CENTRO INTERNACIONAL DE ESTUDOS
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POLYMERIC NANOCAPSULES FOR THE OCULAR DELIVERY OF siRNA

Sofia Mendes Saraiva

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NANOCÁPSULAS POLIMÉRICAS PARA LA ADMINISTRACIÓN OCULAR DE siRNA

Sofia Mendes Saraiva

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Polymeric nanocapsules for the ocular delivery of siRNA

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Nanocápsulas poliméricas para la administración ocular de siRNA

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À minha família,

os meus heróis.

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“Trabalhar, trabalhar, trabalhar, não te esqueças de que nada se consegue sem trabalhar.”

“Olha a formiga a passar…!”

J. Saraiva

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Abstract/Resumen

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Abstract

Ocular pathologies have a great impact on the patients daily living activities and quality of life. Innovative polynucleotide-based therapies are under development for different eye diseases. However, these hydrophilic molecules struggle to overcome the extraordinary eye protection barriers. Nanotechnology offers the possibility to circumvent these barriers, protect polynucleotides from degradation and improve their interaction with corneal and conjunctival epithelia.

The aim of this thesis has been the development of new formulations of siRNA based on the use of protamine nanocapsules as a vehicle for ocular instillation. Due to the very low instillation volume, a prerequisite for these nanocapsules was to have a very high loading capacity with a final targeted concentration higher than 1 mg/mL, preferably 5 mg/mL. To achieve this objective, siRNA was adsorbed onto the surface of a cationic oil core and then coated with a protamine layer. This external layer was intended to protect siRNA from premature degradation and to improve the NCs interaction with the corneal epithelium. In addition, protamine was expected to facilitate the translocation of siRNA through the cells' membrane.

In chapter 1, cationic nanoemulsions were prepared by the solvent displacement technique. Among the selected cationic surfactants (cetrimide, benzethonium chloride and ethyl lauroyl arginate) cetrimide was the one that enabled the most efficient siRNA association. These formulations contained a siRNA concentration of 2.5 mg/mL of siRNA, they maintained their colloidal stability and prevented the premature release of siRNA upon incubation in simulated lacrimal fluid or cell culture medium. They exhibited a low cytotoxicity, however they were not able to induce gene silencing *in vitro*.

In chapter 2, cationic nanoemulsions were prepared by the self-emulsification technique. Among the selected cationic surfactants (cetrimide and oleylamine), oleylamine was the one that led to the greatest loading capacity reaching a final siRNA concentration of 5 mg/mL (corresponding to a final loading of 3.9 %). This formulation maintained its physicochemical properties and prevented the premature release of the loaded-siRNA when incubated with lacrimal fluid. They were stable under storage for 15 days at 4 °C both in liquid state or in

freeze-dried form. HeLa cells' treatment with protamine NCs, loading 100 nM of siRNA, did not compromise cell viability and lead to a 24 % reduction of gene expression, 72 h post treatment, an effect that was similar to the one obtained with the commercial transfection agent DharmaFECT™ 3. In addition, following topical ocular administration of protamine NCs to rabbits, it was found that the nanocapsules have a favorable interaction with the corneal epithelium thanks to their protamine shell.

This work highlights the importance of the formulation process in the development of siRNA nanocarriers with the adequate quality attributes (size, drug loading, stability and controlled release) and with the desired performance. Moreover, the results show that the oleylamine-based protamine NCs loading high concentrations of siRNA might have a potential for topical ocular instillation.

Resumen

Las enfermedades oculares tienen un gran impacto en las actividades diarias de los pacientes que las sufren, influenciando dramáticamente su calidad de vida. Las terapias basadas en polinucleótidos ofrecen interesantes expectativas en el tratamiento de distintas enfermedades oculares. Sin embargo, estas moléculas hidrofílicas presentan dificultades para superar las barreras de protección del ojo. La nanotecnología ofrece la posibilidad de superar esas barreras, proteger los polinucleótidos contra la degradación, así como de mejorar su interacción con los epitelios de la córnea y conjuntiva, y su tiempo de permanencia en la superficie del ojo.

El principal objetivo de esta tesis ha sido el desarrollo de nanocápsulas de protamina como vehículos portadores de siRNA, destinados a ser instilados en la superficie ocular. Un prerrequisito en el diseño de estos vehículos ha sido el de presentar una elevada capacidad de carga de siRNA, permitiendo la administración de una dosis de 1 mg/mL, idealmente 5 mg/mL. Para ello se desarrollaron en primer lugar nanoemulsiones catiónicas sobre las cuales se adsorbieron las moléculas de siRNA y, en torno a ellas, una capa de protamina. De esta forma el siRNA estaría protegido de su degradación prematura por la cubierta de protamina, la cual, además, previsiblemente mejoraría la interacción de la NC con el epitelio de la córnea, incrementando el tiempo de permanencia ocular y la biodisponibilidad del siRNA. Además, se barajó la hipótesis de que la protamina pueda mejorar la capacidad de penetración a través de la membrana celular.

En el capítulo 1, se describe la formación de emulsiones catiónicas por el método de desplazamiento de disolvente, utilizando diferentes surfactantes catiónicos (cetrimida, cloruro de benzetonio y etil lauroil arginato), de los cuales fue la cetrimida el que permitió una mayor asociación de siRNA, alcanzando una concentración final de 2,5 mg/mL. Las formulaciones obtenidas mantuvieron su estabilidad coloidal en fluido lacrimal simulado y medio de cultivo celular y evitaron la liberación prematura de siRNA. A pesar de presentar una biocompatibilidad alta, las NCs no fueron capaces de inducir el silenciamiento esperado del gen de interés.

En el capítulo 2, se describe la formación de emulsiones catiónicas por el método de auto emulsificación utilizando diferentes surfactantes catiónicos (cetrimida y oleilamina) de los cuales fue la oleilamina el que permitió una mayor asociación de siRNA (5 mg/ml, correspondiendo a una carga de 3,9 %). Las NCs mantuvieron sus propriedades fisicoquímicas, así como el siRNA asociado tras su incubación en fluido lacrimal. Las NCs permanecieron estables durante almacenamiento, en estado líquido y liofilizado, a 4 °C durante 15 días. El tratamiento de las células HeLa con las NCs de protamina, cargadas con 100 nM de siRNA, dio lugar a una reducción de 24 % de la expresión del gene de interés, efecto similar al causado por el agente de transfección comercial DharmaFECT™ 3, sin comprometer la viabilidad celular. Tras la administración ocular tópica de las NCs de protamina, se observó que estas nanocápsulas presentan una interacción favorable con el epitelio de la córnea gracias a la cubierta de protamina.

Este trabajo pone de relieve la importancia del proceso de formulación en el desarrollo de nanovehículos de siRNA dotados de los necesarios atributos de calidad (tamaño, carga de siRNA, estabilidad y liberación controlada) y de su capacidad de silenciamiento. Además, los resultados muestran el potencial de las nanocápsulas de protamina conteniendo oleilamina y una alta concentración de siRNA para la administración tópica ocular.

Resumen *in* extenso

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1. Introducción

Las enfermedades oculares suelen tener un gran impacto en las actividades diarias y en la calidad de vida de los pacientes que las sufren. Según las últimas estimaciones del "Vision loss expert group", en 2020 el número de individuos que padecerán ceguera, a nivel mundial, aumentará de 36 (en 2015) a cerca de 39 millones [1, 2] como consecuencia de enfermedades como cataratas, glaucoma, degeneración macular relacionada con la edad y retinopatía diabética. Esta situación pone de manifiesto la urgente necesidad de terapias novedosas y más eficientes. Dentro de las nuevas estrategias terapéuticas se destacan las basadas en el uso de polinucleótidos, algunas de las cuales han sido recientemente aprobadas o están en fase de desarrollo clínico [3].

Entre los distintos tipos de polinucleótidos, el ARN de interferencia (ARNi o en inglés iRNA) descubierto en la década de los 90 [4] marcó una nueva era debido a su alto potencial terapéutico. Un ejemplo de este tipo de nucleótidos es el ARN de interferencia pequeño (ARNip o en inglés siRNA), un ARN de doble cadena con 21-23 pares de bases que tiene la capacidad de silenciar genes específicos responsables por el desarrollo de una enfermedad [5]. A pesar de su potencial y de las modificaciones químicas desarrolladas para mejorar su estabilidad [6-8], estos oligonucleótidos siguen teniendo dificultades para lograr su penetración en las células diana y evitar su degradación prematura en los endosomas [9-12].

En el caso específico del globo ocular, existen importantes barreras que dificultan la llegada del fármaco [13], y especialmente de moléculas hidrofílicas como los nucleótidos, a las células diana. La nanotecnología ofrece la posibilidad de superar esas barreras, además de protegerlos frente a su degradación. Entre los distintos tipos de nanosistemas desarrollados para la aplicación ocular, las nanocápsulas poliméricas han mostrado la capacidad de mejorar la interacción con los epitelios de la córnea y conjuntiva [14]. Además, las NCs tienen la capacidad de cargar moléculas hidrofílicas e hidrofóbicas, posibilitando una terapia combinada [15-17]. Nuestro grupo de investigación ha sido uno de los pioneros en el desarrollo de NCs para la administración tópica ocular de fármacos [18, 19].

El principal objetivo de esta tesis ha sido el desarrollo de nanocápsulas de protamina como vehículos de siRNA para instilación ocular. El requisito esencial para dichas nanocápsulas ha

sido el de presentar una extraordinaria capacidad de carga de siRNA, de modo a obtener dosis superiores a 1 mg/mL, preferentemente de 5 mg/mL.

Para el desarrollo de las nanocápsulas de protamina, se elaboraron en primer lugar nanoemulsiones catiónicas, en torno a cuyos glóbulos se adsorbieron las moléculas de siRNA y a continuación, las de protamina, para proteger el siRNA del ambiente externo (Fig. 1). Además, se pensó que la capa de protamina podría, previsiblemente, interaccionar con el epitelio corneal [14] y mejorar, así, la penetración del siRNA a través de la membrana de las células [20].

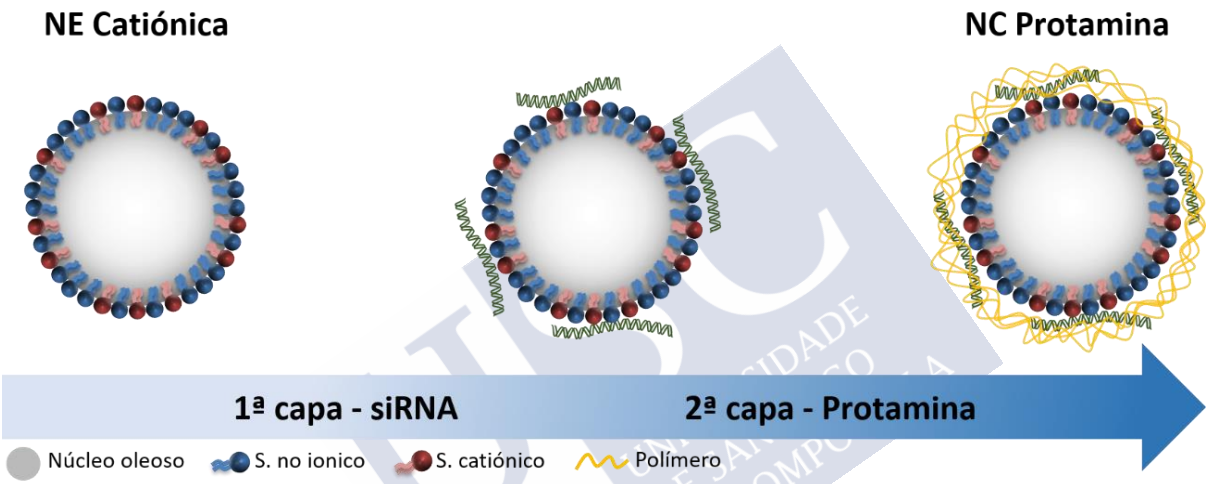

Fig. 1 - Representación gráfica de las nanocápsulas de protamina (S., surfactantes).

2. Resultados y discusión

2.1. Nanocápsulas de protamina como sistemas de liberación de siRNA preparadas por la técnica de desplazamiento de disolvente

Para construir este sistema capaz de adsorber el siRNA y protegerlo de su ambiente externo, inicialmente se desarrollaron nanoemulsiones catiónicas capaces de asociar el siRNA en la superficie de sus glóbulos, los cuales fueron posteriormente recubiertos por una capa de protamina. Las nanoemulsiones fueron preparadas por el método de desplazamiento de disolvente evaluando la influencia de diferentes parámetros de formulación tales como su composición, los disolventes utilizados, la proporción de las fases oleosa y acuosa, y el modo de mezclado de las fases en las características de la formulación obtenida. Teniendo en cuenta

estudios anteriores para lograr la reducción del tamaño de los nanosistemas, la fase oleosa fue inyectada en la fase acuosa.

Con respecto a la composición de las nanoemulsiones se seleccionó el surfactante no iónico, polysorbato 80, presente en diversas formulaciones oftálmicas, debido a su capacidad para estabilizar nanovehículos y de mejorar su capacidad de transfección [21, 22]. Además, al revés de otros surfactantes no interfiere en la adsorción de nucleótidos [17] en la superficie de nanovehículos. Como era previsible, el incremento en la cantidad de este surfactante, dio lugar a un tamaño de partícula menor. Por otra parte, el tipo de aceite utilizado vitamina E (DL-α-tocoferol) o triglicérido de cadena media (Mygliol®), también tuvieron una gran influencia en las propriedades de las nanoemulsiones, siendo la vitamina E el aceite para el que se obtuvo un menor tamaño de glóbulo. La selección de estos aceites se ha debido a que, por una parte, la vitamina E se encuentra en diferentes productos oftálmicos y sus propriedades antioxidantes le confieren la capacidad de proteger las células del estrés oxidativo, implicado en distintas enfermedades [23-25] y que por otra parte, los triglicéridos de cadena media como el Mygliol® son capaces de mejorar la estabilidad de la lágrima [26].

El potencial-ζ positivo de las NEs es conferido por surfactantes catiónicos que suelen ser usados como preservativos en distintas formulaciones comercializadas. Teniendo en cuenta que este tipo de surfactante puede presentar distintos perfiles de toxicidad y asociación de RNA, se seleccionaron tres surfactantes distintos: cetrimida, cloruro de benzetonio (Bz Cl), y etil lauroil arginato (LAE). A pesar de que los tensoactivos presentan grupos catiónicos distintos (amina cuaternaria o grupo guanidina) y diferente longitud de cadena hidrofóbica, las NEs preparadas con los mismos presentaron propriedades fisicoquímicas similares. Sin embargo, las NEs basadas en cetrimida fueron las que permitieron una mayor asociación de siRNA (concentraciones de 3 mg/mL de siRNA; Fig. 2A, NC 0.0) mientras que con las NEs basadas en Bz Cl o LAE se logró una concentración de 1,5 mg/mL (Fig. 2B y 2C, NC 0.0). Estas formulaciones fueron posteriormente recubiertas con concentraciones crecientes de protamina, observando que el ratio de peso 1:0,3 (siRNA:protamina, NC 0.3) era el más adecuado para preservar la capacidad de asociación del siRNA. En la Fig. 2 se representan las propriedades fisicoquímicas de las NCs con los distintos ratios siRNA:protamina p/p testados, así como de su capacidad de asociación de siRNA. La capacidad de desplazamiento del siRNA

se estudió tras la incubación de las NCs de protamina con heparina durante 30 min a 37 °C, comprobándose la capacidad de las NCs con una cubierta igual o superior a 1:0,3 (siRNA:protamina p/p) para liberar siRNA (Fig. 2). Las NCs de protamina mostraron una estabilidad adecuada en fluido lacrimal simulado (FLS), previniendo la liberación prematura del siRNA asociado.

