \times 10^5$ cells/ml) and HaCaT ($1 \times 10^5$ cells/ml) cells were seeded into the 60 central wells of 96-well cell culture plates in 100 μl of complete culture medium. Cells were incubated for 24 h under 5% $CO_2$ at 37 °C and the medium was then replaced with 100 μl of fresh medium supplemented by 5% (v/v) FBS containing the treatments. Unloaded–CS–NPs were assayed in the 3.9–500 μg/ml concentration range, whereas MTX–CS–NPs and free MTX were assessed in the 0.001–50 μg/ml concentration range. The concentration of unloaded–CS–NPs was based on their total composition, and the higher concentration range tested was attributed to their low cytotoxic potential. In contrast, the concentration of MTX–CS–NPs was based on the total amount of MTX encapsulated in the NPs, so that the results could be compared with those obtained using free MTX. Each concentration was tested in triplicate and control cells were exposed to medium with 5% (v/v) FBS only. The cell lines were exposed for 4, 24 or 48 h to each treatment, and their viability was assessed by three different endpoints, as described below.

To assess the pH-dependent cytotoxicity of NPs and free MTX, we also exposed the cells to treatment at pH 6.6, which mimicks the acidic extracellular pH of tumors ($pH_e$) [23]. DMEM 5% FBS was mixed with 1 M HEPES buffer pH 6.2 at a ratio of 5:1, and the resulting mixture, with an experimental pH of 6.6, was applied during the incubation of the NPs or free MTX (at the same concentration range as that described above) with HeLa or MCF-7 cells for 4 or 24 h.

### 2.8. Cytotoxicity assays–antitumor activity of MTX

The MTT assay is based on the protocol first described by Mossmann [24]. It is a measurement of cell metabolic activity, and correlates quite well with cell proliferation [25]. The assay was performed as previously described [26]. Cell viability was calculated as the percentage of tetrazolium salt reduction by viable cells in each sample and the values were normalized by the untreated cell control (cells with medium only).

The NRU assay is based on the protocol described by Borenfreund and Puerner [27], and reflects the functionality of the lysosomes and plasma membrane [28]. The assay was performed following the previously described protocol [26]. The effect of each treatment was calculated as the percentage of uptake of NR dye by lysosomes against the untreated cell control (cells with medium only).

LDH leakage was evaluated to determine the integrity of the plasma membrane using a commercially available kit (Takara Bio Inc, Otsu, Japan), in line with the instructions provided by the manufacturer. This assay quantifies cytotoxicity by measuring LDH released from dead or plasma membrane-damaged cells into the supernatant [29]. Absorbance was measured in a microplate reader (Sunrise™, Tecan, Switzerland) at 492 nm, with 620 nm set as the reference wavelength. The results are expressed as a percentage of control, with 1% Triton-X used as a positive control.

To determine whether the NPs interacted with the viability assays, UV/Visible absorbance measurements were carried out [30]. Unloaded–CS–NPs at 500 μg/ml and MTX–CS–NPs at 50 μg/ml were suspended in DMEM medium (without FBS and phenol red) containing MTT (0.5 mg/ml) or NR (50 μg/ml) dyes, and the occurrence of dye interference was assessed following the previously described procedure [26].

### 2.9. Apoptosis

NP-induced apoptosis in HeLa and MCF-7 cells was quantified using acridine orange/ethidium bromide (AO/EB) double staining, according to standard procedure [31] and using a fluorescence microscope. Briefly, cells were seeded ($5 \times 10^4$ or $1 \times 10^5$ cells/ml, for HeLa and MCF-7, respectively) in 24-well plates and treated with 1, 10 or 50 μg/ml of MTX–CS–NPs or free MTX. After 24 h incubation, the cells were trypsinized and centrifuged at 1200 rpm for 5 min. Then, fluorescent dyes AO (0.5 μg/ml) and BE (10 μg/ml) were added to the cellular pellet. Freshly stained cell suspension was dropped on a glass slide and covered by a cover slip. Slides were observed with an Olympus BX41 fluorescence microscope equipped with a UV-mercury lamp (100 W Ushio Olympus) and a U-N51004v2- FITC/TRITC type filter set (FITC: BP480-495, DM500-545, BA515-535 and TRITC: BP550-570, DM575-, BA590-621). Images were digitized on a computer through a video camera (Olympus digital camera XC50) and were analyzed with an image processor (Olympus cell-B Image Acquisition Software). The percentage of viable, apoptotic and necrotic cells was determined in at least 100 cells.

### 2.10. Cell cycle analysis by flow cytometry

HeLa and MCF-7 tumor cells were cultured in 60 mm petri dishes for 24 h at a density of $5 \times 10^4$ or $1 \times 10^5$ cells/ml, respectively, and then treated with 1, 10 or 50 μg/ml of MTX–CS–NPs or free MTX. After 24 h treatment, the cells were harvested by trypsinization, washed in cold PBS, fixed in ice-cold 70% ethanol and kept at −20 °C. Fixed cells were centrifuged, resuspended in DNA extraction buffer (0.2 M $Na_2PO_4$ and 0.1 M citric acid, pH 7.8) and incubated for 30 min at 37 °C. Then, the cells were stained with staining solution (20 μg/ml propidium iodide, 200 μg/ml RNAse A and Triton X-100 in PBS). The samples were kept in dark conditions for 1 h and measured with the Beckman Coulter ADC Epics XL flow cytometer (Beckman Coulter, FL, USA) (15,000 events were acquired for each sample). The data obtained were processed for cell cycle analysis with Multicycle® software. The amount of propidium iodide intercalating to DNA was used as the parameter to determine the cell cycle distribution phases.

### 2.11. Lysosomal membrane integrity

Lysosomal membrane stability was assessed using the acridine orange (AO) relocation technique. AO is a lysosomotropic base (pKa = 10.3) that produces a red fluorescent emission when accumulated in acidic lysosomes. Disruption of the lysosomal membrane can be assessed by measuring the change in intracellular AO fluorescence (i.e., loss of the lysosomal red signal and gain of cytoplasmic green) [32]. HeLa and MCF-7 cells were exposed to MTX–CS–NPs or free MTX (final concentrations of 1, 10 and 50 μg/ml) for 24 h. Then, the cells were trypsinized, centrifuged at 1200 rpm for 5 min, and 2 μg/ml AO was added to the cellular pellet. Freshly stained cell suspension was dropped on a glass slide and covered by a cover slip. Slides were observed on an Olympus BX41 fluorescent microscope, as described in Section 2.9.

### 2.12. Hemocompatibility studies

Erythrocytes were isolated from rat blood, which was obtained from anesthetized animals by cardiac puncture and drawn into tubes containing EDTA. The procedure was approved by the institutional ethics committee on animal experimentation. The hemolysis assay was performed following the previously described procedure [17]. Twenty-five microliter aliquots of erythrocyte suspension were exposed to unloaded–CS–NPs at concentrations of 100, 250 and 500 μg/ml, and to MTX–CS–NPs or free MTX at concentrations of 10, 25 and 50 μg/ml. The samples were incubated at room temperature for 10 min or 1 h. For the studies of erythrocyte agglutination, 10 μl of each sample was subjected to a hemolysis assay for 1 h (500 μg/ml of unloaded–CS–NPs and 50 μg/ml of MTX–CS–NPS), placed on a glass slide, covered by a cover slip and analyzed by a phase contrast microscope (Olympus BX41, Olympus, Japan).

### 2.13. pH-dependent membrane-lytic activity of nanoparticles

#### 2.13.1. Hemolysis assay as a model for the endosomal membrane

The pH-dependent membrane-lytic activity of the NPs was assessed using erythrocytes as a model of the endosomal membrane [33,34]. Increasing concentrations of unloaded–CS–NPs and MTX–CS–NPs were added to erythrocytes suspended in PBS buffer of pH 7.4, 6.5 or 5.4. The extent and kinetics of hemolysis were assessed as reported earlier [17].

#### 2.13.2. Cell uptake studies - intracellular release of calcein

Calcein, a membrane-impermeant fluorophore, was used as a model drug molecule and tracer to monitor the stability of endosomes following vesicle uptake [35]. HeLa cells were plated ($5 \times 10^4$ cells/ml) in 24-well plates on round cover glasses (Marlenfeld GmbH & Co.KG, Lauda-Könlgshofen, Germany) and incubated overnight at 37 °C under 5% $CO_2$. Then, calcein (1 mg/ml) was added to the cells with or without (control cells) 250 or 500 μg/ml of unloaded–CS–NPs, or 25 or 50 μg/ml of MTX–CS–NPs in DMEM medium without FBS and phenol red. After 1 h incubation at 37 °C, the cells were washed four times with PBS and incubated in complete medium for 3 h to allow intracellular trafficking. The cells were then washed with PBS and fixed with 4% (v/v) formaldehyde in PBS (pH 7.4) for 15 min at room temperature. Each individual cover glasses was mounted on clean glass slides with Prolong® Gold antifade reagent (Invitrogen) and analyzed on an Olympus BX41 fluorescence microscope equipped with a MNIBA3 filter set type (470–495 nm excitation, 510–550 nm emission and 505 nm dichromatic mirror). Images were digitized and analyzed as described in Section 2.9.

### 2.14. Statistical analysis

All *in vitro* experiments were performed at least three times, using three replicate samples for each formulation concentration tested. The cytotoxicity of each formulation was expressed in terms of its $IC_{50}$ (concentration causing 50% death of the cell population), calculated from concentration–response curves. Results are expressed as mean ± standard error of the mean (SE). Statistical analyses were performed using the Student's *t* test or one-way analysis of variance (ANOVA) to determine the differences between the datasets, followed by Tukey's or Dunnett's *post-hoc* test for multiple comparisons using SPSS® software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ and $p < 0.005$ were considered significant.

## 3. Results

### 3.1. Characterization of nanoparticles

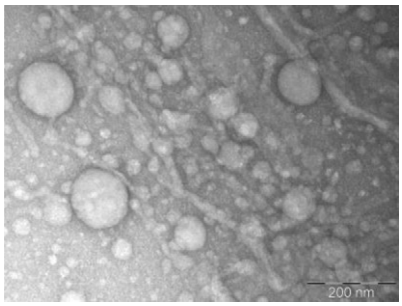
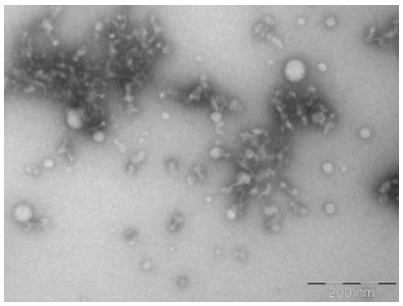
Unloaded- and MTX-loaded NPs were characterized by DLS and TEM. Firstly, we assessed the effects of the dispersion medium (distilled water or culture medium), temperature and exposure time on NP hydrodynamic size. DLS measurements showed that fresh prepared ($t = 0$) unloaded–CS–NPs and MTX–CS–NPs dispersed in distilled water had an average hydrodynamic size of 350 nm and 301 nm, respectively (Table 2). After 24 h incubation ($t = 24$) at 37 °C, the NPs showed a slight size increase. In contrast, when the NPs were dispersed in the cell culture medium (DMEM with 5% FBS), the size increase was moderate by 0 h (579 nm and 483 nm for unloaded–CS–NPs and MTX–CS–NPs, respectively), which indicates that NP agglomeration took place. After 24 h incubation under cell culture conditions, the unloaded–CS–NPs presented a bimodal size distribution, with particles smaller (108 nm) and larger (723 nm) than those obtained at $t = 0$. However, all the MTX–CS–NPs had a hydrodynamic size that was three times smaller (147 nm) after 24 h incubation in cell culture medium. PDI values below 0.3 were obtained in distilled water at both $t = 0$ and $t = 24$, which indicates that the NP population was relatively homogenous in size. In contrast, significantly higher PDI values (>0.4) were found for the NPs dispersed in the cell culture medium. The NPs dispersed in water showed positive ZP values (~30 mV), whereas almost neutral values were obtained in cell culture medium.

A TEM analysis was also performed to assess NP morphology and size (Table 2). The results showed that at $t = 0$ both unloaded–CS–NPs and MTX–CS–NPs dispersed in water had a roughly spherical shape and were much smaller (~20–140 nm and ~20–60 nm, respectively) than the hydrodynamic size determined by DLS (350 and 301 nm, respectively). Finally, the entrapment efficiency achieved for NPs was found to be 63.41 ± 0.65% for MTX. This high incorporation capacity in the designed formulation is probably related to the chemical nature of the drug and to its interactions with the NP structure at the pH value of the experimental conditions.

### 3.2. In vitro release study

The pH-dependent cumulative amount of MTX released from the MTX–CS–NPs is shown in Fig. 1. A control experiment using free MTX was also carried out under similar conditions and complete diffusion across the dialysis membrane was found to occur within 3 h. The release of MTX from NPs at physiological pH showed an initial burst release of 18% after 1 h, whereas about 75% and 100% was released after 8 h and 24 h, respectively. The initial MTX burst release at pH 6.5 was almost the same as at pH 7.4 (~18%), but greater release was achieved at each subsequent time point. In contrast, significantly faster ($p < 0.05$) drug release was obtained at pH 5.4: approximately 42% of the MTX was released

**Table 2**
Characterization properties of the nanoparticles.

| | Unloaded–CS–NPs | MTX–CS–NPs |
|---|---|---|
| Hydrodynamic size (nm) ± SE[a] | | |
| $t = 0$ h water | 350.47 ± 13.31 | 301.17 ± 8.96 |
| $t = 0$ h DMEM 5% FBS | 579.40 ± 18.71/17.39 ± 0.45[c] | 483.50 ± 45.03/14.80 ± 0.78[c] |
| $t = 24$ h water[b] | 403.53 ± 13.23 | 312.57 ± 9.71 |
| $t = 24$ h DMEM 5% FBS[b] | 108.41 ± 14.42/723.15 ± 32.15[c] | 147.73 ± 1.83 |
| PDI ± SE[a] | | |
| $t = 0$ h water | 0.269 ± 0.009 | 0.257 ± 0.008 |
| $t = 0$ h DMEM 5% FBS | 1.00 ± 0.001 | 0.680 ± 0.011 |
| $t = 24$ h water[b] | 0.260 ± 0.018 | 0.269 ± 0.005 |
| $t = 24$ h DMEM 5% FBS[b] | 0.517 ± 0.087 | 0.431 ± 0.029 |
| ZP (mV) ± SE[a] | | |
| $t = 0$ h water | 30.07 ± 1.18 | 26.87 ± 0.90 |
| $t = 0$ h DMEM 5% FBS | −4.41 ± 0.35 | −4.74 ± 0.29 |
| TEM diameter (nm) | | |
| $t = 0$ h water | 20–140 | 20–60 |
| TEM images[d] |   |   |

[a] Mean of three experiments ± SE.
[b] Incubated under cell culture conditions: 37 °C, 5% $CO_2$.
[c] Predominanting size is indicated first.
[d] Scale bars = 200 nm.

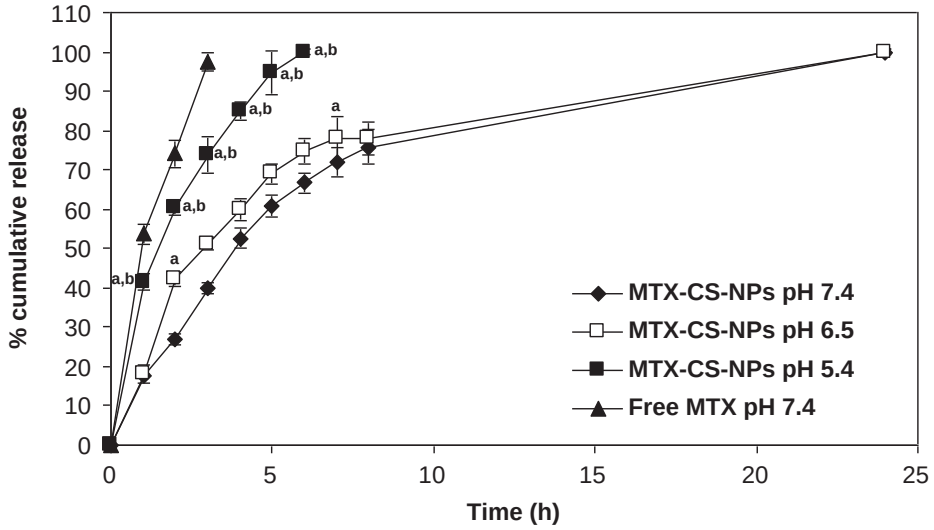



**Fig. 1.** pH-dependent *in vitro* cumulative release of MTX from NPs in PBS buffer at pH 7.4, 6.5 and 5.4. Results are expressed as the mean ± SE of three independent experiments. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test. [a] significantly different from PBS pH 7.4 ($p < 0.05$) and [b] significantly different from PBS pH 6.5 ($p < 0.05$).

from the NPs after 1 h, while total drug release was reached after only 6 h.

### 3.3. *In vitro* cytotoxicity assessments

#### 3.3.1. *Cell viability studies – antitumor activity*

The MTT and NR dye interactions with NPs were studied to test the suitability of MTT and NRU viability assays for the cytotoxicity evaluation of CS-NPs. Our results showed slight interference of the NPs with the viability dyes. The NPs, especially the unloaded–CS–NPs, induced a slight increase in the MTT and NR absorbance values at 550 nm. These data were proved by the UV–vis measurements (data not shown). However, the interferences observed did not result in a further increase in cell viability with increasing NP concentration, which demonstrated that these interactions between NPs and viability dyes were not significant, and also proved that the viability endpoints are suitable for the intended purpose.