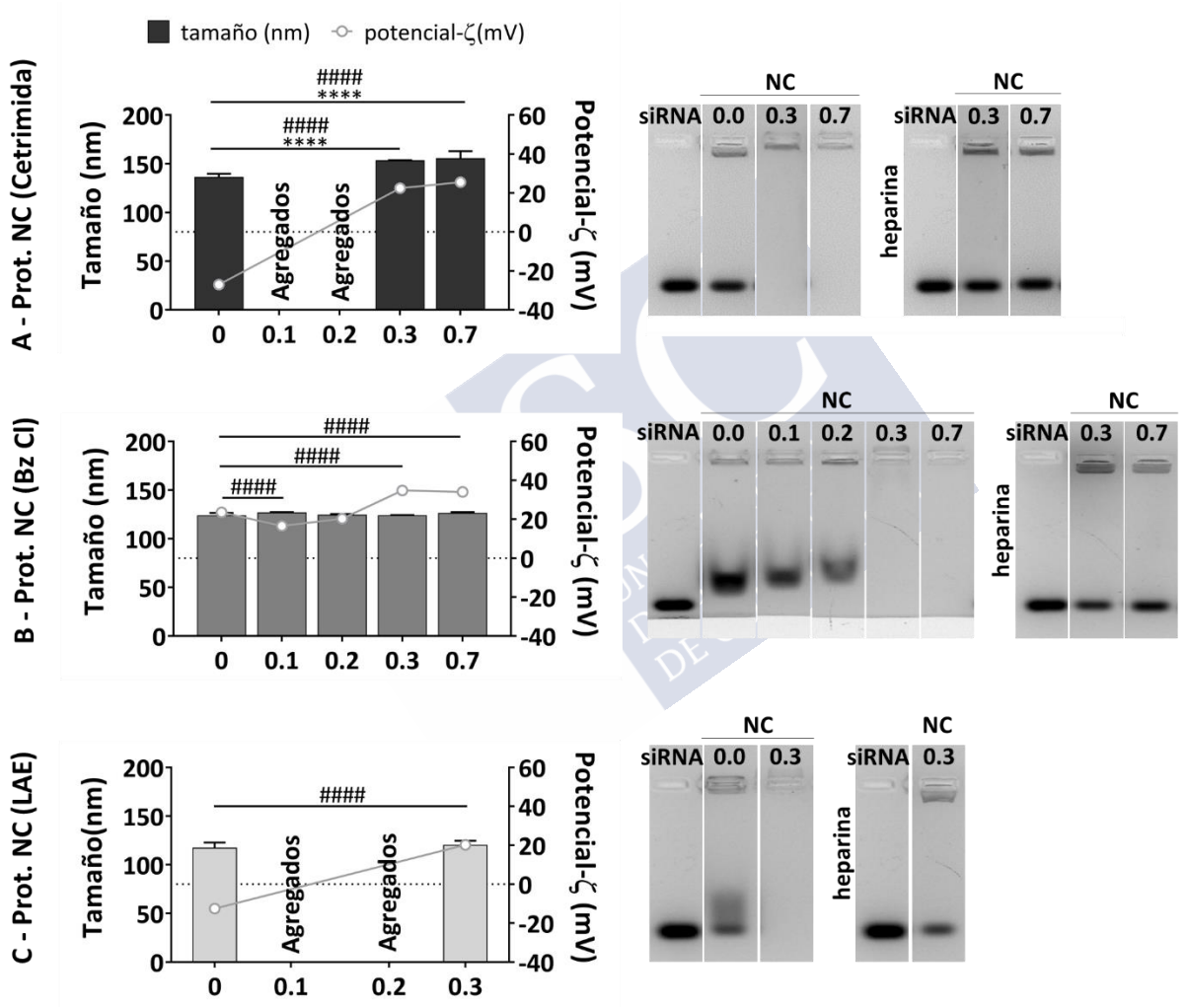

Fig. 2 - Características fisicoquímicas de las NCs de protamina cargadas con siRNA, preparadas con diferentes tensoactivos (Cetrimida (**A**), Bz Cl (**B**), y LAE (**C**)) y recubiertas con diferentes proporciones de protamina (ratio siRNA:protamina p/p desde 1:0,1 hasta 1:0,7). La eficiencia de asociación de siRNA y su desplazamiento mediante adición de heparina se muestran en los geles. NC 0.0 se refieren a las NEs cargadas con siRNA y sin cubierta de protamina. NC 0.1, NC 0.3 y NC 0.7 se refieren a las NCs con una cubierta de protamina de 0,1, 0,3 o 0,7 (ratio siRNA:protamina p/p). Los valores representan la media ± DE (n=3). Los datos han sido analizados por ANOVA de dos vías (**** $p < 0.0001$; #### $p < 0.0001$; * y # se refieren al tamaño de partícula y potencial-ζ, respectivamente).

NCs cargadas con siRNA terapéutico (siRNA*)

En el apartado anterior se presentaron las condiciones idóneas para la asociación de un determinado siRNA, que hemos considerado como modelo, cuya denominación y uso terapéutico son confidenciales. Además, se han explorado las condiciones necesarias para la asociación de otro siRNA terapéutico, distinto del anterior, al que hemos denominado siRNA*. Las características fisicoquímicas de las NEs cargadas con los distintos siRNAs fueron muy similares, sin embargo, la formación de la cubierta de protamina no se logró en las mismas condiciones de formulación. De este modo, se ensayaron distintas condiciones para la asociación del siRNA* (ratios v/v 1:1 hasta 1:4) a las nanoemulsiones, tal y como se refleja en la Fig. 3, las cuales fueron posteriormente recubiertas, utilizando diferentes concentraciones de protamina (proporciones siRNA:protamina p/p de 1:0,1 y 1:0,3). Los resultados muestran que las características fisicoquímicas de las NCs no se vieron afectadas por las condiciones de formulación, sin embargo, al igual que ocurrió con el siRNA modelo utilizado anteriormente, las NCs de protamina que contenían cetrimida, fureon las que permitieron un mayor rendimiento de asociación del siRNA (2,3 mg/mL, correspondiendo a una carga del 7,4 %), mientras que las que contenían Bz Cl y LAE presentaron una concentración final de siRNA* de 0,8 y 1,7 mg/mL (correspondiendo a una carga de 4,7 y 3,8 %), respectivamente.

Surfactante	NE:siRNA* (v/v)	NEs siRNA cargado (mg/mL)	NC siRNA*:Prot. (p/p)	Tamaño (nm)	PDI	Potencial-ζ (mV)
Cetrimida	1:2	2.75	-	131 ± 6	0.2	-18 ± 5
			1:0.3	137 ± 8	0.3	+24 ± 0
Bz Cl	1:4	1	-	112 ± 1	0.2	+9 ± 1
			1:0.3	118 ± 0	0.2	+29 ± 0
LAE	1:1	2	-	115 ± 7	0.1	+5 ± 5
			1:0.1	125 ± 2	0.2	+16 ± 0

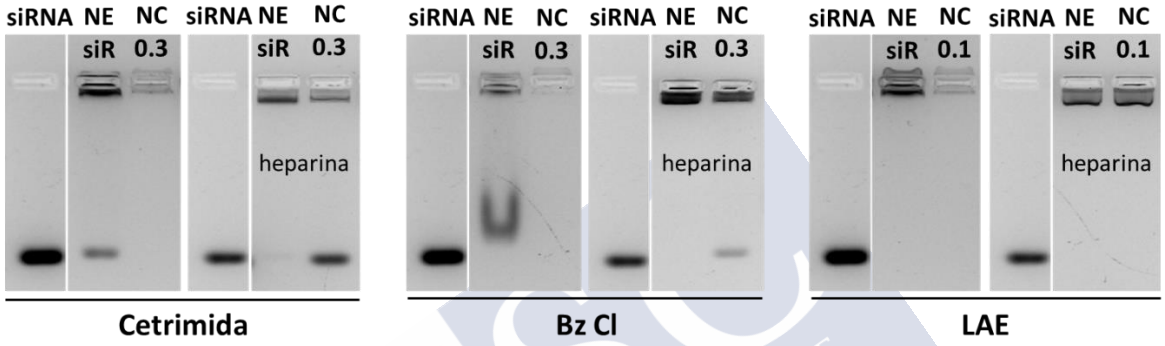

Fig. 3 - Condiciones requeridas para la preparación de las NCs de protamina cargadas con siRNA*. Ensayo en gel de agarosa antes y después de la incubación de los sistemas con heparina. NC 0.1 y NC 0.3 representan las NCs de protamina con una cubierta de siRNA:protamina p/p de 1:0,1 y 1:0,3. Los valores representan la media ± DE (n=3).

El paso siguiente en la caracterización de las formulaciones ha consistido en la evaluación de su estabilidad tras su incubación en fluido lacrimal o en medio de cultivo celular. Los resultados presentados en la Fig. 4 muestran que las NCs de protamina cargadas con siRNA* son estables en estos medios y evitan la liberación prematura del siRNA.

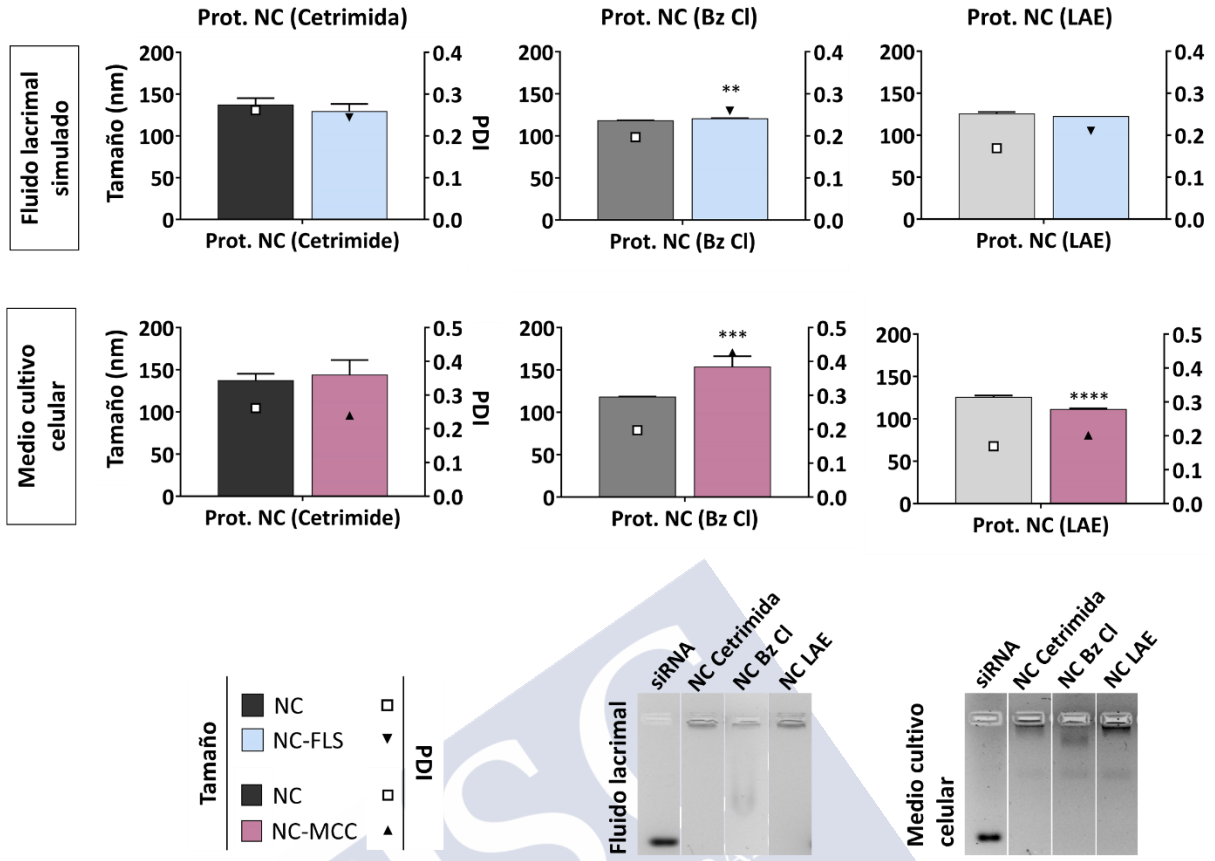

Fig. 4 - Estabilidad coloidal de las NCs de protamina (cargadas con siRNA*) en fluido lacrimal simulado (FLS, 30 min, 37 °C) y en medio de cultivo celular (MCC, 1 h a 37 °C). Las barras grises representan las NCs antes de la incubación con los medios y las barras azules y rosas representan las NCs después de la incubación con fluido lacrimal durante 30 min a 37 °C y con medio de cultivo celular durante 1 h a 37 °C, respectivamente. Los geles muestran la ausencia de liberación del siRNA después de la incubación de las NCs con los respectivos medios. Los valores representan la media ± DE (n=3). Los datos han sido analizados por ANOVA de dos vías (** $p < 0.01$, *** $p < 0.001$. * se refiere al tamaño de partícula).

Finalmente, se estudió el efecto de las NCs de protamina (conteniendo cetrimida, Bz Cl o LAE) en la viabilidad y capacidad de silenciamiento en células HeLa tras su incubación durante un periodo de 1 h. Los resultados de actividad metabólica determinada mediante el método MTS (Fig. 5) indican la ausencia de citotoxicidad en las condiciones ensayadas, lo que permite concluir que la incorporación del agente catiónico en las NCs permite la asociación de altas concentraciones de siRNA sin afectar la viabilidad celular. Sin embargo, el tratamiento de las células con las NCs cargadas con 100 nM de siRNA* durante el mismo periodo de tiempo, apenas ha logrado un silenciamiento de cerca de 19, 10 y 11 %, respectivamente, mientras

que el tratamiento con el agente de transfección comercial DharmaFECT™ 3 ha resultado en un silenciamiento de 52 % (Fig. 5).

La explicación a la insuficiente capacidad de silenciamiento de los prototipos de NCs de protamina puede hallarse en el mecanismo de liberación de las mismas. Para que el siRNA llegue a su destino y haga su función, este tiene que superar distintas barreras celulares. Considerando la eficaz penetración celular de otros prototipos conteniendo protamina [27, 28] o quitosano [29] en tan solo 30 min de incubación, consideramos que el periodo de incubación de 1 h realizado en este estudio ha sido suficiente para que las NCs hayan sido internalizadas, pero no podemos descartar de forma segura que la incubación por un periodo de tiempo más largo no resultaría en una eficacia de silenciamiento más elevada. Otra posible explicación puede ser la degradación endosomal, uno de los obstáculos más grandes en terapias de nucleótidos. Además, las fuertes interacciones del siRNA con el surfactante catiónico y la protamina, que han demostrado dificultar su desplazamiento con heparina, pueden haber resultado en la incapacidad de liberación del siRNA para el citosol.

Por forma a lograr una formulación de NCs de protamina capaz de asociar una alta concentración de siRNA (1-5 mg/mL) y a la vez de liberar el siRNA de forma controlada, y que resulte en el silenciamiento del gen de interés, otras composiciones en específico el uso de otros agentes tensioactivos catiónicos se deberían de explorar.

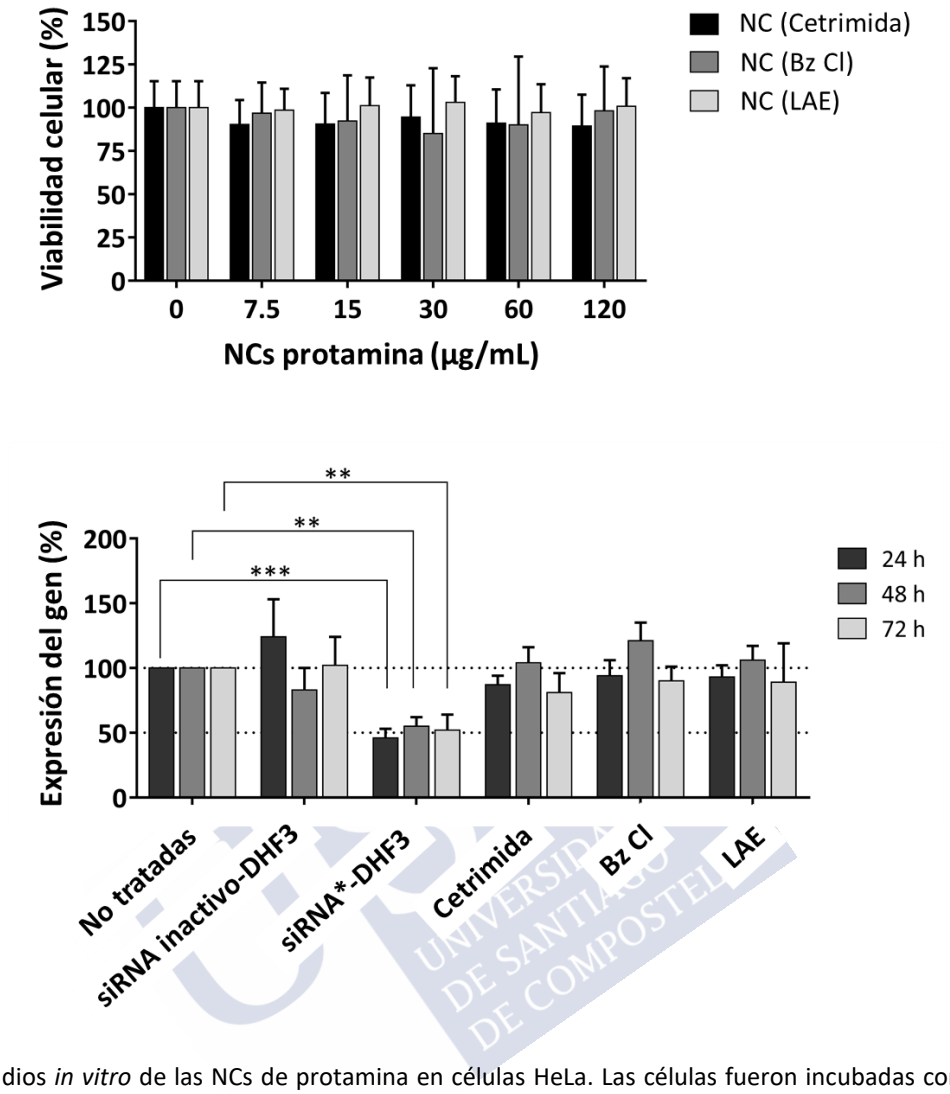

Fig. 5 - Estudios *in vitro* de las NCs de protamina en células HeLa. Las células fueron incubadas con crecientes concentraciones de NCs durante 1 h a 37 °C y 24 h post-tratamiento la viabilidad celular se determinó por MTS. El análisis cuantitativo de expresión génica se hizo por PCR en tiempo real, después de incubar las células con las NCs de protamina cargadas con 100 nM de siRNA*. Los valores representan la media ± DE (n=3). Los datos han sido analizados por ANOVA de dos vías (** $p < 0.01$, *** $p<0.001$).

2.2. Nanocápsulas de protamina como sistemas de liberación de siRNA preparadas por la técnica de auto-emulsificación

Teniendo en cuenta el papel que juegan los agentes tensoactivos catiónicos en la asociación/disociación del siRNA, así como su posible papel en la liberación intracelular y escape endosomal, y que el principal requisito de los nanovehiculos desarrollados en este trabajo es que sean capaces de cargar una concentración de siRNA de 1 mg/mL, preferentemente de 5 mg/mL, en este capítulo se optó por estudiar el efecto de la oleilamina, surfactante que presenta una amina primaria y de la cetrimida. Además, en este trabajo se optó por la técnica de auto-emulsificación para la formación de las nanoemulsiones, por ser una técnica fácilmente escalable.

Preparación de una nanoemulsion catiónica y con alta capacidad de asociación de siRNA

Para la preparación de estas NEs se seleccionaron dos surfactantes, la cetrimida debido a su elevada capacidad de asociación de siRNA en comparación con Bz Cl y LAE (demostrada en el capítulo 1) y por otro lado, la oleilamina, la cual presenta una amina primaria y una cadena carbonada insaturada que le confieren la capacidad de asociar nucleótidos y de mejorar la capacidad de transfección [30-32]. El Labrafac™ lipophile WL 1349 y el polisorbato 80, un triglicérido de cadena media y un surfactante no iónico han sido seleccionados considerando su uso común en formulaciones oculares y en la preparación de sistemas por auto-emulsificación.

La oleilamina fue compatible con el labrafac™ y el polisorbato 80 y resultó particularmente interesante para conseguir una elevada asociación de siRNA, logrando una concentración de 6 mg/mL de siRNA. En el caso de la cetrimida, se utilizaron otros aceites (labrafac, vitamina E o aceite de ricino), que favoreciesen su inclusión en el sistema. De todos ellos, la vitamina E fue la que permitió lograr la más alta concentración de siRNA asociado al sistema (1,6 mg/mL).

Estos resultados parecen contradecir los estudios de Hagigit et al. y Martini et al. que demuestran que la oleilamina, constituida por una amina primaria, presenta una capacidad de asociación de siRNA menor que la de compuestos como el DOTAP que contienen una amina

cuaternaria [30, 31], como el caso de la cetrimida usada en este trabajo. Sin embargo, los resultados aquí obtenidos se pueden deber al carácter menos hidrofóbico de la cetrimida en comparación con el DOTAP y la oleilamina. Así, podría asumirse que la oleilamina presenta una mejor miscibilidad y inclusión en la estructura de la NE, exponiendo su grupo catiónico en la superficie de la NE y permitiendo la adsorción eficaz del siRNA.

Considerando la alta capacidad de asociación de las NEs de oleilamina, este prototipo fue seleccionado para el recubrimiento con protamina. Se testaron distintas concentraciones de protamina (1,5, 3 y 5 mg/mL en la solución de polímero inicial) culminando en la preparación de NCs de protamina con una concentración final de siRNA de 5 mg/mL, correspondiendo a una carga de 3,9 %.

Nanocápsulas de protamina: estabilidad en fluido lacrimal y durante el almacenamiento

Las NCs de protamina (carga 3,9 %) fueron estables en fluido lacrimal durante al menos 1 h a 37 °C, manteniendo su tamaño y evitando la liberación precoz de siRNA (Fig. 6). La liberación del siRNA únicamente se logró en presencia de heparina. Además, estas nanocápsulas presentaron una mejor estabilidad en almacenamiento a 4 °C durante 15 días, que las correspondientes NE-siRNA (sin protamina), manteniendo no solo sus propiedades fisicoquímicas, pero también el siRNA completamente asociado.

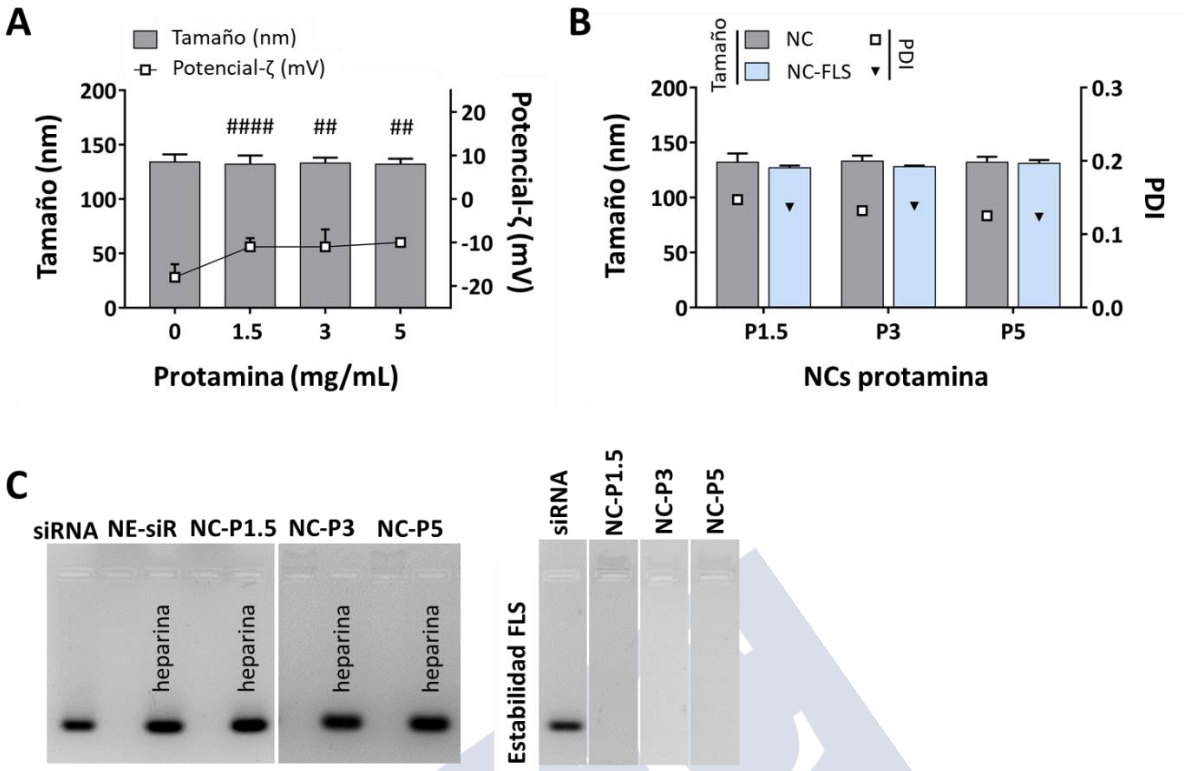

Fig. 6 - Características fisicoquímicas y de capacidad de carga de siRNA de las NCs de protamina (5 mg/mL siRNA). Efecto de la adición de concentraciones crecientes de protamina en el tamaño y potencial-ζ de las partículas (**A**). Estabilidad coloidal de las NCs de protamina (NC P1.5, NC P3 y NC P5 se refieren a las NCs preparadas con una solución de protamina a la concentración inicial de 1,5, 3 o 5 mg/mL, respectivamente) en fluido lacrimal simulado (FLS) durante 1 h a 37 °C (**B**). Las barras grises y azules corresponden a las NCs antes y después de la incubación con el fluido lacrimal, respectivamente. Gel de agarosa de las NE-siRNA antes (NE-siR) y después de la recubierta con protamina (NC-P1.5, NC-P3 7 NC-P5) y su capacidad de libertar siRNA tras la incubación con heparina (**C**, lado izquierdo). Gel representativo de la capacidad de las NCs para mantener el siRNA asociado durante 1 h de incubación con fluido lacrimal (**C**, lado derecho). Los valores representan la media ± DE (n ≥ 3). Los datos han sido analizados por ANOVA de dos vías (## $p < 0.01$, #### $p < 0.0001$. # se refiere al potencial-ζ).

Las formulaciones (NE-siRNA y NCs de protamina) fueron liofilizadas en presencia de trehalosa (10 %, p/v) para intentar mejorar la estabilidad de las formulaciones durante el almacenamiento. La liofilización de las NCs en presencia del crioprotector causó un cambio en el potencial-ζ de las NCs (de negativo a neutro), que se atribuye al reemplazamiento del agua por trehalosa durante el proceso de liofilización [33, 34]. Las condiciones de almacenamiento, notablemente la temperatura, influenciaron la estabilidad de las formulaciones de NE-siRNA, observandose una liberación del siRNA cuando fueron almacenadas a temperatura ambiente, mientras que a 4 °C se mantuvo el siRNA totalmente asociado.

Las estabilidad de las NCs en estado solido (polvo) fue estudiada a 4 °C y temperatura ambiente. La temperatura influyó en la estabilidad de las NCs, de modo que las NCs almacenadas a 4 °C, mantuvieron sus propriedades fisicoquímicas durante 15 días y el siRNA asociado, así como su estabilidad en fluido lacrimal (Fig. 7A y B), mientras que el almacenamiento a temperatura ambiente resultó en la agregación de la formulación. En cuanto a la estabilidad de las formulaciones resupendidas tras su liofilización, se observó un ligero incremento de tamaño tras su almacenamiento a 4 °C durante 15 días (Fig. 7C), manteniendo el siRNA asociado.

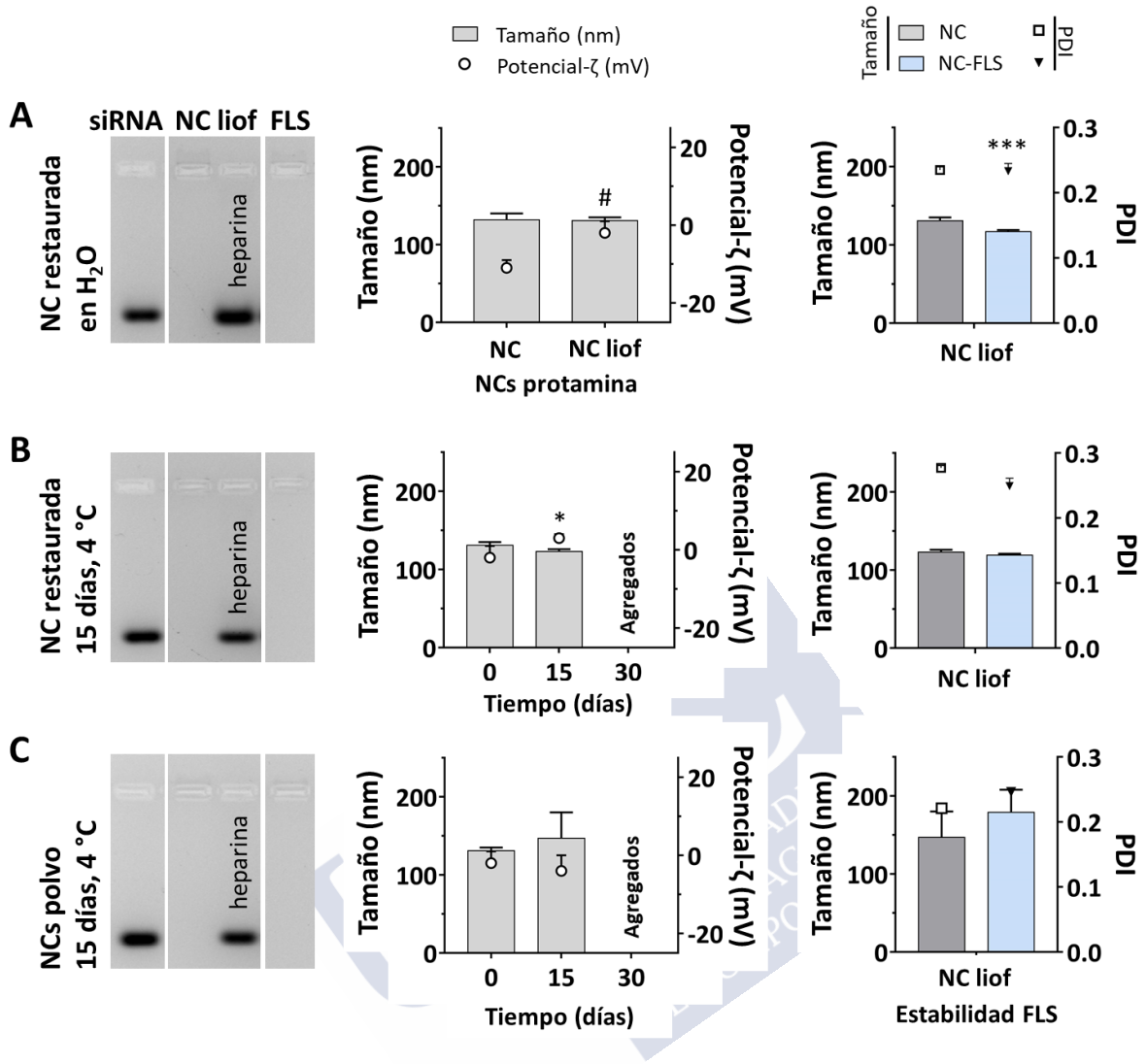

Fig. 7 - Características fisicoquímicas de las NCs de protamina liofilizadas: resuspendidas en agua (**A**), almacenadas a 4 °C tras su resuspensión (**B**) o en estado sólido (polvo) (**C**) y sus respectivas estabilidades en fluido lacrimal simulado (FLS) durante de 1 h a 37 °C (barras azules). Los geles de agarosa muestran el siRNA asociado a las NCs, antes y después de la incubación con heparina, o tras su incubación en FLS. Los valores representan la media ± DE (n ≥ 3). Los datos han sido analizados por ANOVA de dos vías ANOVA (* $p < 0.05$; *** $p < 0.001$; # $p < 0.05$. * y # se refieren al tamaño de partícula y potencial-ζ, respectivamente).

Para la realización de los estudios *in vitro*, se ha utilizado un siRNA terapéutico (siRNA*). Las condiciones previamente optimizadas para la preparación de las NCs de protamina cargadas con siRNA fueron utilizadas con éxito para la encapsulación del siRNA*, presentando características similares, de tamaño y potencial-ζ, estabilidad en fluido lacrimal, así como en almacenamiento a 4 °C (Fig. 8). Así, las NCs de protamina presentaron una carga de siRNA de 3,9 %, que corresponde a una concentración final 5 mg/mL. Además, las NCs mantuvieron su

capacidad de liberar el siRNA tras la incubación en presencia de heparina. La capacidad de mantener el siRNA asociado durante la incubación con el medio de interés o durante el almacenamiento, pero a la vez liberarlo cuando incubado con heparina es importante porque, por un lado, impide que haya una liberación precoz y, por otro, sugiere que el siRNA podrá ser liberado cuando el sistema se desensamble o exista un agente que desplace el siRNA.