The cytotoxicity studies of unloaded–CS–NPs, MTX–CS–NPs and free MTX were performed using tumor (HeLa and MCF-7) and non-tumor (HaCaT) cell lines. Our results clearly proved that the activity of MTX–CS–NPs against the tumor cells was greater than that of the free drug (Fig. 2 and Table 3). The MTT and NRU endpoints showed moderate cytotoxic effects of free MTX (>60% of viability), whereas MTX encapsulated in the NPs significantly reduced the cell growth by up to 17% and 33% of viability in HeLa and MCF-7 cells, respectively (Fig. 2A). The MCF-7 cell line displayed higher resistance than the HeLa cells to both free MTX and MTX–CS–NPs. The non-tumor HaCaT cell line was much less sensitive to the antiproliferative and toxic effects of the MTX–CS–NPs (Fig. 2), with viabilities higher than 56% or 85% as measured by the MTT and NRU assays, respectively. Likewise, the free MTX showed cytotoxicity to the HaCaT cells of less than 20%. Furthermore, the NPs affected cell viability in a time-dependent manner when they were added to the cells in the concentration range of 0.001–50 μg/ml for up to 48 h (Fig. 3A). The free MTX displayed similar cytotoxic behavior, but to a less significant extent. Table 3 shows the $IC_{50}$ values (determined by the MTT assay) of the MTX–CS–NPs and free drug tested at 4, 24 and 48 h. Unloaded–CS–NPs showed low cytotoxic effects against all tested cell lines and displayed, in general, $IC_{50}$ values > 500 μg/ml and at least 60% of cell viability (Table 3). Finally, as determined by the LDH assay, unloaded–CS–NPs, MTX–CS–NPs and free MTX did not affect the plasma membrane integrity of the three cell lines. Viability values above 90% were obtained in all the conditions tested.

Fig. 3B shows the effect of the medium's pH during cell treatment on the cytotoxic responses of free MTX and MTX–CS–NPs in the tumor cell lines. Free MTX did not show any change in its cytotoxic activity on HeLa and MCF-7 cells when it was incubated under mildly acidic conditions (pH 6.6) for 4 and 24 h. In contrast, the higher concentrations of MTX–CS–NPs displayed significantly higher cytotoxicity ($p < 0.005$) to both tumor cell lines after 4 h incubation at pH 6.6. The $IC_{50}$ values decreased from >50 to 21.86 μg/ml and >50 to 33.09 μg/ml in HeLa and MCF-7 cells, respectively. After 24 h incubation, the cytotoxic profile of the NPs was similar at both pH in HeLa cells, whereas in MCF-7 cells, the concentration of 10 μg/ml showed significantly higher cytotoxicity at pH 6.6. The cytotoxic profile of unloaded–CS–NPs did not change significantly when incubated at pH 6.6 (data not shown).

#### 3.3.2. *Apoptosis*

In order to determine whether the initial cell death observed in HeLa and MCF-7 tumor cells exposed to free MTX and MTX–CS–NPs could be due to apoptosis, AO/EB staining was carried out and the samples were analyzed under a fluorescence microscope. Fig. 4 reveals a significant increase ($p < 0.05$ or $p < 0.005$) in the number of apoptotic cells in both cell lines following treatment with free MTX and MTX–CS–NPs. Furthermore, a greater increase in the fraction of apoptotic cells was induced by MTX–CS–NPs than by free MTX, especially in HeLa cells. The effects were dose-dependent for both MTX–CS–NPs and free MTX. The most significant effects were observed with the treatments that were also the most cytotoxic in all the viability assays. A slight to moderate increase was observed in the number of cells undergoing late apoptosis or necrosis, but these results did not differ significantly from those of the control cells. The untreated HeLa and MCF-7 cells were observed with a green intact nuclear structure (Fig. 4c and f, respectively), while after free MTX or MTX–CS–NP treatments we observed cells showing blebbing and nuclear margination (e.g. in Fig. 4d), chromatin condensation (e.g. in Fig. 4e) (early and moderate apoptosis), orange nuclei with normal chromatin distribution (necrosis) (e.g. in Fig. 4g), as well as apoptotic body separation and a reddish-orange color (late apoptosis) (e.g. in Fig. 4h).

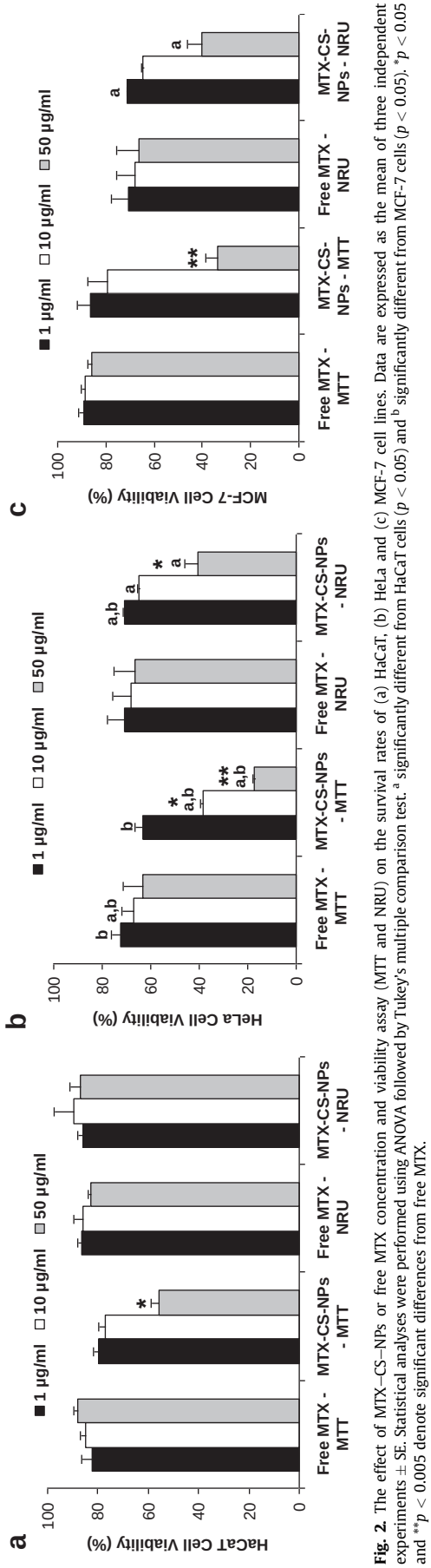



**Fig. 2.** The effect of MTX–CS–NPs or free MTX concentration and viability assay (MTT and NRU) on the survival rates of (a) HaCaT, (b) HeLa and (c) MCF-7 cell lines. Data are expressed as the mean of three independent experiments ± SE. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test. [a] significantly different from HaCaT cells ($p < 0.05$) and [b] significantly different from MCF-7 cells ($p < 0.05$). $^{*}p < 0.05$ and $^{**}p < 0.005$ denote significant differences from free MTX.

**Table 3**
Cytotoxicity expressed as $IC_{50}$ values of free MTX, unloaded–CS–NPs and MTX-loaded–CS–NPs in HeLa, MCF-7 and HaCaT cell lines.

| Cytotoxicity assay – 24 h treatment - $IC_{50}$ ± SE (µg/ml) | | | |
|---|---|---|---|
| Cell line | MTT | NRU | LDH |
| Free MTX | | | |
| HeLa | >50 | >50 | >50 |
| MCF-7 | >50 | >50 | >50 |
| HaCaT | >50 | >50 | >50 |
| MTX–CS–NPs | | | |
| HeLa | 4.37 ± 0.37 | 17.69 ± 0.14 | >50 |
| MCF-7 | 34.72 ± 6.23 | 35.35 ± 5.38 | >50 |
| HaCaT | >50 | >50 | >50 |
| Unloaded–CS–NPs | | | |
| HeLa | 260.39 ± 54.32 | >500 | >500 |
| MCF-7 | >500 | >500 | >500 |
| HaCaT | >500 | >500 | >500 |
| Time-dependent antitumoral activity - $IC_{50}$ ± SE (µg/ml) – MTT assay | | | |
| pH 7.4 | | | |
| | 4 h | 24 h | 48 h |
| Free MTX | | | |
| HeLa | >50 | >50 | 0.0089 ± 0.12 |
| MCF-7 | >50 | >50 | 19.13 ± 2.37 |
| MTX–CS–NPs | | | |
| HeLa | 42.93 ± 2.37 | 4.37 ± 0.37 | 0.0098 ± 0.05 |
| MCF-7 | >50 | 34.72 ± 6.23 | 8.76 ± 1.07 |
| pH 6.6 | | | |
| Free MTX | | | |
| HeLa | >50 | >50 | – |
| MCF-7 | >50 | >50 | – |
| MTX–CS–NPs | | | |
| HeLa | 21.86 ± 1.98 | 6.76 ± 2.54 | – |
| MCF-7 | 33.09 ± 2.31 | 28.97 ± 1,74 | – |

### 3.3.3. Cell cycle analysis

A flow cytometric analysis was performed to clarify the influence of MTX–CS–NPs on cell-cycle distribution in comparison with the free drug, and in addition to the cell viability studies. These experiments showed significant ($p < 0.05$ or $p < 0.005$) suppression of the G1/G0 and G2/M phases with cell cycle arrest in the S phase in both HeLa and MCF-7 cancer cells (Fig. 5). In HeLa cells, this effect was more pronounced after the free MTX treatment. In contrast, the MTX–CS–NPs induced greater accumulation of cells is the S phase in MCF-7 after 24 h exposure. The distinct cell cycle arrest phase observed in cells treated with free MTX or MTX–CS–NPs might be due to the different consequences of unloaded or nanocarrier-loaded MTX in cancer cells. Moreover, a significant increase in the cell population was observed in the sub-G1 phase after treatment with MTX–CS–NPs, which is indicative of apoptotic cells. This corroborates the apoptosis experiments using AO/EB staining.

### 3.3.4. Lysosomal membrane integrity

The ability of free MTX and MTX–CS–NPs to induce lysosomal membrane permeabilization (LMP) was assessed using the AO relocation assay (Fig. 6). In control HeLa and MCF-7 cells, the lysosomes (red-orange granules) can be clearly seen as strong granular AO staining. MCF-7 cells treated with both MTX and MTX–CS–NPs showed little or no LMP by microscopic observation, whereas HeLa cells showed an increased LMP effect induced by 50 µg/ml MTX–CS–NPs, as evidenced by the release of lysosomal contents into the cytoplasm (a reduction in red fluorescence).

### 3.4. Hemocompatibility studies

The hemocompatibility of the NPs and free MTX was studied by hemolysis experiments (Fig. 7A). The release of hemoglobin was used to quantify their erythrocyte-damaging properties. The unloaded–CS–NPs were non-hemolytic (less than 5%) after 10 min

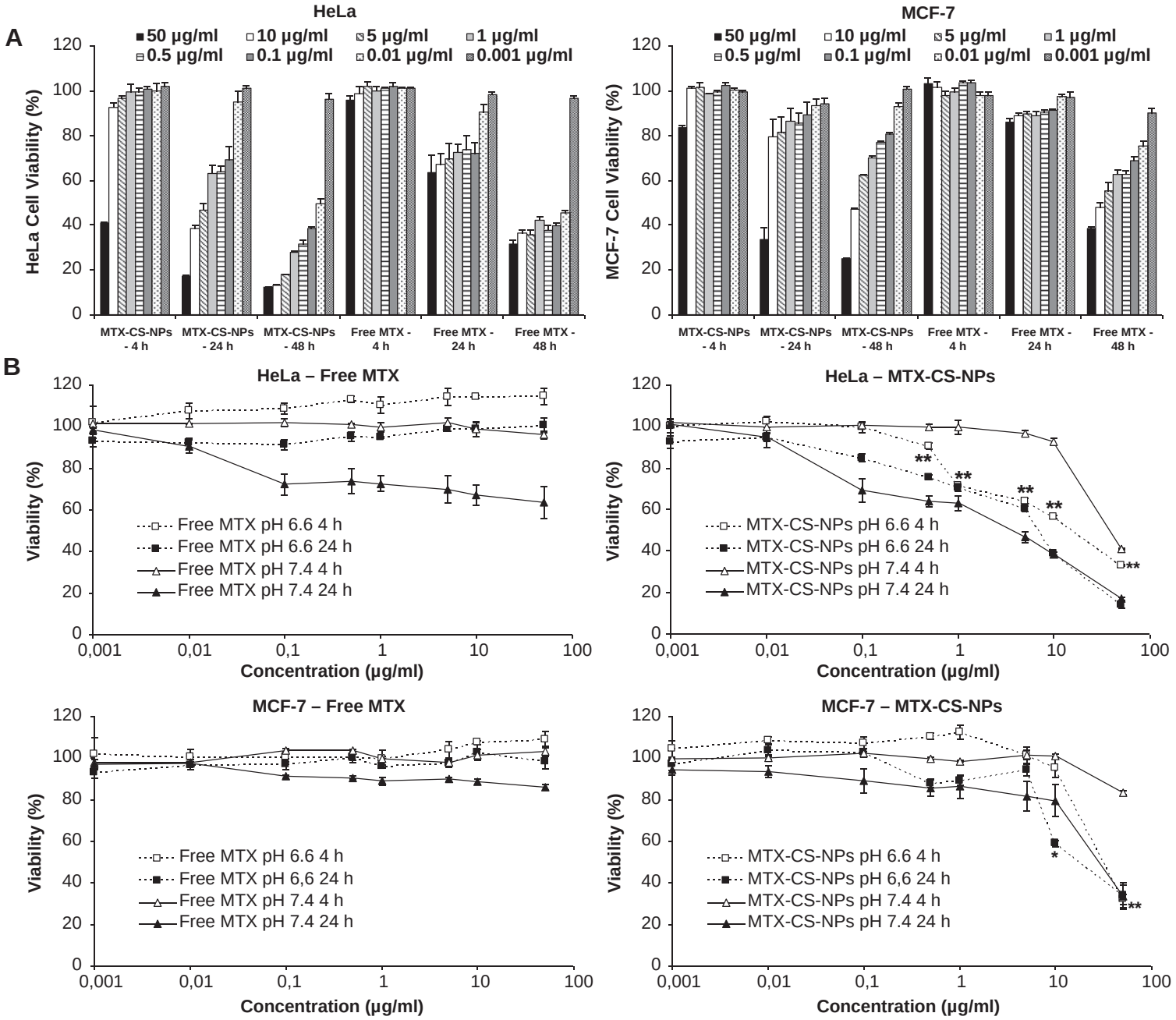



**Fig. 3.** Cytotoxicity of MTX–CS–NPs and free MTX on HeLa and MCF-7 tumor cell lines. (A) Time-dependent cytotoxicity of MTX–CS–NPs and free MTX at the 0.001–50 µg/ml concentration range in tumor HeLa and MCF-7 cells, as determined by a MTT assay. (B) pH-dependent cytotoxicity of MTX–CS–NPs and free MTX against HeLa and MCF-7 cells after 4 and 24 h incubation, as determined by a MTT assay. Data are expressed as the mean of three independent experiments ± SE. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test. $^{*}p < 0.05$ and $^{**}p < 0.005$ denote significant differences from the cytotoxic effects at pH 7.4.