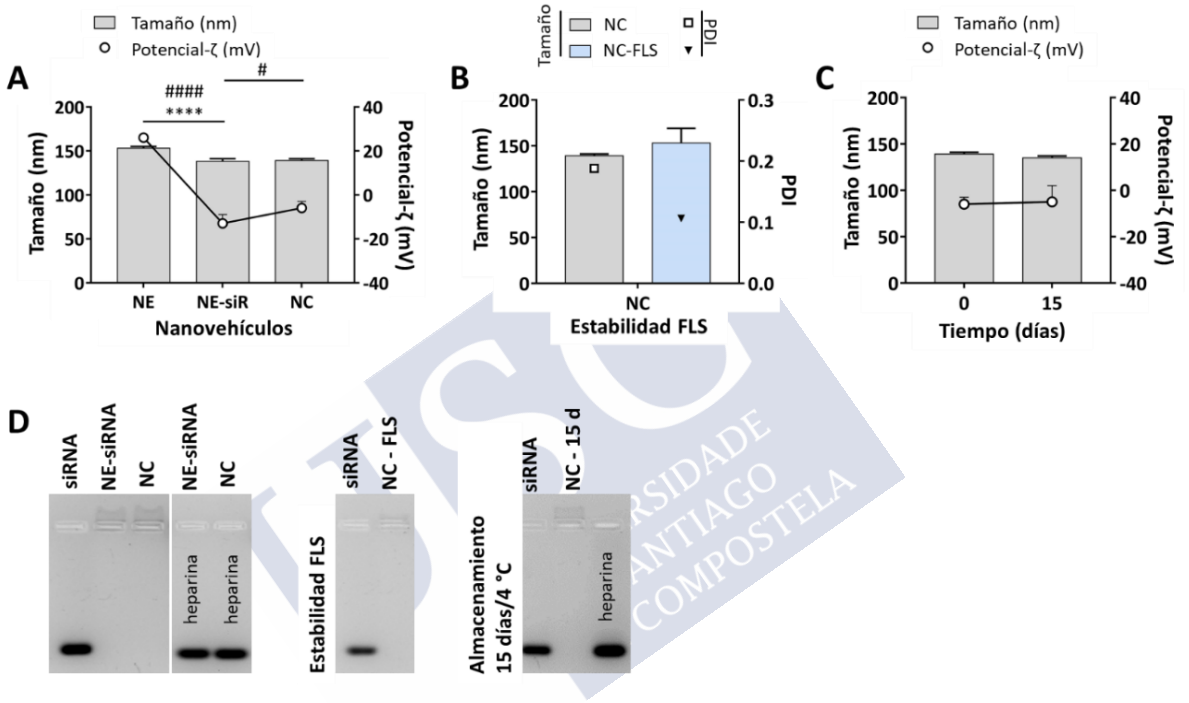

Fig. 8 - Características físicoquímicas de las NCs de protamina cargadas con siRNA*. Efecto de la asociación del siRNA* a las NEs y de la recubierta con protamina (**A**) en el tamaño y potencial-ζ de las partículas. Estabilidad de las NCs de protamina en fluido lacrimal (FLS) durante 30 min a 37 °C (**B**) y en almacenamiento a 4 °C durante 15 días (**C**). Geles de agarosa representativo de las NE-siRNA* y NCs de protamina antes y después de la incubación con heparina; de las NCs incubadas con fluido lacrimal; y después de su almacenamiento a 4 °C (**D**). Los valores representan la media ± DE (n ≥ 3). Los datos han sido analizados por ANOVA de dos vías (**** $p < 0.0001$; # $p < 0.05$, #### $p < 0.0001$. * y # se refieren al tamaño de partícula y potencial-ζ, respectivamente).

Estudios *in vitro en células HeLa*

Los estudios *in vitro* de viabilidad celular y silenciamiento se realizaron utilizando células HeLa como modelo (Fig. 9). Las NCs de protamina conteniendo oleilamina presentaron un buen perfil de biocompatibilidad hasta 30 µg/mL, que corresponden a la concentración de 100 nM de siRNA* usada en el estudio de silenciamiento. La expresión génica de las células HeLa

previamente incubadas durante 1 h con las NCs de protamina, fue analizada a las 24, 48 y 72 h post-tratamiento. Los resultados muestran que las NCs dieron lugar a un nivel de 24 % de silenciamiento del gen de interés a las 72 h post-tratamiento, siendo dicho efecto comparable al obtenido con el agente de transfección comercial DharmaFECT™ 3. Este moderado silenciamiento, logrado en ambos casos, podría ser atribuible al limitado tiempo de tratamiento y las condiciones experimentales. En el caso de las NCs de protamina, la respuesta lenta podrá atribuirse a una liberación gradual del siRNA de las NCs. Aunque no se puede descartar la posibilidad de que el siRNA haya sido degradado en los endosomas. Para corroborar estas hipótesis, se podría incrementar la dosis de siRNA utilizada, incluir en la composición de las NCs compuestos con capacidad fusogénica [35-37] y estudiar la expresión génica durante un periodo más largo.

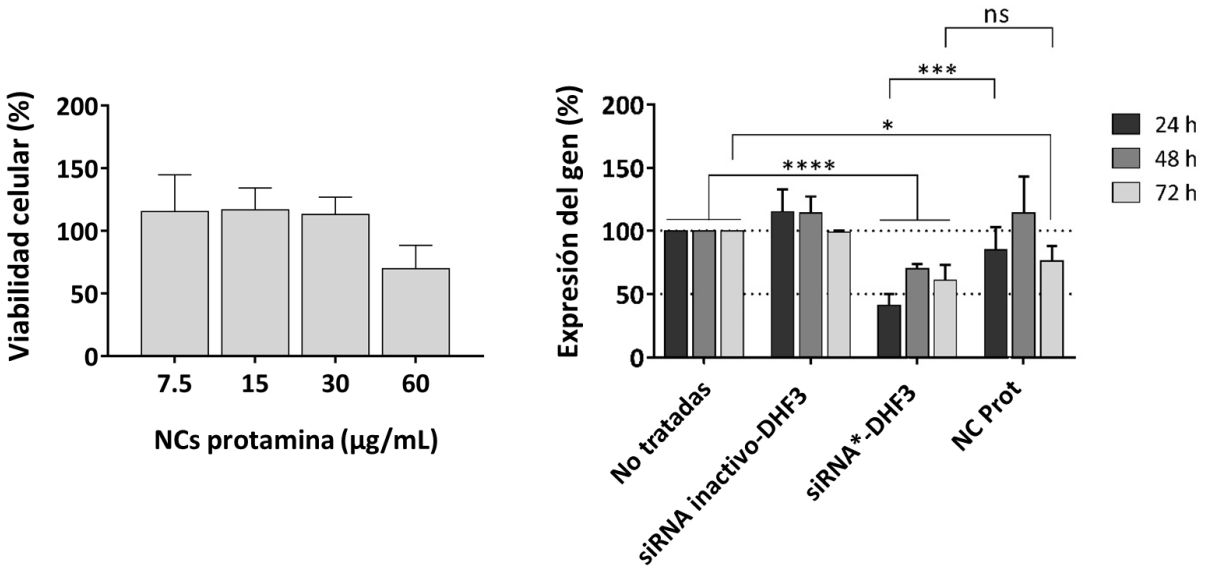

Fig. 9 - Estudios *in vitro* de las NCs de protamina en células HeLa. Las células fueron incubadas con 7.5-60 µg/mL de NCs de protamina durante 1 h a 37 °C y 24 h post-tratamiento la viabilidad celular se determinó por MTS (izquierda). El análisis cuantitativo de expresión génica se hizo por PCR en tiempo real, después de incubar las células con las NCs cargadas con 100 nM de siRNA* (derecha). Los valores representan la media ± DE (n=3). Los datos han sido analizados por ANOVA de dos vías (ns, no significativo, * $p < 0.05$; *** $p < 0.001$, **** $p<0.0001$).

Estudios *in vivo*

La capacidad de las nanocápsulas de protamina para interaccionar y penetrar a través del epitelio de la córnea fue evaluada en ojos de conejos New Zealand. Las nanocápsulas fluorescentes fueran preparadas usando una cubierta de protamina-TAMRA y administradas tópicamente. Transcurrido un periodo de 6 horas, se extrajeron las corneas y se procedió a su observación en el microscopio de fluorescencia confocal. Los resultados mostraron la presencia NCs en la capa externa del epitelio de la córnea (Fig. 10), la cual podría explicarse por la capacidad de penetración celular de la protamina [20] cuyas aminas forman puentes de hidrogeno con los grupos fosfato y sulfato presentes en la membrana de las células [38].

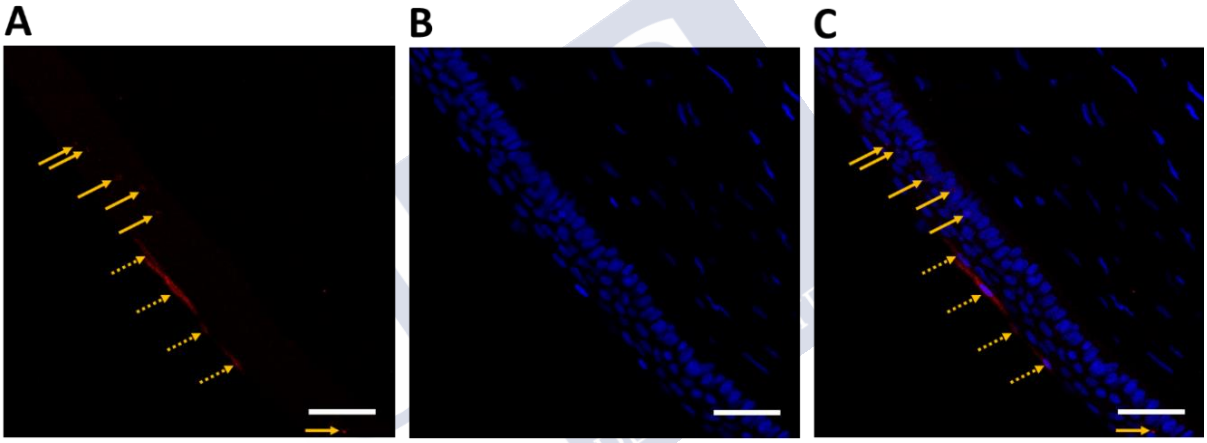

Fig. 10 - Imágenes de microscopia confocal del epitelio de la córnea, representativas de la biodistribución de las NCs de protamina 6 h después de su administración tópica en ojos de conejo saludables. El canal azul corresponde a los núcleos de las células marcados con DAPI y el canal rojo corresponde a las NCs de protamina-TAMRA. Las flechas amarillas punteadas o en línea indican la presencia de NCs en la superficie e interior de la capa externa del epitelio de la cornea, respectivamente. Todas las escalas corresponden a 50 µm.

3. Conclusiones

El principal objetivo de este trabajo ha sido el desarrollo de nanocápsulas de protamina capaces de cargar altas concentraciones de siRNA (hasta cerca de 5 mg/mL) para mejorar su biodisponibilidad y la eficacia del tratamiento cuando aplicado tópicamente en el ojo. Se utilizaron dos tipos distintos de siRNA, el siRNA modelo, el cual se utilizó para optimizar las condiciones de preparación de las NCs y el siRNA* para estudiar la biocompatibilidad y eficacia de silenciamiento de los prototipos desarrollados.

Se estudiaron distintas composiciones, en concreto distintos aceites y surfactantes catiónicos (cetrimida, Bz Cl, LAE y oleilamina) y dos métodos distintos de formulación (desplazamiento de disolvente y auto emulsificación) para la preparación del núcleo oleoso responsable de la asociación del siRNA. El prototipo de NCs de protamina conteniendo oleilamina fue el que premitió obtener la más alta concentración de siRNA en la formulación, 5 mg/mL que corresponde a una carga de 3,9 %. Seguidamente, las NCs conteniendo cetrimida presentaron una carga de 7,4 %, la cual corresponde a una concentración final de siRNA de 2,5 mg/mL en la formulación.

La composición de las NCs, principalmente el surfactante catiónico usado, además de influenciar la eficacia de asociación de siRNA también tuvo impacto en la capacidad de los nanosistemas para liberar el siRNA. De hecho, únicamente la formulación que contenía oleilamina en su composición, resultó eficaz desde el punto de vista de su capacidad de silenciamiento. Finalmente, las NCs de protamina conteniendo oleilamina fueron capaces de interaccionar con el epitelio de la córnea de conejos, demostrando su posible potencial para administración tópica ocular de siRNA.

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Introduction

Synthetic nanocarriers for the delivery of polynucleotides to the eye

This introduction has been adapted from a published review: Saraiva et al. "Synthetic nanocarriers for the delivery of polynucleotides to the eye". European Journal of Pharmaceutical Sciences 103 (2017) p. 5-18

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According to the international Vision loss expert group (VLEG), in 2015 about 217 million of people worldwide suffered from moderate or severe vision impairment and 36 million were blind. By 2020, the number of people suffering from blindness is expected to increase to about 39 million individuals [1, 2], being leading diseases cataracts, glaucoma, age-related macular degeneration (AMD) and diabetic retinopathy. This scenario underlines the necessity of more innovative and effective ocular therapy strategies. Nowadays, approaches based on polynucleotide ocular delivery hold great promise since they may alter gene expression without affecting the structure and sequence of the gene.

The eye is an attractive organ for the development of polynucleotide-based therapies due to the fact that the target tissues are accessible without the need of systemic administration. However, apart from this, the eye is protected by extraordinary barriers, which are very difficult to circumvent, especially in the case of hydrophilic and high molecular weight molecules such as polynucleotides. These barriers are illustrated in Fig. 1, and are briefly described as follows:

In the anterior segment, the first barrier encountered by topically applied molecules is the tear film that is composed of three layers consisting of lipid, aqueous fluid and mucus layers. The presence of different enzymes and mucins in the tear film as well as its constant turnover protect the eye against external pathogens. This is followed by the glycocalyx which is formed by cell surface mucins and covers the surface of the corneal and conjunctival epithelia [3]. The corneal barrier consists of a transparent and avascular multiple layer epithelium, a collagenous layer (stroma) and an internal endothelium. The corneal epithelium continues with the conjunctiva, a transparent and vascularized epithelial membrane that contains goblet cells which are responsible for the production of the mucin MUC5AC [4]. In addition, the presence of tight junctions in both tissues constitutes an obstacle for permeation of drugs, especially through the cornea [5]. The aqueous humor is also part of the anterior segment and it is mostly composed of water and electrolytes, low molecular weight compounds and proteins [6, 7].

The posterior segment is protected by the sclera, which represents the continuation of the cornea, and it is formed by the vitreous humor, retina, choroid and optical nerve. The vitreous humor is a highly dense matrix mainly composed of collagen, hyaluronic acid (HA) and also

proteoglycans that contain negatively charged glycosaminoglycans (GAGs), that can hinder the diffusion of drugs to the retina, even when they are directly injected into this compartment [8]. However, it can also serve as a reservoir for the sustained release of drugs [9]. The retina encompasses different cell layers consisting mainly of nerve cells (ganglion cell layer (GCL)), photoreceptors and retinal pigment epithelium (RPE).

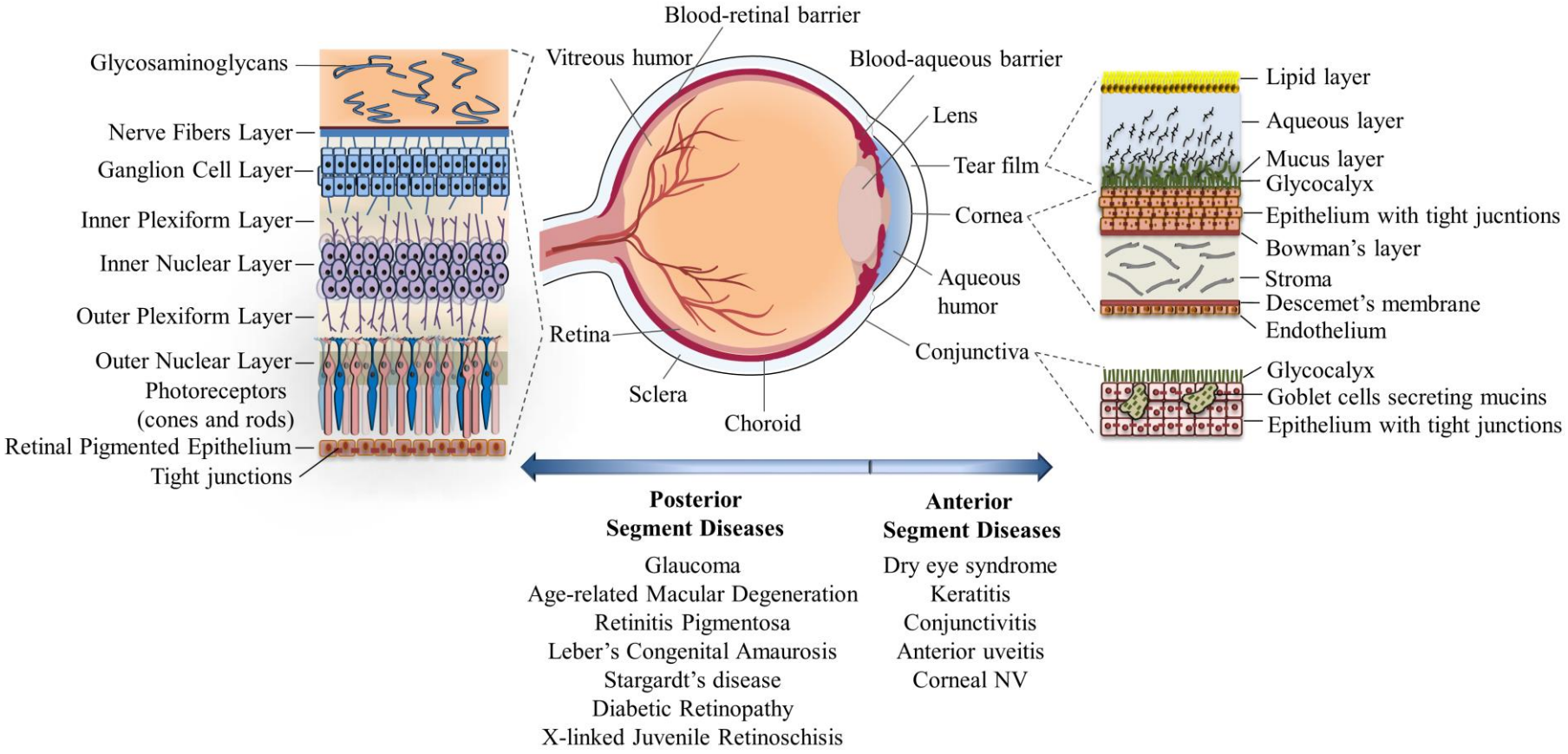

Fig. 1 - Representation of the structure of human eye (in more detail the tear film, cornea, conjunctiva, vitreous humor and retina) and some examples of diseases affecting both anterior and posterior segments.

Both the anterior and posterior segments are also protected by blood-barriers, the blood-aqueous and the blood-retinal barriers, respectively. The blood-aqueous barrier contains the uveal endothelium and ciliary epithelium. This barrier restricts the access of compounds such as plasma albumin and hydrophilic drugs into the aqueous humor, but it is also responsible for the passage of nutrients essential for corneal function [10]. The inner and outer blood-retinal barrier is formed by the retinal vessels' endothelial cells and the retinal pigment epithelium cells, respectively. In both parts of the blood-retinal barrier, the constituent cells are connected by tight junctions. This barrier plays a fundamental role in the regulation of nutrients flux and the restriction of drug diffusion into and out of the retina [11].

There are several routes intended to reach either the anterior or posterior segments of the eye. Topical administration and subconjunctival injections are normally oriented towards treating the anterior segment whereas intravitreal (IVT) and subretinal injections are the most common methods used for the treatment of relevant diseases that affect the back of the eye.

In the next sections we will comparatively analyse the nanotechnology-based strategies that have been reported so far to deliver polynucleotides to both the anterior and posterior segments of the eye. We will highlight their potential for targeting specific tissues and thus, for the treatment of specific ocular diseases. We will conclude with a perspective of the challenges that need to be overcome for the clinical translation and industrial development of these nanomedicines.

1. Polynucleotides used for the treatment of ocular diseases

The polynucleotides that have been studied until now as potential treatments for ocular disorders are described below.

Plasmid DNA (pDNA)

Plasmid DNA-therapeutics aims to express a specific therapeutic gene. Therefore, the plasmid needs to be internalized into the nucleus of the cell (which is still a challenge) where it will be transcripted into a messenger RNA (mRNA). Thereafter, the newly formed mRNA is

transported into the cytoplasm where it is translated into the codified protein. The first nucleotide-based therapy reaching clinical trials was a pDNA construct proposed for the treatment of an immunodeficiency disease caused by an adenosine deaminase deficiency [12]. Since then, according to the clinical trials database (clinicaltrials.gov), several pDNA clinical trials have been conducted although only four of them have been oriented to the treatment of ocular diseases focusing on intraocular melanoma, allergic rhinoconjunctivitis and uveitis (Table 1).

Antisense oligonucleotides (AS-ODNs)

AS-ODNs are synthetic single-stranded RNA fragments (13 to 25 nucleotides) firstly described in 1978 [13], that bind to complementary intracellular mRNA strands by base pairing, forming a short double helix and ultimately blocking its transcription into the undesirable protein. AS-ODNs can also modulate gene expression by enzymatic degradation of targeted mRNA by ribonuclease H [14]. The activity of AS-ODNs is highly limited due to their poor intracellular uptake and poor stability in biological fluids [15].

The only AS-ODNs-based drug (without the association to any type of carrier) approved by the Food and drug administration (FDA) for an ocular condition was registered in 1998 (Table 2). This nucleic-acid based drug, fomivirsen, was marketed as Vitravene® for the treatment of cytomegalovirus (CMV)-induced retinitis in immunocompromised patients [16]. However, in 2004 Novartis Ophtalmics discontinued the product due to the significant decrease of Vitravene® sales as a consequence of the low number of patients infected with CMV. Other AS-ODNs are currently under clinical trials for the treatment of different ocular diseases (see Table 1). For instance, aganirsen (GS-101) has completed a phase III clinical trial for the topical treatment of corneal neovascularization [17, 18] and it is currently in phase II for neovascular glaucoma among other conditions. Other examples of AS-ODNs that have recently entered clinical trials are IONIS-FB-LRx, QR412 and QR-100 for the treatment of geographic atrophy (GA), Usher syndrome type 2 and Leber's congenital amaurosis (LCA), respectively (Table 1).

Small interfering RNA (siRNA)

RNAi-based technology, namely siRNA is a promising alternative for treating eye diseases affecting both, the anterior and posterior segments of the eye. This is a double-stranded RNA (dsRNA) of 21-23 base pairs designed to specifically knockdown target genes [19]. Unlike pDNA, this type of polynucleotide only needs to get into the cytoplasm of the cell where it is loaded into the RNA-induced silencing complex (RISC) [20].

The first clinical trial using a siRNA (Cand5) was conducted in 2004 for the treatment of wet AMD and, since then, other clinical trials based on siRNA have been performed for ocular therapies such as Sirna-027 for choroidal neovascularization (CNV), PF-04523655 for diabetic retinopathy, SYL1001 for dry eye and SYL040012 and QPI-1007 for glaucoma (Table 1). Moreover, several siRNA therapies are under preclinical development for corneal neovascularization (corneal NV), retinitis pigmentosa, diabetic retinopathy, among others. Nonetheless, the majority of the undergoing siRNA studies target diseases affecting the retina (Table 1).

As other polynucleotides, siRNA also suffers from poor stability in biological fluids and restricted capacity to enter cells. Different chemical modifications have been performed in the structure of polynucleotides especially to ameliorate their stability when in contact with biologic fluids thus, improving their bioavailability [21-25].

Aptamers

Aptamers are small molecules synthesized from DNA or RNA sequences that have the capacity to bind to specific proteins, as well as to nucleic acids and other compounds. Due to their unique three-dimensional structure they may act in a similar way as antibodies do but with the advantages of being non-immunogenic and highly stable molecules [26]. The only aptamer that has received marketing approval (Table 2), in 2004, for ocular administration is Pegaptanib sodium (Macugen®), which is an RNA-based aptamer directed against vascular endothelial growth factor (VEGF) [27]. There are other aptamers, i.e. pegpleranib (Fovista®) and ARC1905 (Zimura®) that have been under clinical trials (Phase III and Phase II) for the treatment of AMD and other disease indications (Table 1).

Table 1 - Products under clinical development for both the anterior and posterior segments of the eye.

Product	Indication	Target	Administration route	Developer	Status	ClinicalTrial.gov identifier
pDNA						
CryJ2-DNA-LAMP	Allergic Rhinoconjunctivitis	JRC allergens	Intramuscular injection	Immunomic Therapeutics, Inc.	Completed Phase I	NCT01966224 NCT01707069
Mouse gp100	Intraocular melanoma	Gp 100	Intramuscular/ epidermal jet injection	Memorial Sloan Kettering Cancer Center	Completed Phase I	NCT00398073
pEYS606	Non-infectious uveitis	TNF-α	Electrotransfection in ciliary muscle	Eyevensys S.A.S.	Recruting Phase I/II	NCT03308045
siRNA						
Cand5 (Bevasiranib)	Wet AMD	VEGF-A	Intravitreal injection	OPKO Health	Completed Phase II Terminated Phase III Withdrawn Phase III	NCT00259753 NCT00499590 NCT00557791
	DME				Completed Phase II	NCT00306904
Sirna-027 (AGN211745)	Wet AMD				Completed Phase I/II Terminated Phase II	NCT00363714 NCT00395057
QPI-1007	NAION	Caspase 2	Intravitreal injection	Quark	Completed Phase I, Recruiting Phase II/III	NCT01064505 NCT02341560
	APACG				Completed Phase II	NCT01965106
PF-04523655	Wet AMD	RTP801	Intravitreal injection	Quark/Pfizer	Completed Phase I Completed Phase II	NCT00725686 NCT00713518 NCT01445899
	DME				Terminated Phase II	NCT00701181

Product	Indication	Target	Administration route	Developer	Status	ClinicalTrial.gov identifier
siRNA						
SYL040012 (Bamosiran)	Glaucoma	β2 ADR	Eye drops	Sylentis	Completed Phase I/II Completed Phase II	NCT01227291 NCT01739244 NCT02250612
SYL1001 (Tivanisiran)	Ocular pain associated to DES	TRPV1	Eye drops	Sylentis	Completed Phase I/II	NCT01776658 NCT02455999
	DES				Completed phase III	NCT03108664
RXI-109	Subretinal fibrosis, Wet AMD	CTGF	Intravitreal injection	Rxi	Active Phase I/II	NCT02599064
AS-ODN						
GS-101 (aganirsen)	iCRVO patients at risk of developing NVG	IRS-1	Eye drops	Gene Signal	Not yet recruiting Phase II/III	NCT02947867
ISTH0036	Glaucoma, undergoing trabeculectomy	TGF-β2	Intravitreal injection	Isarna Therapeutics	Completed Phase I	NCT02406833
iCo-007	DME	c-raf kinase	Intravitreal injection	iCoTherapeutics	Completed Phase I Terminated Phase II	NCT00886808 NCT01565148
IONIS-FB-LRx	GA secondary to AMD	Complement factor B	Subcutaneous injection	Ionis Pharmaceuticals	Recruiting Phase II	NCT03815825
QR-421 a	Retinitis Pigmentosa Usher Syndrome Type 2	USH2A exon 13	Intravitreal injection	ProQR Therapeutics	Recruiting Phase I/II	NCT03780257
QR-110	LCA10	ciliopathy gene encoding CEP290	Intravitreal injection	ProQR Therapeutics	Recruiting Phase II/III Recruiting Phase I/II	NCT03913143 NCT03913130

Product	Indication	Target	Administration route	Developer	Status	ClinicalTrial.gov identifier
Aptamer						
Pegpleranib (Fovista®)	Wet AMD	PDGF-B	Intravitreal injection	Ophthotech Corporation	Completed Phase I	NCT00569140
						NCT02591914
					Completed Phase II	NCT01089517
					Terminated Phase III	NCT01944839
						NCT01940887
						NCT01940900
					Terminated Phase II	NCT02214628
	Von Hippel-Lindau Syndrome				Completed Phase I/II	NCT02859441
ARC1905 (Zimura®)	Wet AMD	C5	Intravitreal injection	Ophthotech Corporation	Completed Phase I	NCT00709527
					Completed Phase I	NCT03362190
	Dry AMD				Completed Phase I	NCT00950638
	IPCV				Completed Phase II	NCT02397954
					Withdrawn Phase II	NCT03374670
	GA secondary to Dry AMD				Active Phase II	NCT02686658
	Stargardt disease 1				Active Phase II	NCT03364153

pDNA, plasmid DNA; siRNA, small interfering RNA; AS-ODN, antisense oligonucleotides; NAION, Non-arteritic anterior ischemic optic neuropathy; APACG, Primary angle closure glaucoma; AMD, Age-related macular degeneration; DME, Diabetic macular edema; DES, Dry eye syndrome; iCRVO, Ischemic central retinal vein occlusion; NVG, Neovascular glaucoma; GA, Geographic atrophy; MD, macular degeneration; LCA10, Leber Congenital Amaurosis 10; IPCV, Idiopathic polypoidal choroidal vasculopathy; JCR, Japanese Red Cedar pollen; Gp 100, glycoprotein 100; TNF-α, Tumor necrosis factor α; VEGF-A, vascular endothelial growth factor A; RTP801, hypoxia-inducible factor 1-responsive gene; β2 ADR, β2-adrenergic receptor; TRPV1, transient receptor potential vanilloid 1; CTGF, connective tissue growth factor; IRS-1, insulin receptor substrate-1; TGF-β2, transforming growth factor β2; USH2A, gene encoding usherin; CEP290, centrosomal protein 290; PDGF-B, platelet-derived growth factor subunit B; C5, complement component 5

2. Synthetic nanocarriers for the delivery of polynucleotides to the eye

In general, the delivery of polynucleotides has been attempted using viral vectors (adenovirus (Adv), adeno-associated virus (AAVs), lentivirus, and retrovirus) and non-viral carriers (e.g. nanoparticles, liposomes, dendrimers, nanoemulsions, micelles, etc.). Currently, there are already some approved gene therapy products using viral vectors. These products include a recombinant Ads-p53 gene therapy for the treatment of head and neck squamous cell carcinoma (Gendicine®) [28], an oncolytic virus that promotes cytotoxicity in cancer cells for nasopharyngeal cancer (Oncorine®), a retroviral vector loaded with a human cytocidal cyclin G1 construct for the treatment of solid tumors (Rexin-G®) [29] and the recently approved AAV2, by both FDA and EMA, which contains a functional copy of the RPE65 gene for the treatment of retinal dystrophy caused by RPE65 gene mutation (Luxturna™). These therapies have been approved in China (i.e. Gendicine®, Oncorine®), Philippines (Rexin-G®), USA and Europe (Luxturna™). Glybera®, an AAV1 delivering a human lipoprotein lipase (LPL) variant, approved in Europe for the treatment of LPL deficiency was withdrawn in 2017. Finally in 2019, Zolgensma® an AAV9 delivering a cDNA, was approved by the FDA for the treatment Spinal muscular atrophy (SMA) on children (<2 years)

In addition to these gene therapy products based on the use of viral vectors, during the last years different polynucleotide-based therapies have been approved in the USA and Europe (Table 2) for the treatment of different human conditions. Interestingly, the only nucleotide-based therapy so far available making use of a carrier is Onpattro®, a siRNA therapy indicated in the treatment of polyneuropathy caused by hereditary transthyretin amyloidosis (hATTR) delivered by lipidic NPs.

Table 2 - Oligonucleotide-based therapies approved by the FDA or EMA for the treatment of different human conditions (no vector was used in these therapies except in the case of Onpattro®).

Product	Nucleotide	Carrier	Indication	FDA	EMA
Vitravene® (Novartis)	AS-ODN	-	Cytomegalovirus-induced retinitis in patients with AIDS	1998 - 2004*	1999 - 2002*
Macugen® (Bausch & Lomb)	aptamer	-	Neovascular AMD	2004	2006
Kynamro® (Kastle Therapeutics)	AS-ODN	-	Familial hypercholesterolemia	2013 - 2018*	-
Defitelio® (Jazz Pharmaceuticals)	ODN	-	Hepatic veno-occlusive disease	2016	2013
Exondys 51™ (Sarepta Therapeutics)	AS-ODN	-	Duchenne muscular dystrophy (amenable to exon 51 skipping)	2016	-
Spinraza® (Biogen)	AS-ODN	-	Spinal muscular atrophy	2016	2017
Onpattro® (Alnylam Pharmaceuticals)	siRNA	lipid NPs	hATTR amyloidosis in adults	2018	2018
Tegsedi™ (Akcea Therapeutics)	AS-ODN	-	hATTR amyloidosis in adults	2018	2018

FDA, U. S. Food and drug administration; EMA, European medicines agency; AS-ODN, antisense oligonucleotide; iRNA, interference RNA; NPs, nanoparticles; AIDS, acquired immune deficiency syndrome; AMD, age-related macular degeneration; hATTR, polyneuropathy caused by hereditary transthyretin. * withdrawn year.

Currently, there are other few ocular nanotechnology-based products on the market, that include a few over the counter products for the treatment of dry eye syndrome (DES), such as nanoemulsions (Lipimix™, Soothe XP®, Cationorm®), and liposomes (Lipomil®), as well as drug-containing nanomedicines, such as cyclosporin-A nanoemulsion (Restasis®) indicated for severe DES, a difluprednate nanoemulsion (Durezol®) indicated for the treatment of ocular inflammation and verteporfin liposomes (Visudyne®), a photodynamic therapy approved for the treatment of AMD. Despite of this, the development of synthetic nanocarriers for the delivery of polynucleotides to the eye is still in an early stage. This early development is illustrated in Fig. 2, whereby the number of research articles published per year describing non-viral carriers for ocular polynucleotide therapy has not increased since 2016.

An additional observation is that polymeric nanoparticles/nanocomplexes followed by liposomes/lipoplexes have been the nanostructures that have received most of the attention up to now. Fig. 2 also shows how that research has been concentrated on pDNA and siRNA delivery, with a clear recent tendency to move towards siRNA-based therapies.