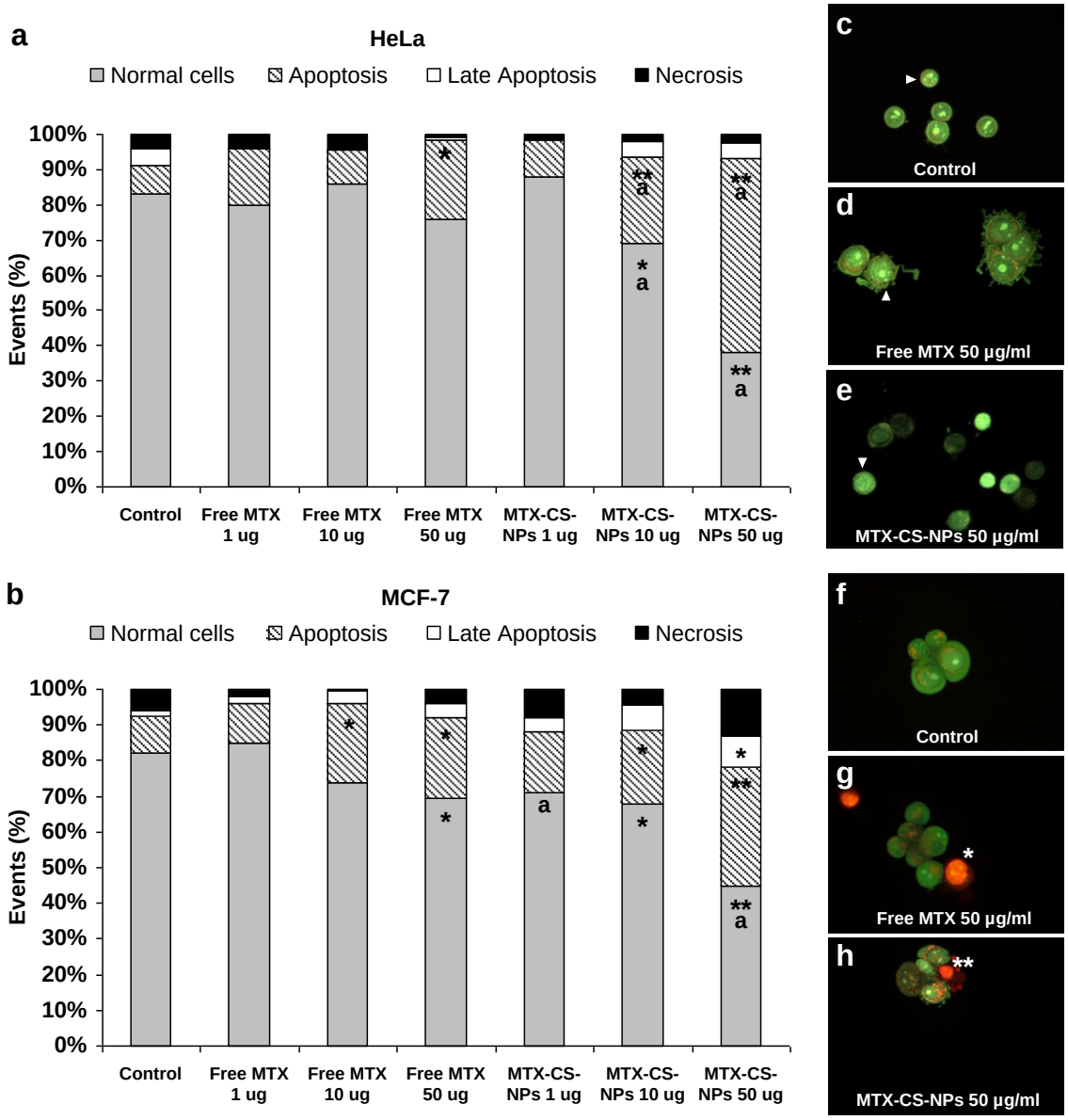



**Fig. 4.** Effect of MTX–CS–NPs and free MTX on apoptosis of (a) HeLa cells and (b) MCF-7 cells determined by fluorescence microscopy after AO/BE staining. Results are expressed as the percentage of viable, apoptotic and necrotic cells after 24 h treatment with 1, 10 or 50 μg/ml of MTX–CS–NPs or free MTX. Fluorescent micrographs of HeLa cells: (c) Untreated control cells, (d) 50 μg/ml free MTX, (e) 50 μg/ml MTX–CS–NPs and of MCF-7 cells: (f) Untreated control cells, (g) 50 μg/ml free MTX, (h) 50 μg/ml MTX–CS–NPs. Legends: (►) typical live nuclei, (▼) chromatin condensation (early apoptosis), (▲) blebbing and nuclear margination (early to moderate apoptosis), (*) necrosis, (**) late apoptosis. Statistical analyses were performed using ANOVA followed by Dunnett's or Tukey's multiple comparison test. $^{*}p < 0.05$ and $^{**}p < 0.005$ denote significant differences from untreated control cells. [a] significantly different from free MTX ($p < 0.05$).

incubation and showed slight hemolysis after 1 h incubation (about 10% at the highest concentration). In contrast, MTX–CS–NPs at 50 μg/ml induced significantly higher hemolysis (>70%) after both 10 and 60 min incubation. The free MTX was non-hemolytic in all the conditions. In addition, neither unloaded–CS–NPs nor MTX–CS–NPs induced agglutination of erythrocytes after 1 h of treatment with the lowest concentrations tested (100 and 250 μg/ml or 10 and 25 μg/ml, respectively) (data not shown). In contrast, significant agglutination was induced by 500 μg/ml of unloaded–CS–NPs and 50 μg/ml of MTX–CS–NPs (Fig. 7B).

### 3.5. pH-dependent membrane-lytic activity of nanoparticles

#### 3.5.1. Hemolysis assay as a model for the endosomal membrane

Fig. 8A shows the membrane-lytic activity of the NPs as a function of concentration with varying pH and incubation time. Negligible membrane lysis was induced by unloaded–CS–NPs after 10 min incubation at physiological pH, while 60 min incubation prompted at most 11% of hemolysis. Interestingly, MTX–CS–NPs at 50 μg/ml induced 70.5% and 94.5% of membrane lysis after 10 and 60 min incubation, respectively. As the pH decreased to 6.5 or 5.4, the membrane-lytic activity of the unloaded–CS–NPs increased significantly ($p < 0.05$) in a dose-dependent manner. The unloaded–CS–NPs reached a maximum hemolysis of 61.2% and 83.5% after 60 min of incubation at pH 6.5 and 5.4, and were 5.5- and 7.6-fold more hemolytic than at pH 7.4, respectively. MTX–CS–NPs showed higher membrane lysis in an acidic environment and were also more active than the unloaded–CS–NPs. MTX–CS–NPs at 25 μg/ml already induced 12.9% and 43.4% of hemolysis after 60 min incubation at pH 6.5 and 5.4, respectively, and were 4.8- and 16.1-fold more hemolytic than at pH 7.4. At 50 μg/ml, the membrane-lytic activity did not differ significantly from that at pH

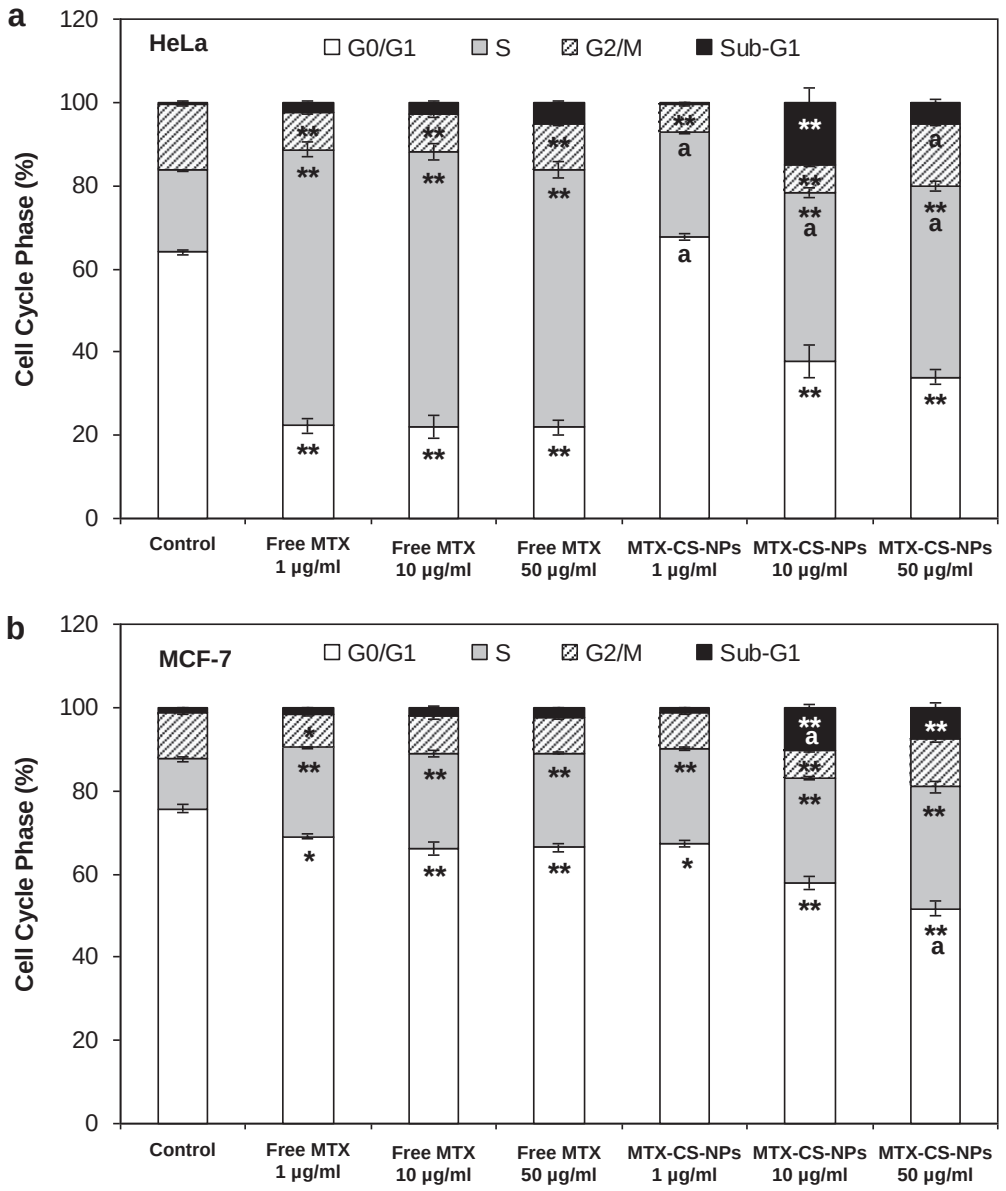



**Fig. 5.** Cell-cycle analysis of (a) HeLa and (b) MCF-7 cells following 24 h treatment with 1, 10 or 50 μg/ml of MTX–CS–NPs or free MTX. Results are expressed as the mean ± SE of three independent experiments, performed in duplicate. Statistical analyses were performed using ANOVA followed by Dunnett's or Tukey's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.005$ denote significant differences from untreated control cells. [a] significantly different from free MTX ($p < 0.05$).

7.4. Finally, unloaded–CS–NPs without 77 KL were prepared to prove that the pH-sensitive membrane-lytic activity of the NPs could be attributed to the surfactant. These NPs showed at most 3.8%, 11.6% and 15.5% of hemolysis at pH 7.4, 6.5 and 5.4, respectively. The hemolysis of free MTX was also assessed at pH 7.4, 6.5 and 5.4, but no membrane lysis was obtained in any of the conditions (data not shown).

*3.5.2. Calcein uptake and endosomal stability*

The ability of the NPs to release endocytosed materials into the cell cytoplasm was examined by fluorescence microscopy, following the uptake of calcein and NPs into HeLa cells. As shown in Fig. 8B, cells treated with calcein alone (control cells) showed a bright punctate distribution of fluorescence, which is consistent with constitutive endocytosis of the external medium. When the cells were co-incubated with calcein and unloaded–CS–NPs or MTX–CS–NPs, diffuse green fluorescence staining was observed in the cytoplasm, which indicates destabilization of the endosomal membrane and release of calcein to the cell cytosol. These results corroborated the hemolysis experiments, in which the NPs showed increased membrane-lytic activity in the pH range characteristic of endosomal compartments.

## 4. Discussion

The ionic gelation method was chosen to form the NPs because of the ability of CS to gel spontaneously on contact with multivalent polyanions. To form ionic crosslinks with TPP and 77 KL, the pH of CS solution was set at 5.5, because at this pH the formation of smaller NPs with higher ZP value was reported [7]. In addition, at pH 5.5, about 90% of the amino groups of CS (p*K*a = 6.5) are protonated [36], which ensures that the crosslinking process takes place to form CS-TPP-NPs. Finally, a pH value of 5.5 would ionize ~50% of the carboxylic groups of MTX (p*K*a = 4.8 and 5.5), which

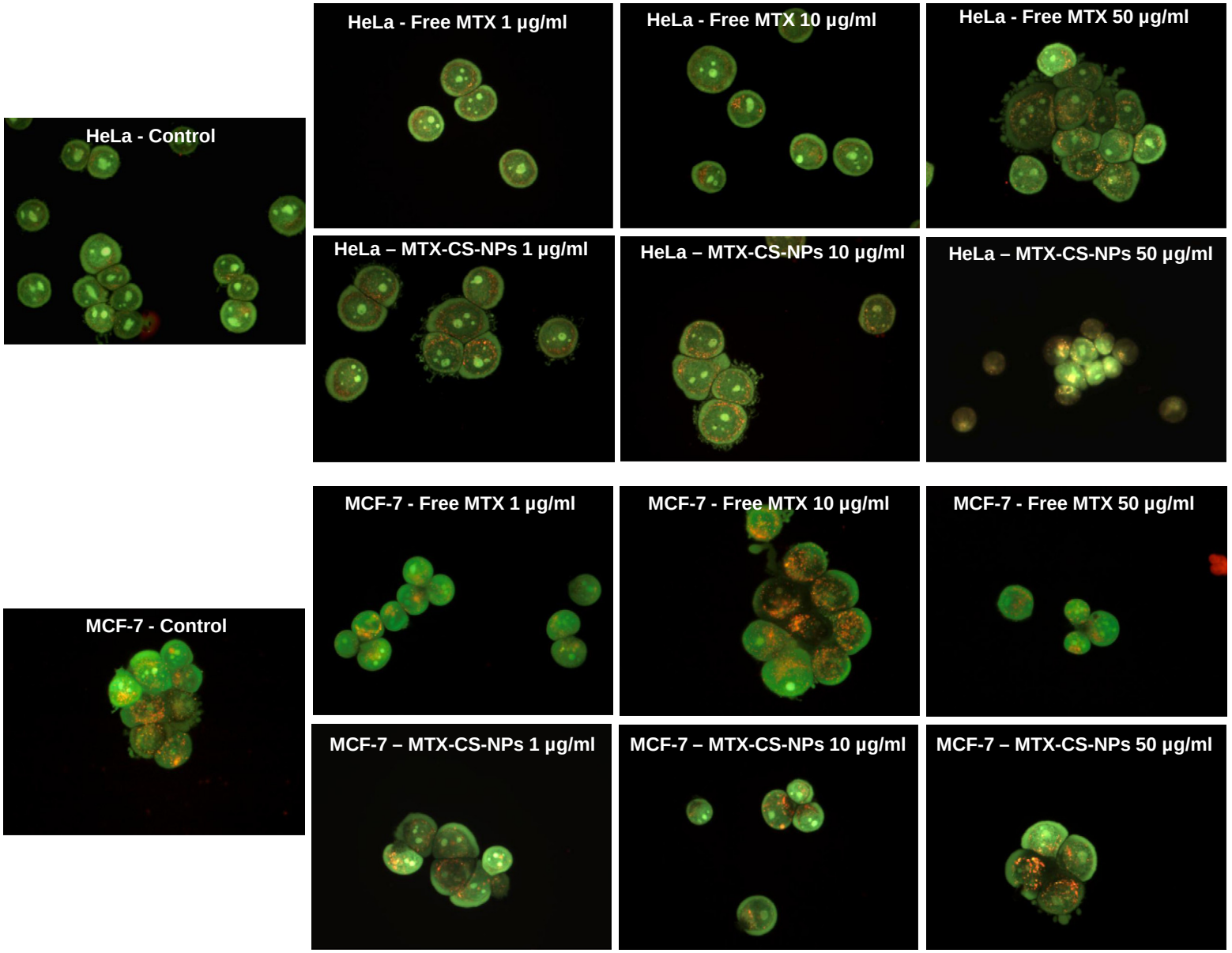



**Fig. 6.** Assessment of MTX–CS–NPs and free MTX effects on lysosomal membrane permeabilization (LMP) in HeLa and MCF-7 cells as visualized via AO staining. In untreated control cells, lysosomes can be seen as red-orange granules and cytoplasm has a diffuse green fluorescence. In cells with lysosomal membrane damage (HeLa cells treated with 50 µg/ml MTX–CS–NPs), lysosomes exhibit a shift from red-orange to a yellow-green fluorescent color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

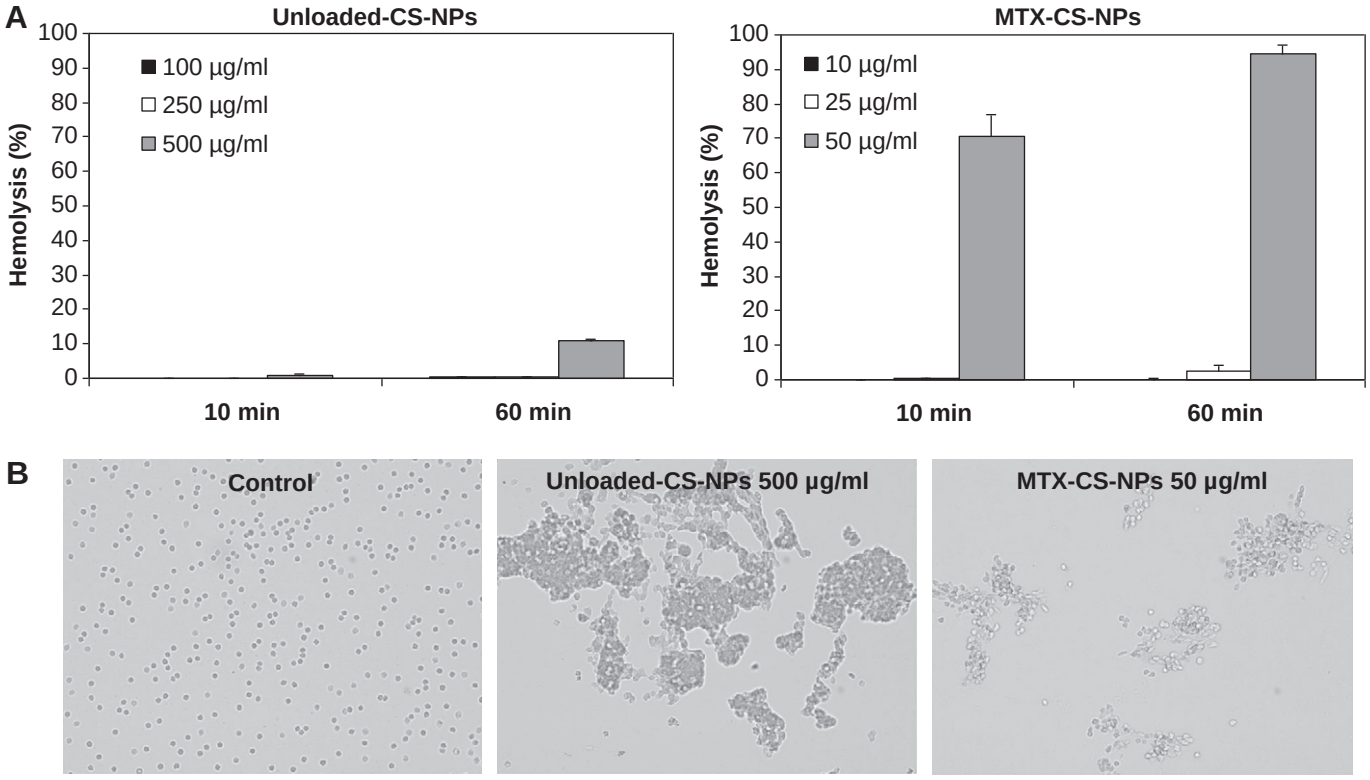



**Fig. 7.** Hemocompatibility of unloaded–CS–NPs and MTX–CS–NPs. (A) Percentage of hemolysis caused by NPs after 10 and 60 min of incubation with rat erythrocytes. Each value represents the mean ± SE of three experiments. (B) Agglutination of rat erythrocytes observed by phase microscopy after 1 h of incubation with 500 µg/ml of NPs.

allows attractive electrostatic interactions between the negatively charged drug molecules and positively charged CS molecules [6]. Therefore, during the process of incorporating the drug into the NPs, we can assume that the MTX molecules are both adsorbed at the particle surface [37] and entrapped/embedded in the CS nanomatrix by hydrogen bonding and hydrophobic forces [6].