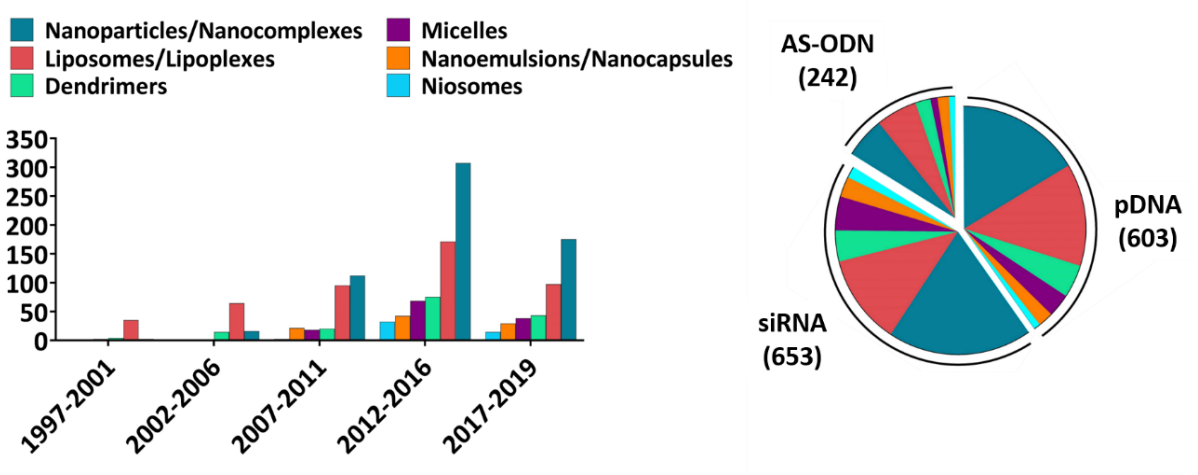

Fig. 2 - Illustration of the evolution of the number of published research articles describing the use of non-viral carriers for ocular polynucleotides delivery from 1997 to 2019. Data obtained from Scopus. Search defined with the combination of keywords and or groups of keywords: [ocular, eye] and/or [pDNA, RNA, siRNA, antisense oligonucleotides, polynucleotides] and/or [nanoparticles, nanocomplexes, liposomes, lipoplexes, niosomes, micelles, nanoemulsions, nanocapsules, dendrimers]. Language: English. (last consult performed on 18/07/2019)

Fig. 3 illustrates non-viral delivery systems currently under development for ocular polynucleotide/drug delivery. Overall, the composition and characteristics of the nanocarriers currently under investigation are discussed below.

Nanocomplexes and Nanoparticles

The design of these non-viral carriers for polynucleotide delivery has evolved over time going from simple nanocomplexes of cationic polymers, e.g. polyethyleneimine (PEI), and polynucleotides [30-33] to more defined nanoparticles. These nanoparticles have been developed using an array of biomaterials which include hydrophobic and amphiphilic polyesters, such as poly(lactic-co-glycolic acid) (PLGA) and PLGA-polyethylene glycol (PLA-PEG) [34, 35], which were investigated at first by our laboratory, as well as proteins such as gelatin [27], albumin [36], and cationic polymers, mainly poly-L-lysine (PLL) [33, 37] and chitosan [38-40].

Our group was among the pioneers in the development of nanoparticles for topical ocular drug delivery [41-44] and reported for the first time the potential of chitosan/hyaluronic acid nanoparticles for the delivery of polynucleotides, i.e. pDNA, to the eye [39, 40]. The inclusion

of HA allowed a better retention and permeation through the rabbits' corneal epithelium, which resulted in a more efficient transfection of the epithelial cells [39, 40]. The combination of different characteristics of these systems, such as biocompatibility, mucoadhesion and targeting of CD44 receptors make them suitable carriers for polynucleotide delivery to the cornea and conjunctiva. In a different study, Urtti's group determined that the HA coating of DNA/PEI complexes decreases PEI's toxicity by shielding the positive charges and reducing non-specific interactions with cell membrane by the CD44 receptor targeting [45].

With regard to the properties that influence the interaction of nanoparticles with the corneal epithelium, we have found that, in addition to the size [41], the surface composition of the nanoparticles and their charge play an important role in their interaction with the corneal epithelium. For example, we observed that chitosan nanoparticles have the ability to interact with the ocular mucosa and be internalized by the corneal epithelial cells [46]. Other authors have explored the functionalization of particles with specific targeting ligands, such as transferrin and arginine-glycine-aspartic acid (RGD), which are expected to improve nanoparticle uptake by ocular cells [47, 48].

Liposomes and lipoplexes

As an alternative to polymers, cationic lipids such as 3-β[N-(N',N' imethylaminoethane)-carbamoyl] cholesterol (DC-cholesterol) and 1,2-dioleoyl-3- trimethylammonium propane (DOTAP) [49-52], have also been used to produce complexes with polynucleotides, named lipoplexes or liposomes. Cationic lipids, in addition to their capacity to complex polynucleotides, are supposed to help the liposomes interacting with the corneal epithelium [53]. Neutral lipids such as 1,2-dioleoyl-3- hosphatidylethanolamine (DOPE) have also been included in liposomal formulations as "helper" lipids since they can change the conformation of these systems to an inverted hexagonal organization thus facilitating their endocytosis and delivering the polynucleotide into the cytoplasm [54, 55]. The surface modification of liposomes or lipoplexes with polymers has also been studied as a way to enhance their stability, e.g. through PEGylation [48, 56, 57] and to facilitate their internalization and

transfection efficiency through the use of peptide penetration enhancers [49, 58] and HA coating [59].

Stimuli-responsive liposomes have also been developed. This is the case of the light-induced liposomal formulation containing verteporfin (Visudyne®), which has been marketed for the treatment of AMD. Due to the different laser applications in ophthalmology, and the time- and site-specific drug release from light-activated liposomes, these systems are of particular interest for the ocular field [60]. More specifically, indocyanine green-liposomes might be an attractive option for ocular drug delivery. Indocyanine green is an FDA approved imaging agent and the only one approved for clinical use under near infrered (NIR) light, which is less damaging that UV light. Moreover, a fast exposure of these light-activated liposomes to NIR light, led to a complete release of the loaded calcein and lluorescein isothiocyanate-dextran (FITC-Dextran) [61].

Niosomes

These nanostructures are made of amphiphilic non-ionic surfactants such as Span® 60, Brij® 35, Brij® 78, Brij® 98 [62, 63], which are known for their penetration enhancer capacity. Several niosome formulations have been developed for ocular drug delivery and some of them have shown promising results for ocular polynucleotide delivery [64-67]. Still, as shown in Fig. 2, these carriers are the least common nanocarriers used for polynucleotide delivery to ocular tissues.

Micelles

Micelles comprise self-assembling diblock or multiblock amphiphilic molecules forming highly ordered monolayer structures. An example of a triblock copolymer micelle approved by the FDA for ophthalmic products is the micellar system formed by the copolymer poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) [68]. This specific composition has been explored for the delivery of pDNA to different ocular tissues [68-70].

Nanoemulsions and Nanocapsules

Nanoemulsions were evaluated in the early 90s by Benita's group for topical ocular drug delivery [71], and a few years later they were suggested for AS-ODN delivery [72]. More recently, Benita's group evaluated cationic nanoemulsions which contain the surfactant DOTAP to enhance the carrier residence time on the ocular surface and efficiently deliver AS-ODNs to the retina [73, 74].

Nanocapsules share common features with nanoemulsions and polymer nanoparticles, as they are formed by oily nanodroplets surrounded by a polymer coating. Our group pioneered the development of nanocapsules for topical ocular drug delivery [43, 75]. Interestingly, working with PEGylated polyester nanocapsules we also observed that the PEG coating was critical in terms of preserving the stability of these nanocapsules in the ocular fluids and, this improved stability was translated into a greater transport of the nanocapsules across the corneal epithelium [76]. More recently, we have found that nanocapsules containing polyarginine and protamine arginine-rich polymer shells, have an improved ocular retention and can be used for corneal wound healing [77].

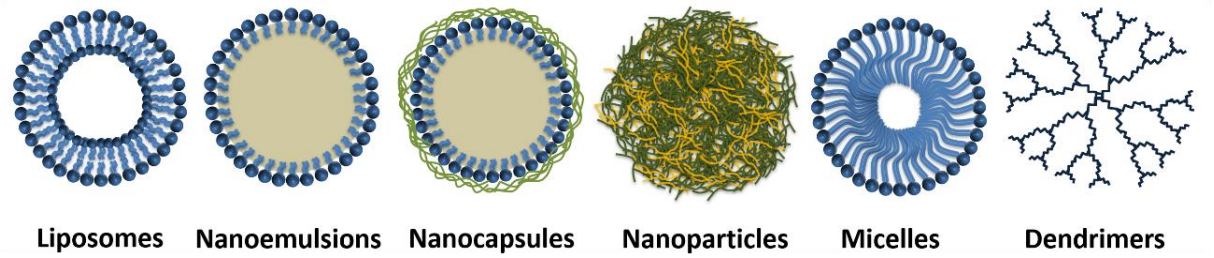

Fig. 3 - Representation of the structure of different types of nanocarriers.

Solid lipid nanoparticles (SLN)

SLN are made of solid lipids such as Compritol®888 ATO, Precirol® ATO 5, Gelucire® 44/14 and stearylamine [78, 79]. These nanoparticles have been shown to facilitate drug penetration into the cornea [78, 80, 81] and were used for the first time for ocular polynucleotide delivery in 2008 by del Pozo-Rodriguez [79].

Dendrimers

Dendrimers are tree-like branched structures that consist of an inner core, repetitive branched units (i.e. different generations) and peripheral multivalent functional groups, which play a key role in the complexation with polynucleotides. The use of Poly(amidoamine) (PAMAM) [82, 83] and PLL [84] based dendrimers for ocular polynucleotide delivery was first reported in 1999 and 2004, respectively. Since then, only a few reports have described the use of these nanocarriers for oligonucleotide [84-86] and pDNA [83, 87] ocular delivery and none for siRNA.

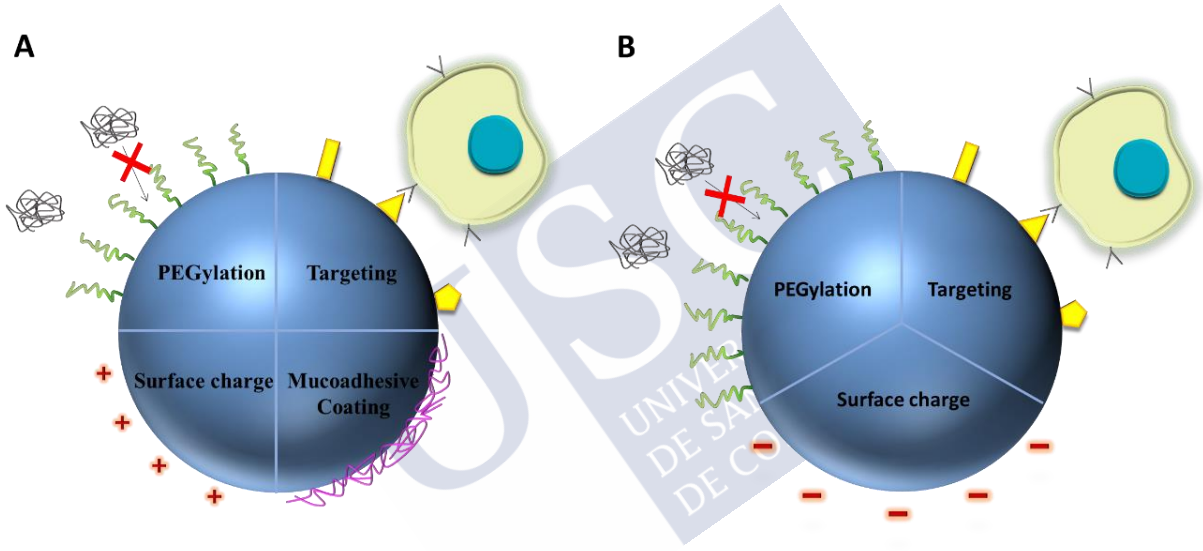

Fig. 4 - Schematic representation of the main strategies used to develop nanocarriers aiming to treat different diseases affecting both anterior (**A**) and posterior eye segments (**B**). When targeting the anterior segment structures, the topically applied carriers usually present a positive surface charge and a mucoadhesive polymeric coating to increase the retention time in the ocular surface. They can also be PEGylated in order to be muco-penetrating and even include specific targeting moieties like arginine-glycine-aspartic acid sequences to target the desired tissue. When targeting the posterior eye segment, nanocarriers administered by intravitreal injection usually present a negative surface charge in order to avoid aggregation with the glycosaminoglycans present in the vitreous humor and a PEGylated surface to improve the diffusion through the vitreous. They might also present targeting moieties to target a specific tissue.

3. Site-specific nanocarriers-based polynucleotide delivery for the treatment of ocular pathologies

The main diseases affecting the anterior and posterior segments of the eye will be discussed in the next sections as well as the nanocarriers developed for polynucleotide delivery to both segments. Tables 2 and 3 summarize some of the nanocarriers used for the delivery of polynucleotides aiming at the treatment of the discussed diseases.

3.1. Nanomedicine approaches for the treatment of anterior segment ocular diseases

There are two main target tissues in the anterior segment of the eye, the cornea and the conjunctiva. The cornea represents a key challenge for many drugs due to its highly organized multilayer epithelium and the presence of tight junctions that limit the permeation of drugs and polynucleotides [4]. Several viral vectors and naked-polynucleotide formulations have reached the clinical development phase for the treatment of ocular disorders, although none of them have been marketed yet.

While the ocular delivery of polynucleotides has been achieved by physical means, such as electroporation [88, 89], iontophoresis [89, 90] gene gun [91, 92] and sonophoresis [93], the use of nanocarriers offers specific advantages, such as (i) their nanometric size and components properties may allow their transport in the conjunctival and corneal epithelium [94], (ii) they may provide a sustained delivery of polynucleotides *in vivo* [30, 95, 96] and, (iii) they may target the cornea, the conjunctiva or both [39, 40].

The most prevalent pharmacological ocular conditions in the anterior segment are DES, ocular inflammation (i.e. keratitis, allergic conjunctivitis, anterior uveitis), corneal wounds and, corneal NV.

Dry eye syndrome

DES is a multifactorial ocular pathology characterized by inflammation, pain and ocular discomfort due to insufficient tear secretion, excessive evaporation and alteration in the composition of the tear film [97]. Current therapies for treating dry eye include drug-free artificial tears and nanosystems such as drug-free cationic nanoemulsions and cyclosporine A loaded nanoemulsions. As an alternative, polynucleotides have been proposed for the treatment of severe dry-eye associated to a deficiency of mucus glycoproteins, such as MUC5AC [98, 99]. For example, Contreras-Ruiz et al. (2013) developed cationized gelatin-based nanoparticles to deliver a plasmid encoding a modified MUC5AC protein (pMUC5AC). This nanoformulation was instilled to a dry eye mouse model and the result of the treatment was a reduction in ocular inflammation accompanied by an improved tear production [99].

Keratitis, conjunctivitis, anterior uveitis

These are diseases related to inflammation in the cornea, conjunctiva and the anterior uvea, respectively. The most common treatment strategies for these types of infections are antimicrobial ophthalmic solutions in the form of eye drops containing different drugs (e.g. anti-histamines, non-steroidal anti-inflammatory drugs, antibiotics or corticosteroids). Polynucleotide-based therapies have also been considered as an alternative for the treatment of severe infectious and inflammatory processes. For example, stromal keratitis and angiogenesis induced by herpes simplex virus-I (HSV) in mice have been reported to be significantly reduced by intravenous injection of cationic polyplexes of PEG-PEI-RGD and anti-VEGF siRNA [100]. In a different study, PEI-siRNA complexes targeting the HSV-1 infected-cell polypeptide 4 gene were evaluated on a mouse model of herpes simplex keratitis. Following topical administration, the said complexes were found to inhibit HSV-I replication *in vivo* for 96 h [101].

Corneal neovascularization

Corneal NV is a pathological event that occurs associated with many ocular diseases that can cause blindness. Corneal NV means the formation of blood vessels within the transparent avascular tissue due to inflammation, infection and hypoxia, among other reasons. The available treatments include topical corticosteroids and non-steroidal anti-inflammatory eye drops and anti-VEGF-A compounds, such as bevacizumab, which were found to have limited clinical efficacy and negative side effects. Based on the critical role that angiogenesis plays in ocular neovascularization diseases, attempts have been made to attack these diseases using new anti-VEGF polynucleotide therapies (targeting VEGF or its receptors). PLGA nanoparticles loaded with a plasmid containing a small hairpin RNA (shRNA) expression cassette against VEGF-A (pSEC.shRNA.VEGF-A) were injected into the corneal stroma in a corneal NV mice model. The plasmid-loaded nanoparticles were found to be effective in reducing the corneal expression of VEGF-A [102]. In another study, human serum albumin (HSA) nanoparticles encapsulating a plasmid (pCMV.Flt23K) were also injected into the cornea of mice and tested for their efficacy against corneal NV [103]. The results showed that HSA nanoparticles provided a 40 % reduction corneal NV after 5 weeks of treatment. A faster response was observed upon subconjunctival injection in mice of PEGylated micelles containing the VEGFR1 plasmid (sflt-1). In this case, at seven days post-injection the corneal neovascularized area was reduced by 45 % [104].

In summary, only a few studies, which are summarized in Table 2, have disclosed the efficacy of synthetic nanocarriers for the treatment of eye-surface diseases either following topical instillation or intra-cornea/conjunctival injection. Nonetheless, and despite their ability to improve the retention time of polynucleotides in the ocular surface and transfect tissues, i.e. cornea and conjunctiva, their efficiency is difficult to judge. In fact, the *in vivo* studies reported so far do not provide a comparison of these new polynucleotides therapies with the currently available treatments.

Table 3 - Polynucleotide-loaded nanocarriers investigated in animal models for the treatment of diseases affecting the anterior segment of the eye.

Nanocarrier	Polynucleotide	Administration route	Animal model	Outcome (PK/PD)	Ref.
Dry eye syndrome					
Gelatin nanoparticles	pDNA (pMUC5AC)	Eye drops	Dry eye mice model	Reduced ocular inflammation; improved tear production	[99]
Vitamin A liposomes	siRNA against HSP47	Eye drops	Chronic GVHD murine model	Improved lacrimal gland fibrosis	[105]
Keratitis					
PEG-PEI-RGD polyplexes	Anti- VEGF siRNA	Intravenous injection	HSK mice model	Reduced stromal keratitis and angiogenesis	[106]
PEI complexes	ICP4-siRNA	Eye drops	HSK mice model	Inhibited HSV replication	[101]
DOTAP/DOPE/DSPE-PEG liposomes	siRNA (siSP, siGP, siGFP)	Eye drops	*Acanthamoeba* keratitis murine model	Combination with chlorohexidine led to 60 % regression of corneal damage	[107]
Corneal NV					
PLGA nanoparticles	pDNA (pSEC.shRNA.-VEGF-A)	Corneal intrastromal injection	Corneal NV mice model	Reduced VEGF-A expression	[102]
HSA nanoparticles	pDNA (pCMV.Flt23K)	Corneal intrastromal injection	Corneal NV mice model	40 % reduction of Corneal NV after 5 weeks	[103]
PEGylated micelles	pDNA (psflt-1)	Subconjunctival injection	Corneal NV mice model	45% reduction of Corneal NV after 7 days	[104]

PEG, polyethylene glycol; PEI, polyethyleneimine; RGD, arginine-glycine-aspartic acid; DOTAP, 1,2-Dioleoyl-3- trimethylammonium propane; DOPE, 1,2-dioleoyl-3- hosphatidylethanolamine, DSPE, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine; PLGA, poly(lactic-co-glycolic acid); HSA; human serum albumin; GVHD, Chronic graft-versus-host disease; HSK, herpes simplex keratitis; Corneal NV, corneal neovascularization; HSV, herpes simplex virus, VEGF-A, vascular endothelial growth factor A

3.2. Nanomedicine approaches for the treatment of back of the eye diseases

Diseases affecting the retina can potentially be treated with polynucleotides-based therapies. The retina is a photosensitive tissue composed of three main layers or cell types. The retinal pigmented epithelium is in the outermost layer followed by the photoreceptors (cones and rods), and the retinal ganglion cells in the innermost layer. Defects in these cell layers can lead to AMD (retinal pigmented epithelium and photoreceptors), retinitis pigmentosa, LCA (photoreceptors), glaucoma and optic neuropathy (retinal ganglion cells). According to the WHO, among the diseases affecting the posterior segment of the eye, glaucoma and AMD are the main causes for blindness.

Subretinal injection is the most effective way to deliver drugs to the photoreceptors and RPE layer of the retina. Additionally, drugs can be injected into the vitreous humor through IVT injection that is less invasive and allows for a broader and more uniform transduction of the retina. Despite their efficacy, these methods are invasive and not acceptable when there is the need for frequent intraocular administrations. Repeated injections may lead to undesired side effects like high risk of infections, cataract development, vitreous hemorrhage and even retinal detachment and endophthalmitis that can potentially cause vision loss. Besides the risks of repeated injections, the poor stability of polynucleotides in biological fluids and their short vitreal half-life urges the need of developing carriers able to protect and deliver them in a specific, efficient and sustained way.

As summarized in Table 4, several types of nanocarriers have been used to deliver polynucleotides to the back of the eye. Such systems have aimed to target different tissues (i.e. choroid, macula, and retina) or cell types (i.e. RGC, photoreceptors, and RPE) of this eye segment, expressing mutations responsible for diseases like AMD, glaucoma, CNV and X-linked juvenile retinoschisis (XLRS), among others. These potential nanomedicines have been injected into the subretinal space or the vitreous humor. In this sense, it has been reported that positively charged particles may aggregate upon interaction with components present in these compartments, i.e. HA and GAGs, and this aggregation would ultimately hamper their cellular uptake [8, 108]. However, the vitreous humor can also act as a reservoir where the carriers can gradually release the compound of interest to yield a sustained delivery of drugs to the retina [9].

Glaucoma

Glaucoma is characterized by the progressive damage of the optic nerve leading to retinal ganglion cells death and permanent vision loss. According to the WHO, glaucoma is the second cause of vision loss worldwide after cataracts, being responsible for 8 % of all blindness cases [109]. Factors like local ischaemia-hypoxia, excessive stimulation of the glutamatergic system, alterations in glial cells, aberrant immunity and mainly high intraocular pressure (IOP) seem to contribute to glaucoma [110].

The current standard treatment for glaucoma involves medication to lower IOP levels by means of either diminishing aqueous humor production (beta blockers) or improving its drainage (prostaglandin analogs). These treatments are chronic and they often suffer from limited patient compliance. In addition, the side effects associated to systemic absorption are no negligible. Therefore, there is a clear need for advanced treatments and delivery technologies.

In vivo studies of nanocarriers loaded with different drugs (e.g. timolol, brimonidine tartrate, pilocarpine) have shown promising results with increased bioavailability, prolonged retention time and sustained drug delivery in addition to minimizing systemic absorption of the associated drugs [111]. Along the same line, a number of patents on ophthalmic nanoformulations such as nanoemulsions [112], and contact lenses delivering nanoemulsions [113], have been recently issued.

Therapies based on polynucleotides could improve the commercially available anti-glaucoma treatments. Trabecular meshwork, ciliary epithelium and muscle and ganglion cell layer are some examples of target tissues for polynucleotide-based glaucoma therapy. However, research in this line is in a very early stage and has mainly made use of naked polynucleotides or viral vectors administered topically or as IVT and subretinal injections.

The studies reported until now to contribute to the development of polynucleotide-based nanomedicines for the treatment of glaucoma have used model polynucleotides associated to lipid nanocarriers. For example, Matsuo and colleagues [52] studied different liposomes loaded with pDNA encoding β-Gal through topical instillation. *In vivo* data in rats showed gene β-Gal expression for up to a month in conjunctival, corneal and retinal ganglion cells after

administration of N-(alpha-trimethylammonioacetyl)-didodecyl-D-glutamate (TMAG) and DC-cholesterol liposomes. A different approach consisted of liposomes with a viral envelope-coating of inactivated hemagglutinating virus of Japan (HVJ, Sendai virus) [114-116], which was supposed to allow the fusion of the liposomes with the cell membrane and deliver the encapsulated nucleotide into the cytoplasm. These liposomes were loaded with LacZ pDNA and a high-mobility group 1 (HMG1) nonhistone nuclear protein, which guides the pDNA into the nucleus. The *in vivo* results following intravitreal and subretinal injections of these formulations to rat and mice presented LacZ expression in the photoreceptors for more than 30 days [114]. Moreover, these liposomes encapsulating FITC-labeled phosphorothioate oligonucleotides were injected into the anterior chamber of rats and monkeys. Fluorescence was detected in the trabecular meshwork of monkeys and in the iris and ciliary body of rats lasting as long as 7 and 14 days, respectively [116].

Johnson et al. [117] synthesized what they called a "peptide for ocular delivery (POD), containing specific amino acids ($GGG(ARKKAAKA)_4$), which theoretically endowed the peptide with cell penetrating properties, and used it for the delivery of pDNA encoding a red fluorescent protein (pCAGRFP) and siRNA to the eye. After topical instillation to mice, the pDNA-POD complexes were found to penetrate the corneal epithelium, sclera, choroid and even the optic nerve. The same nanocomplexes were also found to enter and deliver the associated siRNA in the GCL and RPE, following subretinal and IVT injection, respectively. A different formulation tested *in vivo* was the one combining pDNA with surfactant gemini and "helper" lipids DOPE and DOPE:1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DOPE:DPPC). After topical application, these nanoparticles were found in the limbus, iris and conjunctiva for 48 h and, following IVT injection they were localized within the nerve fiber and GCL of the retina [118].

Among the presented approaches only model polynucleotides and healthy animal models were used, thus no therapeutic effect or comparison with current treatments has been reported.

Age-related macular degeneration

AMD is the leading cause of irreversible blindness in people over 60 years old and it is predicted to affect about 196 million people worldwide by 2020 [119]. This multifactorial disease leads to a progressive degeneration of photoreceptors in the central retina that can result in blindness. There are two forms of AMD, the "dry" and "wet" AMD. Wet AMD, also known as CNV, is caused by the promotion of blood vessels growth mainly by the protein VEGF, which affects the central part of the retina, while dry AMD or nonexudative form is characterized by RPE cell death and consequent photoreceptor degeneration [97]. Currently, AMD has no cure but there are some FDA approved treatments especially for the wet form like pegaptanib (Macugen®) an anti-VEGF aptamer, ranibizumab (Lucentis®) an antibody fragment, and aflibercept (Eylea®) a recombinant fusion protein, which is quickly becoming the gold standard treatment. Bevacizumab (Avastin®) is a monoclonal antibody which has also been used as an off-label treatment.

With the idea of exploring new treatments for AMD, different pDNAs have been associated to different polymers originating nanoparticles and nanocomplexes of various compositions. For example, HSA has been found to be able to condense DNA and protect it from degradation [120, 121]. A plasmid encoding the Cu and Zn superoxide dismutase gene 1 (SOD1), a gene whose deficiency is associated with CNV and RPE dysfunction, was complexed with HSA and injected intravitreally to mice. Unfortunately, *in vivo* results revealed that protein expression was only detected for up to 2 days after IVT injection [121].

In another study, PLGA nanoparticles were used to deliver a shRNA-expressing pDNA targeting hypoxia-inducible factor-1α (HIF-1α), which regulates the transcription of pro-angiogenic factors like VEGF [122]. The i*n vivo* results obtained following IVT injection to a laser-induced CNV rat model showed a decreased of VEGF expression and a reduction on the extent of NV.

A different strategy involving the intravenous administration of PLGA nanoparticles functionalized with an RGD peptide, a ligand of integrin receptors, has been evaluated in mice and also primates. The results indicated that the said nanoparticles loaded with the plasmid pFlt23k.NR were able to get into the retina lesion, promote the plasmid expression for up to 6 weeks and lower the CNV [123]. In another study, the intravenous administration of anti-

VEGF plasmid-loaded PLGA nanoparticles, functionalized with RGD and transferrin peptides, led to a high gene expression in RPE, and the subsequent inhibition of CNV [47]. These positive results obtained following intravenous administration are surprising if we take into account the blood-retinal barrier that considerably limits access to the retina. In our understanding, a high dose will be required when using this modality of administration with the potential undesired off-target effects. Recently, Lajunen and co-workers [51] demonstrated that transferrin-decorated liposomes were able to reach the RPE by topical instillation in Sprague Dawley rats, thus avoiding the possible undesired side-effects associated with IVT and intravenous administrations.

PEGylated liposomes containing protamine sulfate (PS) and HA loaded with a siRNA targeting VEGF-R1 were also tested *in vivo* through IVT injection in a laser-induced mice model of CNV. This system was able to protect the loaded siRNA and decrease the CNV area [57].

Dendrimers have also been tested as polynucleotide carriers in CNV animal models and could be considered as potential strategies for AMD treatment [84, 85]. For example, Marano et al. (2004) developed different lipid-lysine dendrimers that led to a significant reduction in VEGF expression levels and were able to significantly inhibit CNV in a mice model. A long-term study using the laser-induced CNV mice model revealed that a single IVT injection of dendrimers loaded with ODN-1 inhibited up to 95 % the development of CNV in rats and the response lasted for up to six months. This system was able to penetrate through all the retinal cell layers up to the RPE [124].

Finally, the cationic nanoemulsions originally developed by Benita's group [73, 74, 125] and containing DOTAP were used to deliver a 17-mer partially phosphorothioated ODN directed to the VEGF KDR receptor. This cationic nanoemulsion was administered by topical instillation and IVT injection and the results showed that, as expected, only the injected nanoparticles were able to reach the inner nuclear layer (INL) of the retina maintaining therapeutic levels for about 72 h after injection. [74].

Retinitis pigmentosa

This disease affects one in 3,500 to 5,000 people worldwide and can be inherited as autosomal recessive (50-60 %), autosomal dominant (30-40 %) or X-linked (5-15 %). RP can be the result

of mutations in more than 60 genes being one of them rhodopsin, which accounts for 25 % of autosomal dominant RP cases [126].

Based on the PEG stabilizing properties, the PEG-substituted lysine CK30-PEG was used for the formation of complexes with pDNA molecules of interest for RP treatment [127, 128]. The use of this carrier allowed for the efficient delivery of a plasmid containing *Rds* cDNA and a rod opsin (MOP) promoter, and its expression in photoreceptors of a RP mouse model preventing cone degeneration. Gene expression was detected at least one month post-treatment [129]. Han et al. used these particles to compare the efficacy between a genomic DNA (gDNA), which included introns from the rhodopsin gene, and a rhodopsin complementary DNA (cDNA). The introduction of specific genomic sequences improved the rhodopsin expression levels, delaying photoreceptor cells death and improving functionality up to eight months in a rhodopsin knockout (RKO) mouse model [130]. More recently, the group reported the safety and eficacy of the CK30-PEG nanoarticles loaded with pDNA, containing different promoters for transfecting the RPE of healthy baboon animal model [131].

X-linked juvenile retinoschisis

XLRS affects one in 5,000 to 20,000 males who are diagnosed very early within their first years of life. The progression of this disease usually involves vitreous hemorrhage and retinal detachment that ultimately can lead to vision loss. Significant progress has been achieved in understanding the genetic and molecular mechanisms responsible for this disease. Currently, about 190 disease-causing mutations have been identified in the retinoschisis RS1 gene. The RS1 protein is secreted in the retina and it is responsible for maintaining the retina's integrity [132].

Gascón's group developed a hybrid nanostructure consisting of a dextran-protamine sulphate–pDNA complex (i.e. pCMS-EGFP or pCEP4-RS1) adsorbed onto the surface of solid lipid nanoparticles [133]. These plasmid-loaded particles were administered to rats through three different routes: IVT injection, subretinal injection and topical instillation. The results of these studies presented plasmid expression in the cornea after topical instillation, the nanocarriers were also able to transfect the RPE cells and photoreceptors by subretinal

injection while by IVT administration gene expression was mainly detected in the ganglion cell layer. In a subsequent study, the same nanoparticles and a different kind containing HA instead of dextran (HA-protamine-pDNA-SLN) were administered by IVT and subretinal injections to C57BL/6 wild type and Rs1h-deficient mice models. Both carriers were able to transfect different layers of the retina, following both administration methods. However, when subretinal injection was used a higher amount of RS1 expression was detected in the photoreceptors and the expression was maintained for up to two months after injection it in GCL and INL. In addition, both carriers were able to lead to a partial recovery of the retina that consisted of mainly in a decreased loss of photoreceptors and an amelioration of the organization of retina layers [134].

Leber's congenital amaurosis

The prevalence of LCA is between 1 in 81,000 newborns [135]. Fourteen mutated genes have already been identified and they are responsible for photoreceptor cell death at an early age causing blindness. About 6 % of LCA patients present mutations in RPE65 gene that encodes for all-trans-retinyl-ester hydrolase [136].

PEG-Lysine complexed to a pEPI-eGFP and phRPE65, containing a scaffold matrix attachment region (S/MAR) and a macular dystrophy 2 promoter (VMD2), were subretinally administered to wild-type mice and to an RPE65-deficient LCA mice model, respectively [137] S/MAR inclusion was used considering its self-replication capacity residing inside cells for more than 100 generations [138] and increase gene expression [106, 139]. The treatment led to higher DNA expression levels that lasted at least two and a half years in the wild-type mice and despite the fact that only about 20 % of the RPE cells expressed the gene, it still led to improvements in RPE65-deficient LCA disease that were noticed up to six months [137].

More recently, a different strategy combining DOTAP/DOPE/cholesterol liposomes with protamine-plasmid complexes, nuclear localization signaling and cell-penetrating transactivator of transcription (TAT) peptides was developed. These liposomes were administered by subretinal injection in a RPE65-associated blind mice model and the results showed the capacity of these nanocarriers to maintain gene expression for at least three

months. Furthermore, *in vivo* results revealed that the treatment led to blindness correction [49].