The NP hydrodynamic size characterization in water using DLS showed that only a small increase in mean diameter was observed after 24 h, which means that the incubation time and temperature of the cell culture experiments did not directly affect the stability of the NPs, and that the dispersion medium plays a key role in their aggregation behavior. When dispersed in cell culture medium and maintained in the conditions of the cytotoxicity assays, both unloaded–CS–NPs and MTX–CS–NPs displayed unexpected behavior. The increase in measured particle size at $t = 0$ in protein-containing medium may be due to protein coating of the particles and aggregation [7,28]. At the pH of the cell culture medium ($\sim$7.4), there is a considerable reduction in the degree of protonation at the NPs surface (confirmed by the ZP values), which decreases electrostatic repulsion between the particles and, thereby, increases the probability of particle aggregation. However, the decreasing protonation state of the CS molecule in the particle surface might lead to desorption of the superficial molecules, due to reduced ionic interaction with both TPP and 77 KL. This process probably has a slow equilibration, which may justify the shaping of smaller NPs after 24 h incubation in the cell culture medium. The MTX–CS–NPs showed a slight reduction in the ZP value that may be due to the decreased number of free positive groups of CS in the NP surface, which electrostatically attached to the MTX molecules. The TEM experiments displayed NPs with a much smaller diameter than the hydrodynamic size measured by DLS, which could be due to the resolution limitation of DLS [38], to the swelling of the NPs in the presence of water [4] or even to the fact that DLS gives the mean hydrodynamic diameter of the particle core surrounded by the solvation layers, whereas TEM gives the diameter of the particles alone in the dry state [39]. NPs loaded with MTX were even smaller than unloaded–CS–NPs (as also demonstrated by DLS), which suggests that the drug increased the degree of NP compaction. Moreover, in the TEM images we can see some other material that is not condensed in the structure of the NPs, which might be unreacted polymer chains that are not involved in NP formation.

It was shown that the cumulative release rates of MTX from NPs were effectively slower than those of the free drug solution. The observed initial burst release of MTX from the NPs might be due to drug molecules that were loosely incorporated into them, e.g., by electrostatic interaction between the positively charged amino groups of CS in the particle surface and the ionized carboxyl groups of MTX. At physiological pH, we obtained a relatively slow release up to 24 h, which could be attributed to drug diffusion and the swelling/degradation of the polymer [40]. In contrast, the release rate was accelerated by decreasing pH, which confirms the pH-dependent release pattern of this nanoarchitecture. The slightly increased release at pH 6.5 might mean that anticancer drug delivery is triggered at tumor extracellular $pH_e$ (a pH range from 6.5 to 7.2) [23] or early endosomal compartment. Moreover, the MTX release from NPs was significantly increased at pH 5.4, which supports the idea of effectively increased intracellular drug delivery through the late endosomes. The triggered release of payload under acidic conditions might be attributed to the pH-dependent activity of 77 KL [17]. The ionization of 77 KL ($pKa = 6.2$) tends to decrease under acidic conditions, which might contribute to destabilizing the NP structure, due to the reduced amount of available anionic groups that interact electrostatically with the positive amino groups of CS. Finally, high release in an acidic environment might be favored by the lower degree of MTX ionization under this condition, which would decrease the electrostatic interaction of the drug with the positively

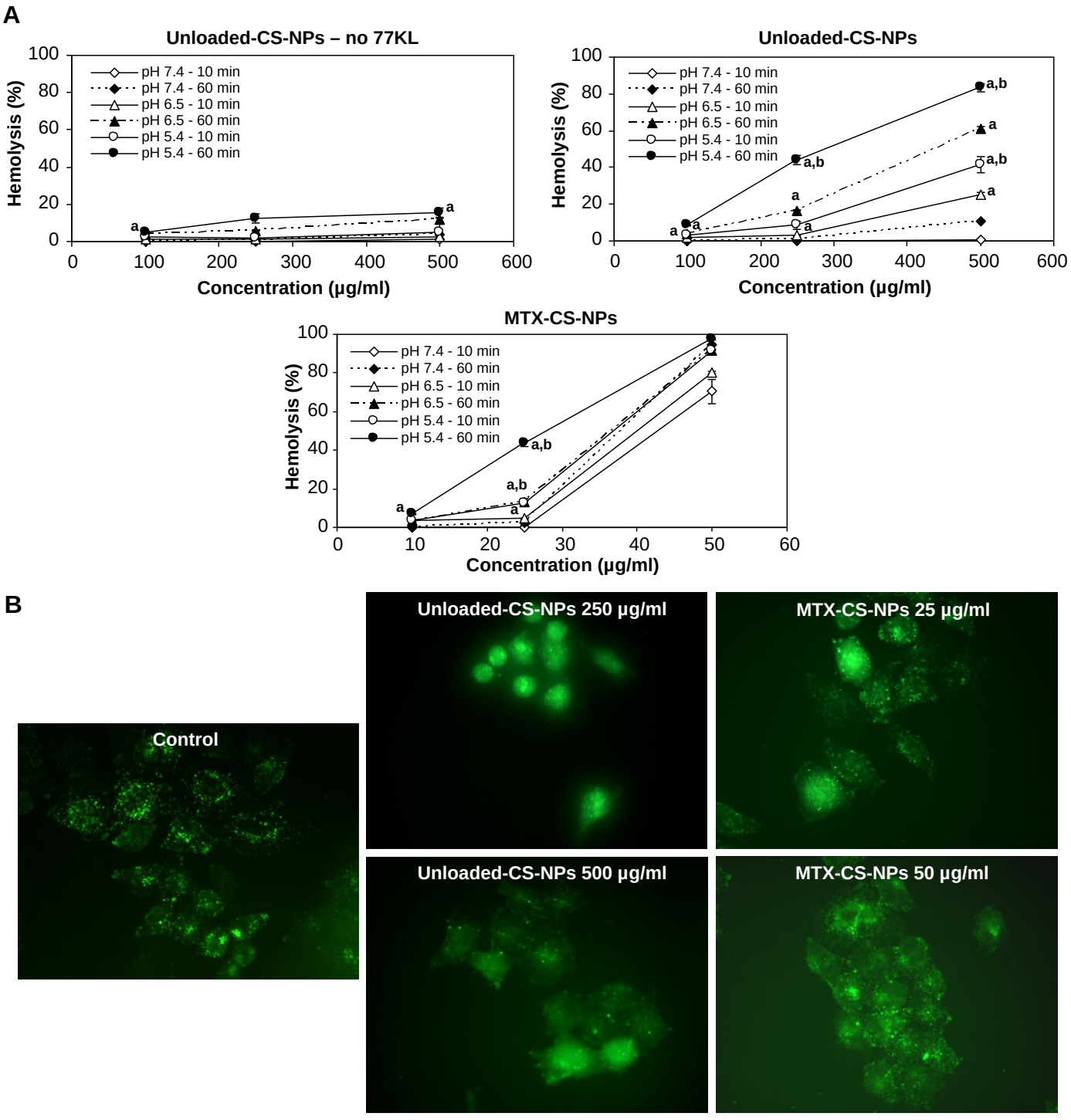



**Fig. 8.** (A) pH-sensitive membrane-lytic activity of unloaded–CS–NPs (with or without 77 KL) and MTX–CS–NPs. NP-induced hemoglobin release from rat erythrocytes was expressed as a function of pH, concentration and incubation time. Results are expressed as the mean ± SE of three independent experiments. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test. [a] significantly different from pH 7.4 ($p < 0.05$) and [b] significantly different from pH 6.5 ($p < 0.05$). (B) Fluorescence microscopy images of HeLa cells showing the subcellular distribution of calcein fluorescence. The cells were treated with 1 mg/ml calcein (control); both 1 mg/ml calcein and 250 or 500 µg/ml of unloaded–CS–NPs or 25 or 50 µg/ml of MTX–CS–NPs. Images were acquired at 3 h after 1 h of uptake.

charged NP surface.The development of a switching carrier in release kinetics, from slow release while circulating to rapid release kinetics once target sites have been reached, is of great importance to avoid the multidrug resistance (MDR) of initially sensitive tumor cells caused by slow release at the site of action [23].

*In vitro* model systems provide a rapid and effective means to assess NPs for a number of toxicological endpoints and mechanism-driven responses. Here, we showed the relative selectivity of the NPs for cancer cells, as the cytotoxic effects of MTX–CS–NPs were significantly lower in the non-tumor control cells (HaCaT). These findings might be related to distinct cell uptake depending on the cell nature or cell proliferative status. Moreover, we clearly showed that the MTX–CS–NPs had greater antitumor effects *in vitro* against the cancer cells than the free MTX. The low antiproliferative effect

of the native drug obtained in our study is very well correlated with the duration of its intracellular retention. Free MTX is found to be pumped off the cell cytosol by P-glycoprotein, which participates in the development of resistance to antifolates [41]. Moreover, the availability of free MTX at its intracellular site of action depends on a passive diffusion mechanism, which can be limited by the high ionization state of MTX at physiological pH. In contrast, the obviously enhanced cytotoxicity of MTX via the nano-sized particles means that there was a significant reverse effect of drug resistance. NPs could reduce the MDR that characterizes many anticancer drugs by a mechanism of cell internalization of the drug by endocytosis [42], by lowering drug efflux from the cells [43] and/or by allowing pH-dependent drug release from the endosomes to cell cytosol [44]. As demonstrated in Section 3.5, the pH-sensitivity of NPs allowed their rapid escape from the endosomal compartment, which may enhance their therapeutic efficiency compared to the native drug. MTT was the most sensitive viability assay for detecting the cytotoxic effects of the NPs, regardless of the cell line. This implies that although NPs primarily exerted toxicity on the mitochondrial compartment after cellular internalization, the plasma membrane remained intact. This finding could be related to the mechanism of action of MTX. MTX is a potent inhibitor of the enzyme dihydrofolate reductase, which plays a key role in the synthesis of DNA precursors and cell growth [10]. Therefore, MTX is a chemotherapeutic agent that mainly inhibits cell proliferation, which justifies the lack of LDH release to the cell cytosol due to membrane rupture (late stage of cell death). We also showed that unloaded–CS–NPs reduced cell viability, especially in HeLa cells. This is most likely due to the combined effect of the free amines of the polymer at the NP surface and the cytotoxic potential of the surfactant by itself. Finally, although our overall results were in line with a previous report [10] and showed that MCF-7 cells displayed resistance to MTX, we also proved the greater activity of MTX–CS–NPs against this cell line.

The cytotoxic activity of pH-sensitive NPs was also investigated to test their feasibility in targeting acidic tumor extracellular $pH_e$. Although free MTX had no enhanced effect in an acidic environment, the MTX loaded into the NPs showed pronounced cytotoxicity at low pH, which might be attributed to the accelerated release of MTX triggered by pH. Moreover, the increased cytotoxicity at pH 6.6 may be due to the combined effect of increased protonation of 77 KL and CS groups on the NP surface at tumor pH, which increases binding to the cell membrane, leading to greater internalization and, thus, greater NP cytotoxicity. These findings support the idea that the MTX–CS–NPs truly discriminate the small difference between the physiological and tumor $pH_e$ by destabilization and the release rate [23].

After we had shown that MTX–CS–NPs exerted cytotoxic effects on the proliferation of cancer cells in a typically dose-dependent manner, we examined whether the induction of apoptosis, LMP or alterations in the normal cell cycle were the possible molecular mechanisms involved in the antitumor activity of NPs in comparison to the free drug. Although the treatment of cancer cells with free MTX significantly inhibited the cell cycle in the S-phase, these cells were essentially more resistant to free MTX-induced apoptosis. In contrast, MTX–CS–NPs induced more significant signs of apoptosis, increased the reduction in cell proliferation and also arrested the cell cycle in the S-phase. Altogether, these data indicated that free MTX acted mainly as a cytostatic drug on HeLa and MCF-7 cancer cells, suppressing DNA synthesis and cellular growth, whereas the MTX loaded into CS-NPs induced both cytostatic and cytotoxic effects [45,46]. Finally, we showed that the LMP effect participated slightly in the mechanism involved in MTX–CS–NPs toxicity in HeLa cells, but not in MCF-7 cells. The LMP induced in HeLa cells by high concentrations of NPs suggests that the lysosomal damage might play some role in the resulting apoptotic effects of MTX–CS–NPs. It was reported that the LMP effect leads to the translocation of lysosomal hydrolases to the rest of the cell, which frequently mediates apoptosis by inducing mitochondrial structural dysfunction [46]. These results indicated that the damage of lisosomal integrity and the early mitochondrial injury (corroborated by the higher sensitivity of MTT assay for detecting NP cytotoxicity) might also support the greater susceptibility of HeLa cells to the toxic effects of MTX.

The assessment of the hemocompatibility of a nanocarrier is indispensable for frequent intravenous dosing. The incorporation of MTX into the NPs was found to increase their hemolytic potential. However, when tested under the same conditions as the NP formulations, free MTX did not induce any undesirable response. Therefore, the main source of hemolysis might be the effects that the drug moiety produces on the NP surface or when encapsulated on it. Indeed, it was recently reported that MTX incorporated in liposomal formulations displayed higher hemoreactivity than its free solution [47]. The agglutination induced by the NPs at the highest concentration tested may be caused by increased binding of the NPs to the erythrocyte membrane, which might deformed cells and hence decrease the repulsion among them [48].

In our previous study [17], we showed the high efficiency of the amphiphile 77 KL at disrupting cell membranes within the pH range characteristic of late endosomes. Therefore, the rationale behind assessing the membrane-lytic behavior of the NPs was to establish the ability of NPs containing this amphiphile to promote triggered release of entrapped molecules in a pH-dependent fashion. The enhanced hemolysis induced by NPs at acidic pH might be due to the increasing protonation state of the carboxylic group of 77 KL in this condition. The protonation of the 77 KL molecule makes it non-ionic and enhances its hydrophobicity, which would increase binding to the membrane and, thus, its lysis. The rather unexpected greater membrane-lytic activity of MTX–CS–NPs could be attributed to a competitive effect between MTX and 77 KL molecules for binding to the positive NP surface. Both structures were negative charged and, thus, MTX molecules could induce faster release of 77 KL molecules from the NP surface, which would result in increased hemolysis.

The membrane-lytic activity of the NPs in an acidic environment might facilitate escape from the endocytotic pathway to lysosomes, and allow efficient intracellular drug delivery to the cell cytosol. To corroborate this pH-sensitive activity of the NPs at intracellular level, we used calcein as an endosomal tracer molecule, which is internalized by the cell through endocytosis and is used to monitor the stability of endosomes following NP uptake [35]. In the absence of NPs, endososomal compartmentalization of calcein was observed, which indicates that the endosome membranes were not damaged [35]. In contrast, the CS-NPs induced efficient release of endocytosed material into the cytoplasm. On the basis of these experiments, we conclude that the NPs deliver calcein to the cytosol of cells by co-endocytosis of this dye and NPs, followed by NP-induced disruption of endosomes and escape of the dye into the cytosol. The cellular uptake of CS-NPs seems to occur by adsorptive endocytosis, as the uptake of similar NPs was previously reported to occur predominantly by adsorptive endocytosis in A549 cells [49] as well as in the HeLa cell line [15].

## 5. Conclusions

In this study, MTX-loaded CS-NPs have been successfully formulated as an intracellular delivery system for improved antitumor activity. The inclusion of the surfactant 77 KL on the NP formulations clearly gives them pH-sensitive membrane-lytic behavior. This facilitates cytosolic delivery of the fluorescent endosomal tracer calcein, and consequently of the encapsulated drug, through

endosomal membrane lysis. Furthermore, the NPs showed high encapsulation efficiency, accelerated release of MTX at a decreasing pH from 7.4 to 5.4, acceptable hemocompatibility and enhanced cytotoxicity on HeLa and MCF-7 tumor cell lines in comparison to free MTX. Another advantage of MTX–CS–NPs was found to be their greater cytotoxic activity around tumor extracellular $pH_e$ compared to that at physiological pH. The combination of the physicochemical characteristics and pH-sensitivity of the NPs resulted in superior cytotoxicity of the entrapped drug, together with generally greater cell cycle arrest and a higher apoptotic response. Based on the overall results, the combined mechanisms of pH-triggered release and cytotoxicity, together with the specific ability to lyse the endosomal membrane after cell internalization, makes MTX–CS–NPs containing the lysine-based surfactant 77 KL a beneficial approach that provides increased anticancer efficacy at the tumor site. However, further *in vivo* studies must be conducted in this field to prove the hypothesis.

## Conflict of interest statement

The authors state that they have no conflict of interest.

## Acknowledgments

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# DISCUSIÓN

Los tensioactivos son moléculas con carácter anfifílo con propiedades fisicoquímicas que les confieren múltiples aplicaciones en la industria farmacéutica y cosmética, además de ser también utilizados en diferentes campos de la tecnología y la investigación. Características de los tensioactivos como su biodegradabilidad, biocompatibilidad y baja toxicidad constituyen propiedades muy importantes para sus innumerables aplicaciones. En la búsqueda de compuestos tensioactivos que presenten estas características y al mismo tiempo conserven las propiedades fisicoquímicas que tienen los tensioactivos comerciales utilizados actualmente, nuestro grupo colabora, desde hace años, en el desarrollo de tensioactivos derivados de aminoácidos, que constituyen una alternativa a los tensioactivos sintéticos convencionales (Infante y col., 1997, 2001; Pérez y col., 2002b; Morán y col., 2004a, 2004b; Colomer y col., 2011).

Entre los compuestos derivados de aminoácidos, este trabajo se ha centrado en los tensioactivos derivados de lisina con carácter tanto aniónico como catiónico. Estudios previos *in vitro* realizados en nuestro laboratorio han demostrado la multifuncionalidad, biodegradabilidad y la baja ecotoxicidad de esta clase de tensioactivos (Sánchez y col., 2007b; Pérez y col., 2009; Colomer y col., 2012), y en particular, su baja toxicidad en diversos modelos celulares (Sánchez y col., 2004, 2006a, 2006b; Pérez y col., 2009; Colomer y col., 2011, 2012). A pesar de que estos estudios avalan la biocompatibilidad y la baja toxicidad de los tensioactivos derivados de lisina, el conocimiento actual relativo a la acción de estos compuestos sobre las membranas celulares en función del pH del medio es escaso. Es importante señalar que los tensioactivos que además de biocompatibles, poseen propiedades de interacción con membranas celulares dependientes del pH, pueden constituir potenciales excipientes bioactivos para el desarrollo de vehículos selectivos destinados a la liberación intracelular de moléculas biológicamente activas. En base a esta premisa, la hipótesis de partida de esta Tesis ha sido postular que los tensioactivos aniónicos y catiónicos derivados de lisina también pueden presentar, en función de su estructura química y características fisicoquímicas, propiedades líticas de membrana variables con el pH del medio y, por lo tanto, pueden ser interesantes como excipientes bioactivos en vehículos nanoestructurados destinados a la liberación intracelular de fármacos en órganos o células dianas. En este sentido, el presente trabajo se ha centrado en establecer, en una primera fase, las propiedades de interacción con membranas celulares, en función del pH del medio, de tensioactivos aniónicos (**Artículo 1**) y catiónicos (**Artículos 2 y 3**) derivados del aminoácido lisina. Posteriormente se han llevado a cabo estudios de citotoxicidad de los tensioactivos aniónicos en diferentes líneas celulares, como propuesta de un modelo predictivo para evaluar los efectos tóxicos potenciales de nuevos excipientes bioactivos desarrollados para aplicaciones farmacéuticas (**Artículo 4**). Finalmente, se han aplicado los tensioactivos previamente caracterizados al desarrollo de sistemas nanoestructurados. Se han formulado nanovesículas

lipídicas basadas en los tensioactivos catiónicos (**Artículos 5 y 6**) y nanopartículas poliméricas basadas en el tensioactivo aniónico 77KL (**Artículo 7**), y en particular, se ha estudiado su toxicidad y comportamiento intracelular *in vitro*.

Las aplicaciones biomédicas de compuestos con propiedades bioactivas requieren un conocimiento previo de sus características y mecanismos de interacción con las membranas celulares. Los tensioactivos pueden interaccionar con la bicapa lipídica provocando un efecto tóxico o beneficioso para el uso propuesto, en función de sus propiedades estructurales, fisicoquímicas y biológicas, y/o de su concentración y modo de aplicación (compuesto en solución o incluido en un vehículo nanoestructurado). En la búsqueda de compuestos biocompatibles y con baja toxicidad, pero con actividad bioactiva específica que les pueda conferir aplicaciones biomédicas especiales, se evidencia el estudio de los tensioactivos derivados del aminoácido lisina realizado en el presente trabajo. Estos compuestos son menos tóxicos, biodegradables, más respetuosos con el medio ambiente, multifuncionales y su síntesis se basa en el uso de materias primas renovables (Infante y col., 1997; Pérez y col., 2002; Morán y col., 2004a). De manera conjunta, estas características plantean vías de investigación de gran interés en el campo farmacéutico y justifican los objetivos trazados en esta Tesis.

En este trabajo se han estudiado dos grupos de tensioactivos derivados del aminoácido lisina: una familia de tensioactivos aniónicos de doble cadena derivados de la $N^{\alpha},N^{\varepsilon}$ -dioctanoil lisina (77K) con diferente contraión en su estructura (dos contraiones orgánicos y voluminosos: lisina y tris; y tres inorgánicos y pequeños: sodio, litio y potasio); y un grupo de tensioactivos catiónicos de cadena simples derivados de la $N^{\varepsilon}$ -acil lisina (con cadena alquílica de 14 o 16 átomos de carbono) o $N^{\alpha}$ -acil lisina (con cadena alquílica de 14 átomos de carbono). Estos tensioactivos se sintetizaron con anterioridad a la realización de esta Tesis, conforme el procedimiento establecido por Vives y col. (1999) y Sánchez y col. (2006a) para los tensioactivos aniónicos, y por Pérez y col. (2009) y Colomer y col. (2011) para los tensioactivos catiónicos.

La lisis celular provocada por los tensioactivos es un proceso de gran importancia práctica. Por eso, uno de los requisitos fundamentales para el desarrollo de tensioactivos con potencial aplicación en sistemas farmacéuticos y biológicos es que presenten una actividad lítica celular muy baja en condiciones fisiológicas (Ryoo y col., 2005). A pesar de que muchos grupos de investigación han realizado diferentes estudios para esclarecer los procesos y mecanismos implicados en la lisis celular (Hägerstrand y Isomaa, 1991; Galembeck y col., 1998; Vives y col., 1999), aún no son del todo conocidos, pero se sabe que influyen muchos factores como la estructura del tensioactivo, la concentración y el tipo de agregado (Berardesca y Distante, 1994; Effendy y Maibach, 1995). En cuanto a los tensioactivos derivados de lisina, se han

propuesto varias características que, combinadas o por separado, podrían estar implicadas en sus propiedades de interacción con las membranas celulares, como p. ej. la CMC, la estructura de la cadena alquílica o los grupos funcionales (Vinardell e Infante, 1999; Pérez y col., 2009; Colomer y col., 2011). En ese sentido, y con el objetivo de profundizar en el conocimiento de la relación estructura-interacción con la membrana, se ha estudiado la influencia de la naturaleza de los contraiones incorporados a la estructura de los tensioactivos aniónicos, y también de la posición de la carga y de la longitud de la cadena alquílica de los tensioactivos catiónicos, en su potencial efecto sobre la membrana celular. Se han evaluado los efectos de la concentración del compuesto, del tiempo de incubación y, especialmente, del pH del medio. Es evidente que una mejor comprensión del fenómeno de lisis celular y de los mecanismos implicados en la interacción de los tensioactivos con las membranas celulares, puede ayudar y guiar en el desarrollo de nuevos tensioactivos que sean más efectivos y menos tóxicos, con el fin de ampliar sus posibles aplicaciones.

En todos los estudios de las propiedades de interacción de los tensioactivos con las membranas celulares, comprendidos en el primer bloque de objetivos de esta Tesis, se ha elegido el eritrocito como modelo de membrana y el ensayo de hemólisis como método de valoración. Por ser una célula que carece de orgánulos internos, se evitan las interferencias que estos podrían suponer en la interpretación de los resultados. Además, constituye el modelo más utilizado para este tipo de estudios debido a su simplicidad y fácil obtención (Svetina y col., 2004). Los eritrocitos también tienen muchas funciones en común con células más especializadas y, por lo tanto, los resultados obtenidos utilizando su membrana como modelo de membrana endosomal pueden ser buenos indicadores de la actividad lítica de los tensioactivos en condiciones fisiológicas (Kleszczynska y col., 2005; Suwalsky y col., 2009). A su vez, el ensayo de hemólisis constituye un buen ejemplo de ensayo que permite evaluar los daños provocados por los compuestos químicos sobre las membranas celulares. Es un método sencillo y específico para valorar tensioactivos o formulaciones que los contengan (Pape y col., 1987; Lewis, 1994), y además sigue un protocolo estandarizado: el protocolo *INVITTOX núm. 99* (Lewis, 1994). De ese modo, el ensayo de hemólisis ha sido seleccionado en esta Tesis como un modelo para valorar la integridad de las membranas endosomales, ya que se ha demostrado previamente una buena correlación entre la actividad hemolítica y la lisis de la membrana endosomal inducida por compuestos bioactivos (Plank y col., 1994).

El primer bloque de objetivos de este trabajo ha consistido en establecer las propiedades de interacción de los tensioactivos con membranas celulares así como los mecanismos involucrados en este proceso. Como primer objetivo específico, se ha postulado evaluar el efecto del pH en la actividad lítica de membrana de los tensioactivos. La comprensión de este efecto es un factor

clave en el desarrollo de sistemas nanoestructurados con capacidad de liberar de forma específica su contenido encapsulado en los compartimentos intracelulares. En las últimas dos décadas, se ha realizado una investigación extensiva centrada en la búsqueda de compuestos bioactivos que tengan la habilidad de desestabilizar la membrana de los endosomas después del proceso de internalización celular (Christie y Grainger, 2003). Especialmente, se han realizado estudios exhaustivos de los polímeros y péptidos en relación a su actividad pH-dependiente y a su potencial aplicación en vehículos para la liberación intracelular de fármacos y material génico (Stayton y col., 2000; Kyriakides y col., 2002; Jones y col., 2003; Kusonwiriyawong y col., 2003; Yessine y col., 2003; Chen y col., 2005, 2008, 2009; Lee y col., 2010; Seo y Kim, 2010). Los tensioactivos también se han estudiado en cuanto a su actividad lítica pH-sensible, pero en menor extensión, ya que existen menos publicaciones en la literatura (Liang y Hughes, 1998; Chen y col., 2003; Asokan y Cho, 2005; Wang y col., 2007; Colomer y col., 2012).

La liberación eficiente de fármacos captados por endocitosis en el citoplasma representa un reto para la liberación intracelular de biomoléculas. Después de entrar en las células por endocitosis pasiva o activa, los sistemas de liberación macromoleculares se dirigen a los lisosomas, donde los fármacos frágiles (p. ej. DNA y proteínas) pueden ser degradados por las enzimas lisosomales (Mellman, 1996). Asimismo, incluso si la carga de la partícula no se degrada, la compartimentación en los endosomas o lisosomas puede impedir que los fármacos lleguen a sus sitios de acción específicos a niveles subcelulares (Sheff, 2004). Por lo tanto, teniendo en cuenta la labilidad de las biomoléculas y la probabilidad de su compartimentación en los endosomas, es necesario desarrollar sistemas de transporte efectivos capaces de liberar su contenido en el citoplasma celular de manera eficiente. Como los endosomas presentan un pH ligeramente ácido (5,0 – 6,5) (Moore y col., 2008), una estrategia para alcanzar ese objetivo consiste en utilizar vehículos nanoestructurados con propiedades pH-sensibles como transportadores intracelulares. Estos vehículos tendrían la capacidad de romper la membrana de los endosomas en condiciones levemente ácidas, evitando así, el tránsito de los sistemas de liberación macromoleculares hasta el lisosoma (Christie y Grainger, 2003; Yessine y col., 2003).

En primer lugar, se han estudiado los tensioactivos aniónicos (**Artículo 1**), y los resultados obtenidos han mostrado que todos los compuestos tienen actividad lítica pH-sensible independientemente del tipo de contraión. El incremento de la actividad hemolítica en función del pH se ha estudiado a dos concentraciones: una por debajo de la $CH_{50}$ (concentración que provoca el 50% de hemólisis) y otra en el rango de la $CH_{50}$. Se ha demostrado que a concentraciones inferiores a la $CH_{50}$, los tensioactivos tienen una actividad lítica de membrana específica a los valores de pH característicos de los compartimientos endosomales. En condiciones fisiológicas no se ha observado

hemólisis; sin embargo, a pH 6,5 (característico de los endosomas primarios) se ha observado un pequeño aumento en el índice de hemólisis, mientras que a pH 5,4 (característico de los endosomas secundarios) la actividad hemolítica ha aumentado de forma significativa, sugiriendo que estos tensioactivos aniónicos tienen la habilidad de romper, especialmente, la membrana de los endosomas secundarios de forma eficiente y especifica. Por otro lado, a concentraciones en el rango de la $CH_{50}$, estos tensioactivos también han demostrado, de manera general, valores de hemólisis significativos a pH fisiológico. Este hecho hace evidente que su actividad lítica pH-dependiente se mantiene hasta una concentración límite, por encima de la cual los efectos tóxicos de estos compuestos pasan a ser significativos y su actividad lítica de membrana deja de ser específica a valores de pH más ácidos. Sin embargo, se puede resaltar que el tensioactivo 77KL, que a concentraciones más elevadas ha mantenido su actividad pH-sensible, resulta bastante eficiente en romper la membrana al pH de los endosomas primarios (pH 6,5). El 77KL y el 77KT han sido los menos hemolíticos a pH fisiológico, mientras que a pH 5,4 el 77KL fue el compuesto más activo con menor valor de $CH_{50}$. Además, es importante señalar que al hacer una comparación de los valores de $CH_{50}$ con los valores de la CMC, se observa que la hemólisis ocurre a concentraciones inferiores a la CMC a todos los pH estudiados, indicando que la presencia de micelas no es un prerrequisito para la lisis celular. Es decir, el tensioactivo en forma de monómero es responsable de inducir la desestabilización de las membranas. Yue y col. (2005) describen que los polímeros derivados del aminoácido lisina pierden el comportamiento pH-sensible al formar micelas estables, lo que nos lleva a suponer que la estructura monomérica del compuesto es importante en la actividad hemolítica pH-dependiente. Los tensioactivos también se han valorado en cuanto a su citotoxicidad a las concentraciones propuestas como pH-sensibles, y se ha observado que especialmente el 77KK y el 77KL inducen efectos tóxicos leves a estas concentraciones.

El hecho de que el contraión no influye directamente en la actividad lítica pH-dependiente, indica que la variación del potencial hemolítico de los tensioactivos en función del pH es debido a un cambio en el balance hidrófilo/hidrófobo de su molécula. Es evidente, que el grupo carboxilo (grupo cargado negativamente) sufre un cambio de protonación con la variación del pH, lo que podría explicar el aumento en el potencial lítico de membrana de estos compuestos a medida que se disminuye el pH. Se ha determinado el p*K*a del tensioactivo 77KL, que se ha aplicado al desarrollo de nanopartículas poliméricas en combinación con el quitosano (**Artículo 7**). Se obtuvo un valor de p*K*a = 6,6, lo que corrobora la hipótesis de que el grupo carboxilo del aminoácido lisina sufre un aumento en su p*K*a una vez incorporado en la molécula del tensioactivo (p. ej. para el 77KL, el p*K*a original del ácido carboxílico ha pasado de 2,2 a 6,6). Por lo tanto, la protonación del grupo carboxilo a pH ácido, con el consecuente aumento de la hidrofobicidad y anfifilicidad de la molécula (Wang y col., 2007), parece ser un factor clave en el incremento de la actividad lítica, especialmente, a pH 5,4.

A continuación, se ha procedido al estudio de la actividad lítica dependiente del pH de los tensioactivos catiónicos (**Artículo 2**). Se ha observado que la actividad pH-sensible depende directamente de la posición de la carga catiónica. Sólo los compuestos con la carga positiva en el grupo α-amino de la lisina tienen la capacidad de cambiar su potencia hemolítica en función del pH del medio. A concentraciones sublíticas, aproximadamente siete veces por debajo de la $CH_{50}$, los tensioactivos MKM y PKM son no hemolíticos en condiciones fisiológicas, mientras que en el rango de pH característico de los endosomas, presentan una actividad lítica significativamente superior. El MKM es el más activo tanto a pH 6,5 como a pH 5,4, demostrando que el aumento en la hidrofobicidad del tensioactivo se correlaciona inversamente con su potencial lítico a valores de pH que simulan las condiciones endosomales. Además, tal y como se ha observado con los tensioactivos aniónicos, los compuestos catiónicos también pierden su actividad lítica pH-sensible a concentraciones en el rango de la $CH_{50}$. Por el contrario, el tensioactivo MLM, con la carga positiva en el grupo ε-amino de la lisina, no ha presentado actividad hemolítica variable en función del pH. Por lo tanto, a la vista de los resultados de hemólisis obtenidos a los diferentes valores de pH, cabe destacar que la actividad lítica pH-sensible se puede modificar especialmente por la variación de la posición de la carga catiónica, y en menor medida, por la alteración de la longitud de la cadena alquílica. La especificidad de los tensioactivos MKM y PKM a los pH endosomales, les confiere propiedades de gran interés en el campo de la nanotecnología que, a su vez, sugieren su aplicación en vehículos para liberación intracelular de biomoléculas, tal como se ha demostrado tras su incorporación en sistemas nanovesiculares (**Artículo 6**).

Los resultados obtenidos en este estudio han evidenciado que el p*K*a del tensioactivo y, por lo tanto, su índice de protonación a determinado pH, está directamente relacionado con su actividad lítica pH-dependiente. Los compuestos MKM y PKM tienen valores de p*K*a = 5,3 y 4,5, respectivamente, lo que corrobora su protonación superior a pH ácidos y, por lo tanto, una actividad lítica superior en dichas condiciones. Por el contrario, el MLM tiene p*K*a = 8,1, lo que determina que esté casi completamente protonado en todo el rango de pH estudiado. A su vez, dicha protonación, aproximadamente constante, justifica su actividad hemolítica independiente del pH.

Se ha demostrado una mejor cinética de actividad hemolítica a pH 6,5 y 5,4, tanto para los tensioactivos aniónicos (**Artículo 1**), como para los catiónicos con la carga positiva en el grupo α-amino de la lisina (**Artículo 2**). Pack y col. (2005) mostraron que las macromoléculas captadas por endocitosis son transportadas de los endosomas primarios a los lisosomas en varias horas. Por lo tanto, la actividad hemolítica tiempo-dependiente observada con los tensioactivos derivados de lisina, sugiere que estos compuestos tienen la capacidad de desestabilizar la membrana de los endosomas antes de la evolución vesicular de endosomas a lisosomas, una característica que

condiciona sus potenciales aplicaciones en vehículos para la liberación intracelular de fármacos.

Por último, se ha comparado la actividad hemolítica de los tensioactivos derivados de lisina con la de los tensioactivos convencionales disponibles comercialmente. Tanto el tensioactivo aniónico SDS (**Artículo 1**) como el catiónico HTAB (**Artículo 2**) no han mostrado actividad lítica dependiente del pH. Estos resultados corroboran y acentúan las ventajas de los tensioactivos derivados de lisina estudiados en esta Tesis. Además de biocompatibles y biodegradables, se ha demostrado que presentan propiedades específicas de interacción con las membranas celulares, lo que les confiere un abanico superior de potenciales aplicaciones biomédicas, tal y como también se ha propuesto en esta Tesis (**Artículos 5, 6 y 7**).

Generalmente, se asume que a concentraciones suficientemente elevadas, el principal mecanismo de lisis celular es la solubilización de los lípidos y proteínas de la membrana debido a la formación de micelas mixtas (Jones, 1999). Por el contrario, a concentraciones bajas, el principal resultado de la interacción de los tensioactivos con la membrana, es un cambio en su permeabilidad, que provoca penetración de agua, hinchazón de la célula y lisis osmótica de la membrana (Bielawski, 1990). Como en este trabajo se ha visto que los tensioactivos aniónicos (**Artículo 1**) inducen hemólisis a concentraciones inferiores a la CMC a todos los pH estudiados y, por lo tanto, a concentraciones no muy elevadas, se ha asumido que la lisis de la membrana celular podría ocurrir a través de un mecanismo osmótico. Esta hipótesis se ha corroborado; sin embargo, se ha observado que a pH 5,4 la protección osmótica de la hemólisis es inferior, lo que indica que la gran potencia hemolítica de los compuestos a este pH puede estar induciendo la solubilización parcial de la membrana. En cambio, los experimentos de protección osmótica han mostrado apenas inhibición parcial de la hemólisis a todos los pH estudiados tras la incubación con los tensioactivos catiónicos (**Artículo 2**). Por lo tanto, se asume que el mecanismo osmótico no es el único proceso involucrado en la lisis de la membrana, y que existe algún efecto de interacción más complejo por parte de los compuestos catiónicos. Algunos de estos mecanismos de interacción se han dilucidado en el estudio realizado sobre los aspectos más específicos de la interacción de los tensioactivos catiónicos con los lípidos y las proteínas de la membrana (**Artículo 3**).

Las alteraciones estructurales en la membrana de los eritrocitos, como etapa previa a la lisis celular, corroboran que los tensioactivos se intercalan en la bicapa lipídica. Los tensioactivos aniónicos (**Artículo 1**) han inducido la formación de formas crenadas (equinocitos) a pH fisiológico, mientras que los compuestos catiónicos (**Artículos 2 y 3**) han provocado la formación de estomatocitos, lo que está de acuerdo con la hipótesis de la bicapa lipídica (Sheetz y Singer, 1974). En cambio, a pH 6,5 se ha observado que todos los

tensioactivos han inducido la formación de estomatocitos, lo que indica una interacción de los compuestos con la monocapa interna de la membrana, mientras que a pH 5,4 se han formado esferocitos, como resultado de una mayor incorporación en ambas monocapas de la membrana (Vermehren y Hansen, 1998). La mayor penetración de los tensioactivos en la membrana hasta la monocapa más interna a pH ácidos, parece ser un condicionante importante del aumento de la actividad hemolítica. Por lo tanto, el grado de perturbación de la bicapa fosfolipídica y de incorporación del tensioactivo se considera un factor clave en la potencia hemolítica de un tensioactivo.

En la búsqueda de una mejor comprensión de los mecanismos involucrados en la actividad pH-dependiente de los tensioactivos catiónicos, se han evaluado sus propiedades de interacción con biomembranas y las alteraciones en la bicapa lipídica tras el tratamiento con cada compuesto a diferentes valores de pH (**Artículo 3**). Se ha buscado especialmente entender como las características estructurales de los diferentes compuestos pueden afectar su interacción con la membrana y estar relacionadas con la actividad lítica pH-dependiente. Se ha partido de la hipótesis que, a concentraciones sublíticas, la interacción de sustancias bioactivas con las membranas podría dar lugar a alteraciones moleculares significativas, que a su vez, podrían estar directamente relacionadas con la lisis selectiva en función del pH del medio.

El papel que juegan los tensioactivos en la protección frente a la hemólisis hipotónica se puede utilizar como un índice de las interacciones con la membrana celular. Los tensioactivos tienden a interaccionar con la membrana del eritrocito de manera bifásica, es decir, protegiendo frente a la hemólisis hipotónica a concentraciones bajas y provocando hemólisis a concentraciones elevadas (Isomaa y col., 1986; Isomaa y Hägerstrand, 1988; Galembeck y col., 1998; Sánchez y col., 2007a). Este trabajo ha demostrado que los tensioactivos catiónicos tienen diferente perfil antihemolítico dependiendo de la posición de la carga positiva. El MLM, con la carga en el grupo ε-amino de la lisina, tiene una actividad que se correlaciona con la tendencia general de los tensioactivos, tal y como se ha demostrado en los estudios mencionados anteriormente, mientras que los tensioactivos MKM y PKM mantienen la actividad antihemolítica en un amplio rango de concentraciones. Además, se ha demostrado que la potencia antihemolítica se correlaciona inversamente con la hidrofobicidad del tensioactivo y directamente con el volumen hemolítico crítico de las células. Se ha postulado que cuando los tensioactivos se intercalan en la membrana celular, incrementan el área de la membrana respecto al volumen celular, permitiendo a la célula alcanzar un volumen hemolítico crítico restrictivo que le permitiría alcanzar un gran volumen antes de lisarse en medio hipotónico (Machleidt y col., 1972). En resumen, estos estudios han confirmado que los tensioactivos tienen la capacidad de incorporarse en la membrana a concentraciones sublíticas (en el

mismo rango de aquellas que han demostrado actividad lítica pH-sensible) y, además, han corroborado que la posición de la carga catiónica afecta el tipo de interacción de los compuestos con la bicapa lipídica.

Para explicar la posible reorganización y movilidad de los lípidos de la membrana celular en función del tensioactivo y pH del medio, se han estudiado los cambios en la fluidez de la bicapa lipídica, parámetro este que nos informa de la estructura y el estado de la membrana (Shinitzky y Barenholz, 1978; Xia y Onyuksel, 200). Se han seleccionado dos sondas fluorescentes que se sitúan en diferentes regiones de la bicapa: la DPH y la TMA-DPH. De estos experimentos, cabe destacar que sólo los tensioactivos MKM y PKM, con la carga catiónica en el grupo α-amino de la lisina, han aumentado significativamente la fluidez de la membrana en condiciones levemente ácidas, tanto en su extremo polar como en su porción interna. De hecho, el MKM ha sido efectivo en desorganizar la membrana a pH 6,5 y 5,4, mientras que el PKM sólo ha afectado la organización de los lípidos al pH de los endosomas secundarios (pH 5,4). Estos resultados muestran el mecanismo de la actividad pH-dependiente de estos compuestos, y además corroboran los resultados previamente discutidos: el MKM es mucho más potente en romper la membrana a pH 6,5 y 5,4, mientras que el PKM sólo aumenta significativamente su actividad lítica a pH 5,4 (**Artículo 2**). Dando continuidad al estudio de los mecanismos involucrados en la actividad lítica pH-sensible, se ha evaluado la influencia de los tensioactivos sobre el perfil proteico de la membrana. En contra de sus efectos sobre los lípidos, se ha demostrado que todos estos compuestos interaccionan con algunas proteínas de la membrana (banda 3, banda 6 y anquirina) independientemente de la posición de la carga catiónica, induciendo pérdidas significativas de las mismas. Este hecho indica que la interacción simultánea con los lípidos y las proteínas de la bicapa fosfolipídica está involucrada en la actividad lítica pH-sensible de estos tensioactivos. Además, estas alteraciones en las proteínas de membrana y en la estructura de los lípidos suelen inducir cambios en la superficie externa de las células, lo que a su vez corrobora las alteraciones morfológicas demostradas en los estudios de microscopía electrónica de barrido, tal y como se ha discutido anteriormente.

Basado en los resultados de los **Artículos 2** y **3**, se puede concluir que la actividad lítica pH-dependiente de los tensioactivos catíonicos derivados de lisina depende de características estructurales específicas, especialmente de la presencia de la carga positiva en el grupo α-amino de la lisina. A la vista de los resultados obtenidos, se puede postular que esta actividad específica puede ser explicada por el aumento del estado de protonación de la molécula del tensioactivo a medida que disminuye el pH. Además, se puede evidenciar por alteraciones en las propiedades normales de la membrana en condiciones levemente ácidas, como la desorganización de los lípidos, con el consecuente aumento en la fluidez de la bicapa lipídica, y la pérdida significativa de

proteínas estructurales de la membrana. A su vez, estos resultados corroboran la hipótesis de que la actividad lítica pH-sensible existe debido a mecanismos específicos de interacción con la bicapa lipídica en función del pH del medio.

Del mismo modo que se han valorado los mecanismos de interacción de los tensioactivos catiónicos con las membranas celulares, los tensioactivos aniónicos estudiados en esta Tesis fueron previamente evaluados por nuestro grupo de investigación en cuanto a algunos de los posibles procesos involucrados en su proceso de incorporación en la membrana y posterior lisis celular (Martínez y col., 2007; Sánchez y col., 2007a). Se estudió el efecto de estos compuestos y la posible influencia de su contraión en la protección frente a la hemólisis hipotónica (antihemólisis) y las alteraciones en la fluidez de membrana. No se observó ninguna relación entre el tipo de contraión y el grado de protección inducido por los tensioactivos en frente de la hemólisis hipotónica, mientras que las variaciones de la potencia antihemolítica no afectaron de modo directo el volumen hemolítico crítico. En los estudios de fluidez de membrana se demostró que sólo los tensioactivos 77KK, 77KT y 77KP fueron capaces de aumentar la fluidez en la región polar de la membrana, mientras que no se observaron efectos significativos en el interior de la bicapa lipídica. En resumen, los autores concluyeron que no se puede establecer ninguna relación entre el tipo de contraión de estos tensioactivos y su actividad antihemolítica o su capacidad de inducir cambios en la fluidez de la membrana y, por lo tanto, estos compuestos presentan diferentes mecanismos de interacción con la membrana que no se pueden atribuir únicamente a la presencia de los contraiones. Del mismo modo, en esta Tesis se ha demostrado que el contraión influye sólo en la potencia hemolítica, pero no en el perfil lítico dependiente del pH (**Artículo 1**).

Actualmente, la valoración inicial de los efectos tóxicos de nuevos productos se hace cada vez más utilizando métodos *in vitro*, alternativos al uso de animales y de acuerdo con el principio de las 3Rs. Los métodos basados en cultivos celulares presentan muchas ventajas respeto a los ensayos *in vivo*: ausencia de cuestiones éticas, coste inferior, mayor rapidez de ensayo, posibilidad de ensayar varios compuestos a la vez, etc (Gartoff, 2005). La utilización de líneas celulares establecidas como sistemas *in vitro* es preferible respeto a los cultivos primarios. Las líneas establecidas son homogéneas y de fácil manejo (Benassi y col., 1999; Lee y col., 2000), mientras que las células provenientes de cultivos primarios presentan una variabilidad potencial inherente que hace con que sea más difícil la comparación de los resultados (Guillot, 1992).

Teniendo en cuenta la variabilidad inherente entre las diferentes líneas celulares y su diferente sensibilidad a los compuestos, en esta Tesis se ha propuesto un modelo de ensayo predictivo de la citotoxicidad de tensioactivos

bioactivos con potenciales aplicaciones farmacéuticas (**Artículo 4**). Se ha planteado la utilización de seis líneas celulares, dos tumorales y cuatro no tumorales, y de dos ensayos de citotoxicidad basados en diferentes mecanismos de viabilidad celular. Con esta batería de ensayos se ha corroborado la baja toxicidad de los tensioactivos aniónicos derivados de lisina, previamente estudiados utilizando el tensioactivo comercial SDS como referencia (Sánchez y col., 2004, 2006a). De hecho, los resultados obtenidos en este estudio, de manera conjunta con los obtenidos en el **Artículo 1**, también han apoyado la selección del tensioactivo 77KL para ser incluido como excipiente bioactivo en formulaciones de nanopartículas poliméricas con propiedades pH-dependiente (**Artículo 7**).

La comparación de las curvas dosis-respuesta y de los valores de $CI_{50}$ ha revelado que los tensioactivos presentan distintos niveles de citotoxicidad dependiendo de la línea celular. En general, se han observado efectos citotóxicos menores en las células tumorales HeLa y MCF-7, y en los fibroblastos 3T3, mientras que los queratinocitos NCTC 2544 y los fibroblastos 3T6 fueron las células más sensibles a todos los compuestos estudiados. De hecho, la distinta sensibilidad de las líneas celulares a los tratamientos aplicados podría ser el principal condicionante de las diferentes respuestas citotóxicas, tal y como se ha observado en otros estudios con distintos compuestos (Burlando y col., 2008; Tan y col., 2008; Backorová y col., 2011). La mayor resistencia de las células tumorales se explicaría por las diferencias estructurales y funcionales existentes entre las células sanas y las tumorales, como p. ej., en su actividad metabólica y su composición molecular (Frey y col., 2007).

Ya se ha demostrado que los tensioactivos aniónicos tienen actividad lítica pH-dependiente independientemente del contraión; sin embargo, la presencia de diferentes contraiones influye en la potencia hemolítica (**Artículo 1**). Con el objetivo de profundizar en el conocimiento de la relación estructura-actividad, se ha utilizado el modelo de cribado propuesto en este trabajo para estudiar también la influencia de la naturaleza del contraión en la potencial citotoxicidad de los tensioactivos. En todas las líneas celulares seleccionadas, los tensioactivos con contraiones orgánicos en su estructura (77KK y 77KT) presentaron una tendencia a ser menos citotóxicos. Asimismo, entre los compuestos con contraión inorgánico, el tensioactivo 77KL presentó una citotoxicidad similar o incluso inferior. La baja toxicidad del 77KL, especialmente en las líneas celulares tumorales, sugiere una interacción específica del contraión litio con estas células. En resumen, según estos resultados parece ser que la presencia de diferentes contraiones en las moléculas de tensioactivo influye directamente en su acción citotóxica. En muchos estudios ya se mencionó la influencia del contraión en las propiedades biológicas de los tensioactivos (Kleszczynska y Sarapuk, 1998; Benrraou y col., 2003; Haldar y col., 2005; Morán y col., 2012). Es evidente que el tipo de

contraión afecta, al menos en parte, la capacidad del tensioactivo en interaccionar con los sistemas biológicos.

Tras la evaluación de las propiedades y mecanismos de interacción de los tensioactivos con membranas celulares en función del pH, se ha procedido realizar el segundo bloque de objetivos de esta Tesis: desarrollar sistemas nanoestructurados basados en estos tensioactivos, estudiar su potencial toxicidad *in vitro* y evaluar su efectividad en promover una liberación intracelular eficiente. Para ello, se han desarrollado dos modelos de formulaciones nanoestructuradas: nanovesículas (NVs) lipídicas conteniendo los tensioactivos catiónicos (MKM, PKM o MLM) (**Artículos 5 y 6**) y nanopartículas (NPs) poliméricas conteniendo el tensioactivo aniónico 77KL (**Artículo 7**).

Las NVs catiónicas se han desarrollado con el objetivo de corroborar la potencial aplicación de los tensioactivos catiónicos derivados de lisina como excipientes bioactivos en sistemas nanoestructurados. Se ha utilizado el fosfolípido dimiristoilfosfatidilcolina (DMPC), con o sin la adición de colesterol, como matriz lipídica básica, y en cada formulación se ha incluido un tensioactivo catiónico (MKM, PKM o MLM) (**Artículos 5 y 6**). En primer lugar, se ha procedido al estudio de su potencial aplicación tópica (**Artículo 5**), utilizando células epidérmicas para la valoración de sus potenciales efectos tóxicos. Por otra parte, se ha avanzado en el estudio de la internalización celular de estas NVs y también en los diversos mecanismos que podrían estar involucrados en sus efectos tóxicos, lo que a su vez, ha demostrado su potencial aplicación sistémica (**Artículo 6**).

Inicialmente, se ha valorado la influencia de la inclusión de los tensioactivos en las características fisicoquímicas de las NVs resultantes (**Artículos 5 y 6**). La caracterización fisicoquímica de estos sistemas ha demostrado la formación de NVs estables, con carga neta positiva y diferente distribución de tamaños, y que además presentan una gran incorporación del tensioactivo en su estructura. Las mediciones realizadas por DLS en agua han demostrado que las NVs conteniendo el tensioactivo PKM, con la mayor longitud de la cadena alquílica, tienen un mayor tamaño hidrodinámico. Por el contrario, los análisis por TEM han mostrado que las NVs que contienen los tensioactivos con la carga positiva en el grupo α-amino de la lisina presentan, generalmente, tamaños muy inferiores y morfología de membrana unilamelar, mientras que las NVs con el MLM (carga positiva en el grupo ε-amino de la lisina) tienen membrana multilamelar y mayor tamaño. Se ha demostrado que cada técnica analítica proporciona informaciones distintas, especialmente, en cuanto al tamaño de los nanomateriales (Ojogun y col., 2009; Gao y col., 2010; Mehrotra y col., 2011), lo que evidencia la importancia de la combinación de dos o más métodos de análisis para una caracterización

fisicoquímica completa y fiable (Jones y Grainger, 2009). Al analizar estos resultados, se ha demostrado que las características fisicoquímicas de las NVs dependen, especialmente, de la posición de la carga catiónica del tensioactivo, y en menor extensión, de la longitud de su cadena hidrocarbonada.

La agregación de los sistemas nanoestructurados en soluciones acuosas, especialmente, en aquellas ricas en iones y proteínas como son los medios de cultivo, puede afectar a su toxicidad (Holl, 2009; Horie y col., 2012). Por lo tanto, este efecto se debe evaluar antes de proceder con cualquier valoración toxicológica *in vitro* (Boberhof y David, 2010). En los estudios de agregación de las NVs en las condiciones que simulan un ensayo toxicológico *in vitro*, se ha demostrado una vez más que la posición de la carga catiónica del tensioactivo influye directamente en el comportamiento de los sistemas resultantes (**Artículos 5 y 6**). Los tensioactivos con la carga en el grupo α-amino de la lisina tienen valores de p*K*a más ácidos, lo que disminuye drásticamente su protonación a pH 7,4 (pH del medio de cultivo) y, por lo tanto, reduce la carga neta de la NV y favorece su agregación. En cambio, el MLM tiene un p*K*a = 8,1 y consecuentemente tiene un mayor índice de protonación a pH 7,4, lo que mantiene la carga positiva de las NVs y previene su aglomeración. A la vista de estos resultados, el estado de agregación de las NVs podría ser uno de los condicionantes de su distinto comportamiento intracelular después de la internalización por endocitosis (**Artículo 6**), y también de la distinta sensibilidad de los ensayos de viabilidad celular a los efectos tóxicos de estos sistemas, tal y como se discute a continuación (**Artículos 5 y 6**).

Además de la influencia del estado de agregación de los nanomateriales en su toxicidad subyacente, está ampliamente descrito en la literatura que los métodos utilizados en los procedimientos *in vitro* (p. ej. los colorantes MTT y rojo neutro) pueden influir en la respuesta toxicológica (Worle-Knirsch y col., 2006; Monteiro-Riviere y col., 2009, 2010). A través de mediciones espectrofotométricas, se ha demostrado que estos colorantes interaccionan de modo muy débil con las NVs (**Artículos 5 y 6**). Asimismo, considerando que no se ha observado incremento de la viabilidad celular a medida que se aumentó la concentración (Monteiro-Riviere y col., 2009, 2010), se puede considerar que los ensayos de viabilidad MTT y NRU son adecuados para valoración de la citotoxicidad *in vitro* de las formulaciones de NVs desarrolladas en esta Tesis.

Cuando se desarrolla una nueva formulación para administración tópica, es de gran interés conocer sus potenciales efectos adversos utilizando ensayos de citotoxicidad *in vitro* y células representativas de la piel. En ese sentido, se han elegido líneas celulares de queratinocitos (HaCaT) y fibroblastos (3T3), que son modelos comúnmente utilizados para la valoración de la irritación dérmica (Sánchez y col., 2004), y también monocitos humanos leucémicos (THP-1), considerados sustitutos de células dendríticas cutáneas (Sakaguchi y col.,

2006). Se han observado niveles diversos de citotoxicidad dependiendo de la línea celular, del ensayo de viabilidad y del tensioactivo estudiado. No se ha podido obtener una conclusión clara de cuales células son más sensibles a los efectos tóxicos de las NVs, ya que las respuestas han variado entre las diferentes formulaciones. Sin embargo, estos resultados están de acuerdo con estudios previos (Schöler y col., 2001; Xia y col., 2008), que demostraron que los sistemas nanoestructurados inducen efectos tóxicos variables dependiendo de la línea celular.

La utilización de diferentes ensayos de viabilidad *in vitro* permite comprender algunos mecanismos involucrados en la citotoxicidad de nuevos compuestos y formulaciones. En este trabajo se han utilizado tres ensayos: MTT, NRU y LDH. El MTT detecta cambios en la actividad metabólica de la célula y se correlaciona con la proliferación celular (Venkatesan y col., 2011), mientras que el NRU y el LDH proporcionan información respecto a la integridad de membrana, siendo el primero más específico de la membrana lisosomal (Fröhlich y col., 2012) y el segundo de la membrana plasmática (Yang y col., 2009). El MTT ha sido el ensayo más sensible en detectar los efectos citotóxicos de las NVs formadas con MKM y PKM, lo que indica que estas formulaciones afectan primero el metabolismo celular, mientras que son necesarias concentraciones más elevadas para afectar las membranas lisosomal y plasmática. Además, aquellas formulaciones con el tensioactivo más hidrófobo (PKM), se han mostrado más tóxicas en todas las líneas celulares. Por el contrario, las NVs con el MLM han afectado del mismo modo a la actividad metabólica y a la integridad de las membranas celulares, tal y como se ha demostrado por los valores similares de $CI_{50}$ obtenidos con los tres ensayos de viabilidad. En conjunto, estos resultados demuestran un hallazgo clave de este estudio: los efectos tóxicos de las NVs están influidos directamente por la posición de la carga catiónica del tensioactivo y, en menor extensión, por su hidrofobicidad.

En muchas ocasiones, la simple determinación de la citotoxicidad no siempre permite predecir qué compuestos o formulaciones son irritantes y cuáles no irritantes para la piel (Fentem y col., 2001) y por eso, es necesario utilizar otros marcadores adicionales, como la distribución diferencial (liberación y almacenamiento) de citocinas. El complejo fenómeno de la irritación dérmica comprende diversos factores y tipos celulares, como las células epidérmicas, los fibroblastos, las células endoteliales y los leucocitos que invaden la zona interactuando con diferentes células bajo el control de citocinas y de mediadores lipídicos. En ese sentido, es evidente que las reacciones inflamatorias e inmunitarias de la piel son mediadas por varias sustancias químicas que influyen en la comunicación célula-célula (Coquette y col., 2000).

Los queratinocitos tienen como función principal proporcionar y mantener la integridad estructural del estrato córneo y además, juegan un papel muy importante en el inicio y el mantenimiento de las reacciones inflamatorias e inmunológicas a través de la producción de citocinas, que pueden ser liberadas tras el estímulo de los queratinocitos por luz UV o por agentes químicos (Mckenzie y Sauder, 1990). De todas las citocinas producidas por los queratinocitos, solo la citocina proinflamatoria IL-1α y el TNF-α activan un número suficiente de mecanismos efectores que desencadenan el proceso de inflamación dérmica (Kupper, 1990). Por ese motivo, se ha elegido la línea celular de queratinocitos HaCaT para llevar a cabo las determinaciones de IL-1α (intra y extracelular) como marcador inflamatorio tras someter las células al tratamiento con las formulaciones de NVs catiónicas. Del mismo modo, se han combinado la línea celular THP-1 (monocitos humanos leucémicos) como modelo celular y la IL-8 como mediador inflamatorio con el objetivo de constituir un ensayo para el cribado de nuevos nanomateriales con potencial actividad inflamatoria. Las células THP-1 se han considerado sustitutas de las células dendríticas cutáneas en estudios de sensibilización dérmica *in vitro* (Sakaguchi y col., 2006; Mitjans y col., 2008, 2010), pues tienen la capacidad de producir y secretar la citocina proinflamatoria IL-8. Además, este ensayo, tal y como se ha propuesto, puede ser utilizado como modelo *in vitro* para valorar el potencial alergénico de nuevos compuestos y formulaciones. La IL-8 constituye un marcador biológico utilizado para discriminar sensibilización de contacto de irritación, eventos estos que tienen diferente impacto sobre la salud humana (Mitjans y col., 2008, 2010). Al incrementar su concentración, las NVs con los tensioactivos MKM y PKM han sido capaces de incrementar de modo concentración-dependiente la cantidad de IL-1α intra y extracelular y también de IL-8, alcanzando respuestas significativas respecto al control negativo. Sin embargo, a altas concentraciones, los niveles más elevados de citocinas parecen ser debidos a la citotoxicidad y no únicamente a una respuesta inflamatoria significativa. Además, estos niveles de IL-1α y IL-8 son generalmente inferiores en comparación a los controles positivos SDS y lipopolisacárido, respectivamente. Por el contrario, aquellas NVs con el MLM no han inducido un aumento de IL-1α intracelular, mientras que la liberación de IL-8 sólo ha ocurrido a una concentración altamente citotóxica. En resumen, se ha demostrado que las NVs tienen un potencial inflamatorio débil, ya que se han observado niveles bajos de IL-1α y IL-8 a concentraciones que tampoco fueron muy citotóxicas (> 75% de viabilidad). A través de estos resultados, también se ha podido demostrar la influencia de la posición de la carga catiónica en la toxicidad de las NVs. En este caso, la carga en el grupo ε-amino de la lisina está relacionada con un potencial inflamatorio inferior.

Además, una nueva formulación desarrollada para administración tópica tiene que ser evaluada en cuanto a su potencial efecto fotoirritante. El riesgo de lesiones en la piel como consecuencia de la exposición solar se ha convertido en un hecho preocupante en los últimos años debido a un incremento en dicha exposición y a la disminución de la capa de ozono. Por lo

tanto, la evaluación de la fototoxicidad de nuevas formulaciones aplicadas por vía tópica es esencial para valorar el posible daño que se podría producir en la piel tras la exposición solar. Actualmente, existe un método aceptado para evaluar la fototoxicidad *in vitro* utilizando fibroblastos, es el ensayo conocido como 3T3-NRU-PT (OECD, 2004). Este método está aprobado para productos químicos y farmacéuticos, pero teniendo en cuenta la falta de ensayos validados para los nanomateriales, se considera aceptable para la valoración de sistemas nanoestructurados (Hartung, 2010; OECD, 2011). En este estudio, se ha realizado una valoración más amplia de la fototoxicidad, usando además de fibroblastos 3T3 y el ensayo NRU, los queratinocitos HaCaT y el ensayo de viabilidad MTT. Se ha demostrado que ninguna de las NVs ha sido capaz de inducir fototoxicidad significativa. Asimismo, respecto a las características estructurales, cabe destacar que las NVs con el tensioactivo MLM (carga positiva en el grupo ε-amino de la lisina) presentan una menor tendencia a inducir efectos fototóxicos.

Además de su propuesta como vehículos para administración tópica, las NVs conteniendo los tensioactivos catiónicos derivados de lisina también se han desarrollado según la hipótesis de que la inclusión de compuestos bioactivos con actividad lítica pH-sensible, podría dar lugar a unos vehículos con propiedades eficientes y específicas para la liberación intracelular de biomoléculas (**Artículo 6**). Sin embargo, un vehículo ideal para administración sistémica y liberación intracelular de fármacos debe presentar, además de propiedades bioactivas, baja toxicidad y biocompatibilidad con los componentes sanguíneos. En ese sentido, este trabajo también se ha centrado en realizar una amplia evaluación toxicológica *in vitro* de los sistemas nanovesiculares propuestos, y el posterior estudio de su comportamiento intracelular en función de las características estructurales del tensioactivo incorporado. Con el objetivo de mejorar el conocimiento de los posibles efectos adversos de los nanomateriales para la salud humana, se han estudiado las condiciones óptimas de los ensayos *in vitro* para su valoración toxicológica.

La nanotecnología es considerada una de las tecnologías clave del siglo XXI y que además está avanzando muy rápidamente, con más de 1000 nanoproductos, con las más diversas aplicaciones, ya disponibles en el mercado (Arora y col., 2012). Considerando que la exposición intencional, así como la accidental, a los nanomateriales viene aumentando continuamente, y que actualmente no hay protocolos claramente establecidos que reglamenten la evaluación toxicológica de los mismos, los estudios toxicológicos *in vitro* resultan extremamente relevantes y de gran interés en este contexto.

Los mecanismos involucrados en la toxicidad de los nanomateriales son complejos y actualmente poco conocidos. Debido a la creciente preocupación social y ética respecto al uso de animales de experimentación, y también

debido al alto coste que suponen estos ensayos, la valoración de los efectos tóxicos de nuevos productos nanotecnológicos, tiende cada vez más a realizarse en modelos *in vitro*. Teniendo en cuenta el gran desconocimiento respeto al comportamiento y efectos adversos de estos productos, no hay razones éticas y científicas para empezar los estudios de cribado de nuevos sistemas nanoestructurados con modelos superiores y más complejos como los animales de experimentación (Hartung, 2010). No obstante, muchas veces es difícil de obtener una reproducción experimental fiable de los fenómenos que ocurren en condiciones fisiológicas, por lo que resulta esencial seleccionar una batería de ensayos *in vitro* que abarquen el mayor rango posible de los fenómenos que se producen *in vivo*. Efectivamente, es necesario utilizar diferentes parámetros *in vitro* que incluyan distintos mecanismos de acción para que la situación se parezca lo máximo posible a los procesos que tienen lugar *in vivo*. En ese sentido, los métodos basados en cultivos celulares se han ido introduciendo progresivamente con ese fin, ya que permiten estudiar con rapidez y de modo relativamente económico, los efectos adversos de determinados productos junto con sus mecanismos de acción (Pappinen y col., 2005; Arora y col., 2012).

Basado en la búsqueda de posibles mecanismos involucrados en la toxicidad de los nanomateriales y de la comprensión de las relaciones estructura-actividad, se ha propuesto una batería de ensayos *in vitro* que evalúan diferentes características celulares. Además de los fibroblastos 3T3, también se ha seleccionado la línea celular tumoral HeLa como modelo de estudio. Tal y como se ha demostrado con las células 3T3 y HaCaT, las células HeLa han mostrado diferente sensibilidad a las formulaciones de NVs, corroborando que la combinación de varios modelos celulares es un abordaje necesario para un conocimiento más fiable de los efectos tóxicos de nuevos nanomateriales. Entre los ensayos de viabilidad (MTT, NRU y LDH) estudiados, el MTT ha sido siempre el más sensible en detectar la toxicidad de las NVs con MKM y PKM, independientemente de la línea celular. Este efecto parece estar relacionado con la habilidad de estas NVs, con propiedades pH-dependientes, de internalizarse y liberarse al citoplasma tras la ruptura de la membrana de los compartimientos endosomales. Tras este proceso, la consecuente liberación de las NVs y/o sus constituyentes al citoplasma parece estar directamente relacionada con sus efectos tóxicos sobre la mitocondria, lo que, a su vez, afecta primeramente la actividad metabólica de la célula (Yang y col., 2009; Horie y col., 2012).

Tras el estudio previo de la citotoxicidad de las NVs en los fibroblastos 3T3, se ha elegido esta misma línea celular como modelo para elucidar los posibles mecanismos involucrados en el proceso. Se ha observado que todas las formulaciones han inducido efecto apoptótico; sin embargo, las respuestas más significativas se han obtenido con las NVs conteniendo PKM. Por lo tanto, se puede demostrar que hay una correlación directa entre la citotoxicidad y la

apoptosis, ya que las NVs con PKM también han presentado efectos citotóxicos superiores. Del mismo modo, estas NVs, y también aquellas conteniendo MKM, han inducido peroxidación lipídica significativa, lo que indica su capacidad de provocar el estrés oxidativo a nivel intracelular. Estos mecanismo parecen estar relacionados, ya que los efectos apoptóticos pueden ser atribuibles al daño oxidativo (AshaRani y col., 2009). Además, la disfunción mitocondrial, corroborada por el ensayo de viabilidad MTT (Fisher y col., 2003), se considera una vía intrínseca de la apoptosis y un marcador de niveles intracelulares elevados de ROS (Choi y col., 2007; Jones y Grainger, 2009) y, por lo tanto, también parece estar involucrada, al menos en parte, en la inducción de los efectos apoptóticos y del estrés oxidativo (peroxidación lipídica).

Como mecanismo de citotoxicidad de las NVs que contienen los tensioactivos con la carga positiva en el grupo α-amino de la lisina (PKM y MKM), se puede proponer que la inducción del estrés oxidativo produce una disfunción de la mitocondria que, a su vez, lleva la célula a sufrir apoptosis. Una vez se alcanzan niveles críticos de concentración en contacto con la célula, esta finalmente pierde la integridad de su membrana y muere. Es importante señalar que la perturbación del balance oxidativo de la célula parece estar directamente relacionada con el desarrollo de innumerables mecanismos de desestabilización de la homeostasis intracelular (Horie y col., 2012). Por el contrario, este mecanismo propuesto no puede ser aplicado a las NVs que contienen MLM en su formulación, pues estas no han inducido un estrés oxidativo significativo. A la vista de los resultados obtenidos, se demuestra una vez más el efecto crítico que tiene la posición de la carga catiónica del tensioactivo en los efectos tóxicos de la formulación resultante. La carga en el grupo ε-amino de la lisina supone un mecanismo de citotoxicidad independiente del estrés oxidativo y de un daño mitocondrial significativo (demostrado por el menor índice de citotoxicidad detectado por el ensayo MTT).

La posible administración por vía intravenosa de los nanomateriales requiere una valoración de su compatibilidad con los constituyentes de la sangre. Los componentes superficiales y la carga neta de estos sistemas son críticos en su hemoreactividad (Eliyahu y col., 2002; Kuznetsova y col., 2012). Sin embargo, se ha demostrado que las NVs catiónicas no son hemolíticas y tampoco inducen aglutinación de los eritrocitos a las concentraciones poco citotóxicas. Además, estos sistemas no adsorben proteínas plasmáticas, lo que indica que este proceso no tendría influencia sobre sus mecanismos de captación celular y toxicidad (Dutta y col., 2007).

Como hallazgo clave de este trabajo, se ha corroborado la hipótesis que ha motivado el desarrollo de las NVs catiónicas: la inclusión de los tensioactivos catiónicos con actividad lítica pH-sensible (**Artículos 2 y 3**) ha dado lugar a unos vehículos con propiedades líticas eficientes y selectivas de

las membranas endosomales. Los análisis por microscopía de fluorescencia, utilizando las células HeLa como modelo celular y el colorante fluorescente calceína como marcador de endocitosis y de la estabilidad endosomal, han demostrado la capacidad de las NVs con los tensioactivos de carga positiva en el grupo α-amino de la lisina, para romper la membrana de los endosomas y liberar el material al citoplasma. Además, la eficiencia superior de las NVs con MKM para liberar la calceína al citoplasma tras la ruptura de la membrana endosomal corrobora su actividad hemolítica superior a pH 6,5 y 5,4 (**Artículo 2**). Las NVs conteniendo MLM han sido estudiadas como vehículos control con propiedades independientes del pH, y su comportamiento intracelular ha corroborado su actividad lítica no sensible a los cambios de pH del medio (**Artículos 2 y 3**). Muchos compuestos terapéuticos tienen su sitio de acción en los compartimientos intracelulares y, por lo tanto, su eficacia terapéutica depende de un mecanismo eficiente de internalización y liberación en el citoplasma (Plank y col., 1998). En ese sentido, se destaca la relevancia de los resultados obtenidos en este trabajo, que indica que las NVs conteniendo los tensioactivos MKM y PKM son potenciales vehículos para la administración intracelular de biomoléculas.

Dando continuidad al segundo bloque de objetivos de esta Tesis, se han estudiado los efectos del tensioactivo aniónico 77KL tras su incorporación en formulaciones de NPs poliméricas (**Artículo 7**). La actividad lítica pH-sensible (**Artículo 1**), juntamente con su biocompatibilidad y baja toxicidad (**Artículo 4**), han motivado la elección de este tensioactivo como excipiente bioactivo en formulaciones de NPs poliméricas conteniendo el fármaco metotrexato (MTX), y también su propuesta como potencial vehículo para la terapia antitumoral.

Las NPs se han desarrollado a través del método de gelificación iónica (Calvo y col., 1997), pero con algunas modificaciones tras incluir el 77KL en el proceso de complejación con el polímero quitosano. Se demostró que la incorporación del tensioactivo no ha afectado el proceso de formación de las NPs y tampoco ha cambiado el potencial zeta positivo típico de las partículas formuladas con el polímero quitosano (Gan y col., 2005; Fan y col., 2012). Como parte de la caracterización fisicoquímica, se ha valorado el comportamiento de estas formulaciones en medio de cultivo. Tal y como se ha mencionado para las NVs (**Artículos 5 y 6**), este tipo de valoración es de gran importancia para una mejor comprensión de las respuestas obtenidas en los estudios toxicológicos *in vitro*. Se ha observado una cinética un tanto inesperada de estas NPs, ya que inicialmente se han agregado en medio de cultivo, mientras que después de 24 h de incubación en las condiciones que simulan un ensayo de citotoxicidad, se ha observado una disminución de su tamaño hidrodinámico. Este proceso se podría justificar por la protonación reducida del quitosano a pH fisiológico, lo que supone una posible reducción en

la complejación de este polímero con el polianión TPP y con el tensioactivo 77KL tras el equilibrio de las NPs en un medio acuoso rico en iones y proteínas.

En este estudio se ha corroborado la hipótesis de que la encapsulación de un fármaco antitumoral en un vehículo nanoestructurado con propiedades pH-dependiente puede aumentar su potencia terapéutica. Se ha demostrado claramente que el MTX ha sido más activo en inhibir la proliferación de las células tumorales en comparación con el fármaco libre no encapsulado. La actividad superior del fármaco encapsulado se atribuye en parte a las propiedades fisicoquímicas de la formulación de NP. El pequeño tamaño de partícula y la carga superficial son parámetros que facilitan la internalización celular y, por lo tanto, tienden a favorecer la acción terapéutica del fármaco en los compartimientos intracelulares. Además, la actividad pH-dependiente de las NPs ha proporcionado un aumento en la liberación del fármaco encapsulado a medida que se disminuye el pH del medio, lo que sugiere una mayor especificidad de estas formulaciones para liberar el fármaco a nivel endosomal o del tejido tumoral extracelular (pH ~6,6), así como una menor liberación inespecífica a pH fisiológico. Es importante destacar que, de forma conjunta, estas características contribuyen a reducir los efectos adversos de los fármacos antitumorales, además de evitar el desarrollo de resistencia a estos fármacos (efecto MDR) debido a una liberación muy lenta en el sitio de acción (Na y col., 2003). Por otra parte, los estudios de hemólisis, con el eritrocito como modelo de membrana endosomal, complementados por los estudios de internalización celular utilizando el colorante fluorescente calceína como marcador de la estabilidad de los endosomas (Hu y col., 2007), han corroborado la actividad lítica pH-sensible de las NPs y, por lo tanto, su capacidad para romper la membrana endosomal y liberar intracelularmente el fármaco encapsulado. En conjunto, estos efectos justifican la actividad antitumoral superior del fármaco incorporado en las NPs. Además, se ha probado que la presencia del tensioactivo bioactivo 77KL es el principal condicionante de la actividad lítica pH-sensible de las NPs.

El MTX no encapsulado tiende a ser bombeado hacia el exterior de la célula por la glicoproteína-P (Kusnetsova y col., 2009) y, además, su entrada en la célula por difusión pasiva puede estar limitada debido a su alto grado de protonación a pH fisiológico o a pH del tejido tumoral, por lo que se demuestra la ventaja de los sistemas nanoestructurados para una mayor acción terapéutica del MTX. Los sistemas nanoestructurados se han mostrado eficientes en la terapia antitumoral, por superar la resistencia de muchos tejidos cancerígenos a los fármacos (efecto MDR) y por mejorar el efecto EPR en los tumores (Jabir y col., 2012).

Se han estudiado los mecanismos involucrados en la citotoxicidad de las NPs conteniendo el MTX en la búsqueda de procesos de interacción celular específicos que justifiquen la mayor actividad de los sistemas

nanoestructurados en comparación con el fármaco no encapsulado. Se ha observado que las NPs inducen efectos superiores sobre la proliferación celular, la apoptosis y el ciclo celular. Se ha demostrado que las células se acumulan en la fase S del ciclo celular tras el tratamiento con el MTX, lo que indica una inhibición de la síntesis de ADN. La progresión del ciclo en las células HeLa se ha afectado en menor proporción, pero las respuestas han sido igualmente significativas. Como mecanismo de la actividad antitumoral del MTX y, especialmente, del MTX encapsulado en las NPs, se ha propuesto que tras la internalización y liberación intracelular del fármaco, la inhibición de la progresión del ciclo celular lleva a una disminución de la proliferación celular y además está asociada con daño en el ADN, lo que a su vez causa los efectos apoptóticos. El MTX asociado a las NPs también ha afectado la integridad de la membrana lisosomal en las células HeLa, pero no en las células MCF-7. En consecuencia, estos resultados han demostrado que el daño de la membrana lisosomal y la lesión mitocondrial precoz (demostrada por la mayor sensibilidad del ensayo MTT) también son mecanismos involucrados en los efectos apoptóticos sobre las células HeLa del MTX encapsulado en las NPs. Se ha descrito previamente que el daño en la integridad de la membrana lisosomal lleva a la liberación de las enzimas hidrolasas del lisosoma al resto de la célula, lo que generalmente provoca la apoptosis tras la inducción de daño en la mitocondria (Joanitti y col., 2010). En conjunto, estos mecanismos parecen estar directamente relacionados con la sensibilidad superior de estas células tumorales a los efectos tóxicos del MTX. Por el contrario, la línea celular MCF-7 ha sido menos sensible, lo que corrobora su mayor resistencia a la actividad antitumoral del MTX (Yoon y col., 2010). Sin embargo, pese a esta resistencia, el MTX encapsulado en las NPs ha sido más eficaz en inhibir la proliferación celular, además de inducir efectos superiores sobre el ciclo celular y la inducción de apoptosis, lo que sugiere una mayor actividad terapéutica del fármaco encapsulado. Además, la baja citotoxicidad obtenida en una línea celular no tumoral (HaCaT), utilizada como control, permite postular que existe una potencial especificad de las NPs conteniendo MTX por las células tumorales.

Por último, como resumen de todos los resultados obtenidos, es importante destacar la actividad lítica pH-sensible de los tensioactivos aniónicos y catiónicos derivados de lisina, tal y como se ha demostrado a través de los estudios de su interacción con la membrana de los eritrocitos. Además, pese a estos efectos sobre este modelo de membrana endosomal, estos compuestos también han demostrado actividad lítica pH-dependiente tras su internalización celular cuando son incorporados en sistemas nanoestructurados, por lo que se considera que podrían ser potenciales excipientes bioactivos en vehículos para la liberación intracelular de moléculas biológicamente activas. Del mismo modo, el amplio rango de métodos *in vitro*

utilizado en esta Tesis se ha mostrado eficaz en demostrar los potenciales efectos tóxicos de los nanomateriales, así como los principales mecanismos involucrados en dichos procesos.

# CONCLUSIONES

Los resultados obtenidos en el presente trabajo han permitido extraer las siguientes conclusiones, que se han agrupado según los dos bloques de objetivos generales planteados:

**Respecto a las propiedades de interacción con membrana celulares:**

- Los tensioactivos aniónicos derivados de la $N^{\alpha},N^{\varepsilon}$ -dioctanoil lisina tienen actividad lítica pH-sensible independiente del contraión. El tipo de contraión influye únicamente en la potencia de esta actividad. El tensioactivo 77KL ha sido el más eficaz en mantener la actividad pH-dependiente a concentraciones más elevadas y, por lo tanto, tiene la capacidad de conservar la actividad lítica selectiva a pH ácidos para un amplio rango de concentraciones. Del mismo modo, este compuesto ha demostrado efectos citotóxicos leves a las concentraciones propuestas como pH-sensibles.

- La actividad lítica pH-sensible de los tensioactivos catiónicos derivados de la *N*-acil lisina depende directamente de sus características estructurales. Sólo los compuestos con la carga positiva en el grupo α-amino de la lisina (MKM y PKM) tienen la capacidad de cambiar su actividad lítica en función del pH del medio. Además, la hidrofobicidad de estos tensioactivos se correlaciona inversamente con su potencia lítica pH-dependiente.

- Desde el punto de vista mecanístico, la actividad lítica pH-sensible de los tensioactivos catiónicos con la carga positiva en el grupo α-amino de la lisina se evidencia por las alteraciones en las propiedades normales de la membrana en condiciones levemente ácidas. Provocan la desorganización de los lípidos, con el consecuente aumento en la fluidez de la bicapa lipídica, y la pérdida significativa de proteínas estructurales de la membrana. A su vez, estos resultados corroboran la hipótesis de que la actividad lítica pH-sensible es debida a mecanismos específicos de interacción con la bicapa lipídica en función del pH del medio.

- La combinación de líneas celulares tumorales y no tumorales y de ensayos de citotoxicidad basados en diferentes mecanismos de viabilidad celular se ha propuesto como un modelo predictivo para evaluar los efectos citotóxicos de nuevos compuestos bioactivos con potencial aplicación cosmética y/o farmacéutica. A través de esta batería de ensayos, se ha visto que la presencia de diferentes contraiones en las moléculas de los tensioactivos aniónicos derivados de la $N^{\alpha},N^{\varepsilon}$ -dioctanoil lisina influye directamente en su acción citotóxica. En general, los tensioactivos con contraiones orgánicos han presentado una tendencia a ser menos citotóxicos. Sin embargo, el tensioactivo 77KL ha presentado una citotoxicidad similar o incluso inferior, especialmente en las células tumorales.

- En conjunto, estos resultados demuestran la potencial aplicación de los tensioactivos aniónicos y de los catiónicos con la carga positiva en el grupo α-amino de la lisina como excipientes bioactivos en sistemas nanoestructurados para la liberación intracelular de fármacos. Su actividad lítica pH-sensible supone una ruptura selectiva de la membrana endosomal a pH levemente ácido, lo que a su vez llevaría a la liberación al citoplasma de las biomoléculas encapsuladas en dichos vehículos.

**Respecto al desarrollo de sistemas nanoestructurados, su evaluación toxicológica y su comportamiento intracelular *in vitro*:**

- Se han desarrollado nanovesículas incluyendo los tensioactivos catiónicos derivados de lisina en su matriz lipídica básica. Estas nanovesículas son estables, con gran incorporación de los tensioactivos en su estructura, carga neta positiva y diferente distribución de tamaños. Además, se ha demostrado que las formulaciones con los tensioactivos con la carga positiva en el grupo α-amino de la lisina tienden a agregarse en medio de cultivo tras 24 h de incubación en las condiciones que simulan un ensayo de citotoxicidad *in vitro*.

- La potencial aplicación tópica de las nanovesículas catiónicas se ha propuesto tras la valoración de su citotoxicidad utilizando células representativas de la piel como modelo de ensayo *in vitro*. Se han observado efectos citotóxicos moderados, respuesta inflamatoria leve y ausencia de fototoxicidad. De manera conjunta, los resultados obtenidos han demostrado la aplicabilidad de las líneas celulares y métodos propuestos como un potencial modelo de ensayo, por lo que se propone su utilización para una valoración toxicológica eficiente durante el cribado de nuevos sistemas nanoestructurados para administración tópica.

- Las nanovesículas formuladas con los tensioactivos con la carga positiva en el grupo α-amino de la lisina se proponen como potenciales vehículos para la administración sistémica y liberación intracelular de biomoléculas. Los estudios de internalización celular, utilizando la calceína como marcador de endocitosis y estabilidad endosomal, corroboraron la capacidad de estas nanovesículas para romper la membrana de los endosomas y, por lo tanto, la actividad pH-dependiente de los tensioactivos MKM y PKM. Por otra parte, el amplio rango de métodos *in vitro* utilizados ha demostrado que estas formulaciones inducen una citotoxicidad moderada, siendo el estrés oxidativo, la disfunción mitocondrial y la apoptosis los principales mecanismos involucrados en su citotoxicidad.

- Los resultados de los estudios de citotoxicidad utilizando diferentes líneas celulares (3T3, HaCaT, THP-1 y HeLa) y ensayos de viabilidad (MTT, NRU y LDH) han demostrado que los efectos tóxicos de las nanovesículas dependen directamente de la posición de la carga catiónica del tensioactivo y, en menor extensión, de su hidrofobicidad**.**

- Se han desarrollado nanopartículas poliméricas incluyendo el tensioactivo aniónico 77KL como excipiente pH-sensible. Se encapsuló el fármaco anticanceroso metotrexato y se demostró su mayor actividad en inhibir la proliferación de las células tumorales en comparación con el fármaco libre no encapsulado, lo que se atribuye, especialmente, a la actividad pH-dependiente de las nanopartículas. Tras la internalización y liberación específica del fármaco a nivel intracelular, la inhibición del ciclo celular, la apoptosis y, en menor medida, el daño mitocondrial y lisosomal, se han demostrado como los mecanismos involucrados en la actividad antitumoral del metotrexato encapsulado. A la vista de estos resultados, se propone la utilización de estas nanopartículas pH-sensibles como potencial vehículo en la terapia antitumoral.

- En conclusión, es importante destacar que el desarrollo de vehículos con actividad pH-dependiente es una propuesta interesante para alcanzar una eficiente liberación intracelular de biomoléculas y aumentar sus efectos terapéuticos. Por lo tanto, se considera que los tensioactivos derivados de lisina estudiados en esta Tesis son potenciales excipientes bioactivos en formulaciones nanoestructuradas. Por otra parte, los métodos *in vitro* utilizados fueron eficaces para demostrar los mecanismos involucrados en los potenciales efectos tóxicos y/o terapéuticos de los nanomateriales. Los modelos de ensayo propuestos para la evaluación toxicológica *in vitro* de nuevos nanomateriales contribuyen para la consolidación de una nueva disciplina, la Nanotoxicología, y también para disminuir las incertidumbres relacionadas con los efectos tóxicos de los nanomateriales.

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