Stargardt's disease

This autosomal recessive disease has no cure yet and it is the most common inherited juvenile macular degeneration affecting one in 8,000-10,000 people worldwide. Stargardt's disease causes problems to adapt to darkness and a progressive and irreversible loss of central vision. The disease is caused by mutations in the ABCA4 gene that encodes a protein of the ABC lipid transporter family [140]. Hundreds of disease-causing mutations have been identified in this gene that can lead to Stardardt's or to cone-rod dystrophy and several forms of autosomal recessive RP [141]. To the extent of our knowledge, only PEG-Lysine complexed to pDNA have been assessed for this disease and gene expression was detected for about eight months improving mice's recovery of dark adaptation [142].

Diabetic retinopathy

This ocular pathology is the most frequent complication of diabetes mellitus, a disease that according to the WHO affects more than 422 million individuals around the world. Diabetic retinopathy which affects more than 93 million people results from vascular abnormalities causing glial dysfunction and death of retinal neurons, and ultimately, blindness [143]. Monoclonal antibody-based therapies, such as Eylea® and Lucentis® are some of the FDA approved treatments for this disease. As an alternative, different siRNA therapies, such as those targeting the connective tissue growth factor (CTGF) [144], VEGF [145], fibronectin, collagen and laminin [146], HIF-1α [145], or hypoxia-inducible gene RTP801 [147] have been proposed. However, these siRNAs have not been used in association with nanocarriers so far.

Different approaches making use of anti-angiogenic microRNAs [148] and plasminogen fragments [149] associated to PEG-Lysine have also been explored. These nanocomplexes were intravitreally injected in a mouse model for late-onset diabetic retinopathy. A single administration reduced the expression of VEGFR-2, suppressing angiogenesis for at least three

months after treatment [148]. In another study, PLGA nanoparticles loaded with a plasminogen fragment Kringle 5 (K5) plasmid were administered by IVT injection in oxygen-induced retinopathy (OIR) and streptozotocin-induced diabetic mice models. Similarly, a single injection led to a significant reduction of retinal vascular leakage and attenuated VEGF over-expression and retinal NV for at least one month [149].

Despite the ability of synthetic nanocarriers for ocular polynucleotide delivery and all the potential advantages and the promising results observed in animal models, their delivery efficiency is still low. On the other hand, the majority of the clinical trials using polynucleotides for ocular diseases have used naked polynucleotide treatments and about 20 % have combined them with viral vectors, especially AAVs,. These clinical trials have been oriented to the treatment of macular degeneration, LCA and Leber´s hereditary optic atrophy. Despite of this, it should be recognized that major advances in ocular polynucleotide-based therapies have already been achieved and that this field is now opening opportunities for future developmental programs. The main approaches used to improve non-viral carriers performance in the treatment of the previously discussed diseases affecting both anterior and posterior segments are summarized in Fig. 5 A and B, respectively.

Table 4 - Polynucleotide-loaded nanocarriers investigated in animal models for the treatment of conditions affecting the posterior segment of the eye.

Nanocarrier	Polynucleotide	Administration route	Animal model	Outcome (PK/PD)	Ref.
Glaucoma					
TMAG-DC-Cholesterol, liposomes	pDNA (pCMV-β-Gal)	Eye drops	Healthy rat	β-Gal expression in conjunctiva, cornea and RGC for 1 month	[52]
HVJ-AVE liposomes	pDNA (pCMV-β-Gal)	IVT and subretinal injections	Healthy rat	β-Gal expression in neural retina for 1 month	[114]
HVJ-AVE liposomes	Phosphorothioated oligonucleotides	Injection into the anterior chamber	Healthy rat and monkeys	Fluorescence in trabecular meshwork for 7 (monkeys) and 14 days (rat)	[116]
Gemini-DOPE nanoparticles	pDNA (pCMV-tdTomato)	Eye drops and IVT injection	Healthy mice	pDNA detected in retina's nerve fiber and GCL 48h after IVT injection	[118]
siRNA-peptide Y-DOTMA/DOPE liposomes	siRNA against MRTF-B	Subconjunctival injection	Glaucoma filtration surgery rabbit model	30 % MRTF-B silencing in conjunctiva; significant decrease of conjunctival scaring	[150]
Age-related macular degeneration					
HSA NPs	pDNA (pSOD)	IVT injection	Healthy mice	Protein expression detected 2 days after injection but not after 7 days	[121]
PLGA NPs	pDNA (pshHIF-1α)	IVT injection	CNV rat model	Decreased VEGF expression and reduced CNV for 1 month	[122]
RGD-functionalized PLGA NPs	pDNA (Flt23k)	Intravenous injection	Primate and mice CNV models	Lowered CNV; improved 40 % mice's vision	[123]
RGD/transferrin functionalized PLGA NPs	Anti-VEGF intraceptor plasmid	Intravenous injection	CNV mice model	47-73 % reduced CNV area, 2 weeks after treatment	[47]
Lipid-lysine dendrimers	Anti-VEGF oligonucleotide	IVT injection	CNV mice model	Inhibited by 95 % the development of CNV for 4-6 months	[124]
PS/HA PEGylated liposomes	siRNA targeting VEGF-R1	IVT injection	CNV mice model	Decreased CNV area	[57]
Cationic NEs (oleylamine, DOTAP, AOA)	AS-ODN against VEGF KDR	Eye drops, IVT injection	Healthy rabbits	Injected nanoemulsions reached the retina's INL	[74]

Nanocarrier	Polynucleotide	Administration route	Animal model	Outcome (PK/PD)	Ref.
Age-related macular degeneration					
PEG-SS-POD NPs	pDNA (pCAGLuc, pCAGLacZ, pCAGhFLT1)	Subretinal injection	Laser-induced CNV mice model	50 % reduction of CNV area, 7 days post-treatment	[151]
siRNA hydrogel-PEI-HA NPs	Anti-VEGF siRNA	IVT injection	laser-induced CNV mouse model	26 % reduction of CNV area for at least 2 weeks	[152]
Retinitis pigmentosa					
CK30PEG NPs	gDNA	Subretinal injection	RKO mice model	Improved rhodopsin levels and improved photoreceptors functionality for 8 months	[130]
CK30PEG NPs	MOP-NMP vector carrying Rds gene	Subretinal injection	Rod-dominant RP mice model	Prevention of cone degeneration	[129]
CK30PEG-TAT NPs	pDNA (pCAGIG-co-sgRho-EGFP pEPI-MOP-co-sgRho pshRho-(1, 2 or 3))	Subretinal injection	homozygous P23H knock-in mouse model	Partial structural and functional recovery of rod photoreceptor cells	[153]
X-linked juvenile retinoschisis					
Dextran/PS SLN	pDNA (pCMS-EGFP pCEP4-RS1)	Eye drops Subretinal, IVT injections	Healthy mice	Transfection of different ocular tissues	[133]
Dextran/PS SLN HA/PS SLN	pDNA (pCAG-GFP-CMV-RS1)	Subretinal, IVT injections	Healthy mice Rs1h-deficient mice model	Partial recovery of retina for 2 months	[134]

Nanocarrier	Polynucleotide	Administration route	Animal model	Outcome (PK/PD)	Ref.
Leber's Congenital Amaurosis					
CK30PEG nanoparticles	pDNA (pEPI-EGFP pEPI-hRPE65 (S/MAR and VMD2 promoters))	Subretinal injection	Healthy mice RPE65-deficient mice model	Gene expression lasted for 2.5 years in the healthy mice; Improved the phenotype of RPE65-deficient mice	[137]
DOTAP/DOPE/cholesterol/PS liposomes containing NLS and TAT	pDNA (pcDNA3; pGFP) chicken Rpe65 cDNA	Subretinal injection	RPE65-deficient mice model	Gene expression in RPE for 3 months Blindness correction	[49]
Ret-PEG-ECO NPs	pDNA (pRPE65)	Subretinal injection	RPE65-deficient mice model	Increased function of rod and photoreceptors; slowed cone degeneration for at least 4 months	[154]
Stargardt's disease					
CK30PEG nanoparticles	pDNA (pEPI-CMV-EGFP with ABCA4 cDNA and MOP-ABCA4)	Subretinal injection	ABCA4 deficient mice model	Gene expression in the retina for 8 months; improved recovery of dark adaptation	[142]
Diabetic retinopathy					
CK30PEG nanoparticles	pDNA pCAG-miR200-b-IRES-eGFP)	IVT injection	Ins2Akita mice	Angiogenesis marked suppression	[148]
PLGA nanoparticles	pDNA (pK5)	IVT injection	OIR mice model Diabetic mice model	Reduced retinal NV for 1 month	[149]

TMAG, N-(alpha-trimethylammonioacetyl)-didodecyl-D-glutamate; HVJ-AVE, hemagglutinating virus of Japan - artificial viral envelope; DC-cholesterol, 3-β[N-(N',N' imethylaminoethane)-carbamoyl] colesterol; HVJ-AVE, inactivated hemagglutinating virus of Japan-artificial viral envelope; DOTMA, 1,2-di-O-octadecenyl-3-trimethylammonium propane; DOPE, 1,2-dioleoyl-3-hosphatidylethanolamine; HSA, human serum albumin; PLGA, poly(lactic-co-glycolic acid); RGD, arginine-glycine-aspartic acid; PEG, polyethylene glycol; PS, protamine sulfate; HA, hyaluronic acid; NPs, nanoparticles, NEs, nanoemulsions, SLN, solid lipid nanoparticles; DOTAP, 1,2-Dioleoyl-3- trimethylammonium propane; AOA, arginine octadecyl amide; PEG-SS-POD, polyethylene glycol-disilfide-peptide for ocular delivery; TAT, cell-penetrating transactivator of transcription; NLS, peptide of nuclear localization signaling; Ret, retinylamine; ECO, (1-aminoethyl)iminobis[N-(oleicylcysteinyl-1-amino-ethyl)propionamide]; siRNA; small interference RNA; AS-ODN, antisense oligonucleotides; VEGF, vascular endothelial growth factor; gDNA, genomic DNA; IVT, intravitreal; CNV, choroidal neovascularization; RKO, rhodopsin knockout; Rs1h, retinoschisis; RPE65, Retinal pigment epithelium-specific 65 kDa; ABCA4, ATPbinding cassette, subfamily A; OIR, oxygen-induced retinopathy; MRTF-B, myocardin-related transcription factor; INL, inner nuclear layer; RPE, retinal pigmented epithelium

4. Translational aspects of ocular delivery of polynucleotides

Despite the multiple reports centered on the benefits of nanotechnology for treating ophthalmic conditions it is surprising that only a small number of these products have obtained marketing authorization. As indicated in section 3, besides a variety of emulsions and liposomes, which are over-the-counter products for the treatment of DES, there are nanoemulsions containing specific drugs, such as cyclosporine A (Restasis®, Lipomil®) and difluprednate (Durezol®) for the treatment of ocular inflammation, and liposomes containing verteporfin (Visudyne®) and the PEGylated anti-VEGF aptamer, pegaptanib (Macugen®), both for the treatment of AMD. The majority of these products are emulsions composed of well-known oils (castor oil or medium-chain triglycerides oil), emulsifiers (polysorbate 80, pemulen, poloxamer 188, tyloxapol or cetalkonium chloride) and excipients to maintain tonicity and pH. However, the application of more sophisticated technologies for the development of oligonucleotide-based nanomedicines is associated to significant challenges as highlighted below.

The term nanomedicine includes an ample range of products with enormous variations in size, shape, materials and other characteristics. This heterogeneity makes consensus definitions and classifications of these products difficult to establish from the regulatory standpoint, thus complicating the generation of appropriate regulatory guidance. Both, the FDA and the EMA, however, consider that the development of a product that falls within the definition of nanotechnology may require special attention in terms of toxicity and safety.

Market authorization of pharmaceuticals requires a full physical and chemical characterization of the drug. The increasing complexity of nanomedicines indicates that characterization has most likely to be tailored for the specific product in development. The initial phases of product development may require a thorough characterization to select the specific tests that will subsequently be used to release batches and test the quality of the final product. Therefore, as with other medicinal products, it is important to identify the critical quality attributes (CQAs) that maintain a direct relationship with products quality, efficacy and toxicity; these CQAs should be the basis for the product's quality control throughout its lifecycle. CQAs for ophthalmic nanomedicines may include aspects related to nanotechnology such as particle

size, size distribution, surface charge, hydrophilicity; but also attributes related to pharmaceuticals, such as purity, stability, sterility, and manufacture controls. Among the ophthalmic products approved so far, only the cationic nanoemulsion containing cyclosporine A specifically lists CQA related to nanotechnology: particle size and zeta potential. This is important as it is well known that size has a direct impact on the biodistribution and clearance of the nanomedicine. For instance, subconjunctival administration of fluorescent polystyrene nanoparticles has shown that 20 nm nanoparticles exhibit a rapid clearance from ocular and periocular tissues while 200 nm nanoparticles made of the same material are retained in the eye for a period of two months [94]. It should also be considered that the nanomaterial administered to the eye can aggregate, thereby influencing the *in vivo* behavior of the nanomedicines.

On the other hand, although small scale manufacturing of different types of nanomedicines in research laboratories is fairly common, with the exception of nanoemulsions, the experience in large-scale manufacturing is still limited. Large-scale manufacturing requires standardization and manufacturing under Good Manufacturing Practices (GMP). Factors affected by the scale-up include changes in particle size, drug loading, quality and quantity of impurities, structural alterations, among others. In particular, for oligonucleotides changes in manufacturing processes may lead to decreased stability or even degradation of the product, as a consequence, a very well controlled small-scale process may turn out to be unreliable or non-reproducible at a larger scale if the process has not been efficiently controlled during the scale-up. For multi-step processes it is usually useful to establish mid-process controls that can inform of the quality of the intermediate products in order to better control the process as a whole.

An additional specific requirement of ocular pharmaceuticals is sterility. Many materials used to produce nanomedicines are susceptible to routine sterilization techniques such as gamma irradiations or autoclaving. For small or malleable particles double filtration may be an option for sterilizing the final product but for bigger rigid particles aseptic manufacturing may be the only option.

Many nanocarriers are specifically designed to improve delivery of the active ingredient, thus it is expected that biodistribution of the nanomedicine and its unformulated counterpart

should be significantly different. In fact, for ocular administration, it is crucial to determine whether the formulation changes the ability of the drug to enter systemic circulation. Many approaches to develop nanomedicines for eye conditions seek precisely this aim; to reduce the amount of compound that enters systemic circulation reducing the potential systemic side or off-target effects of the drug. One of the main challenges of pharmacokinetic (PK) studies of nanomedicines is to address the fate of the free drug following administration of a nanomedicine. For efficacy assessment the amount of free drug reaching the intended site should be measured. On the other hand, the distribution of the complete nanomedicine and its fate following delivery should be studied in order to address eventual toxicities associated to the nanocarrier. For larger molecules, such as oligonucleotides specific methods used to assess the free drug should be used to quantify the amount of drug reaching the target site. Subtle variations in the characteristics of nanomedicines may result in altered patterns of biodistribution; therefore, it is generally expected that PK profiles of nanomedicines have greater variability than PK profiles obtained with unformulated drugs. In addition, for ocular pharmaceuticals administered in eye drops the percentage of entrapment, which is the amount of drug in one eye drop, is difficult to control further increasing the variability of the PK profiles. These inherent characteristics of nanomedicines for ophthalmic use should be taken into account when designing PK studies to avoid unnecessary duplication of experimental work. The surface characteristics of the nanomedicine have a strong impact on the absorption and distribution of the drug and can be modified by surface coatings in order to obtain particles with the desired characteristics.

It is generally accepted that specific types of nanomedicines may raise concerns in terms of toxicity, but the general battery for assessing toxicity of drugs in a preclinical setting should be able to identify these toxic effects and their potential relation to dose. Knowledge about the differences in biodistribution between the unformulated drug and the nanomedicine are helpful to pinpoint possible target tissues for toxicity. Also, additional tests may be needed if components of the nanomedicine are known to have dose-limiting toxicities or if any of the components are not naturally degraded and excreted. Particular attention should be made on potential immunotoxic effects. These toxicities are not necessarily deduced from the unformulated drug as they may arise from specific interaction of the drug with other

components of the nanocarrier or by interactions of the nanomedicine with proteins present in biological fluids. The physical characteristics of the nanomedicines may also increase their potential to interact with biological components. In the particular case of the eye, interaction with melanin may alter the PK profile of the drug in the eye generating locally high concentration of the drug upon release from melanin [155]. Formulations reaching the bloodstream may also potentially interact with components of the complement system or coagulation inducing side effects [156]. The assessment of these toxicities is not necessarily straightforward as animal models are not necessarily good predictors of human immune systems. In these cases, *in vitro* tests with human cells can be used to complement animal studies.

The production of nanosystems usually requires the use of surfactants. Surfactants lower the surface tension between the nanosystem and the dispersion liquid acting as stabilizing agents. Usually there is a correlation between the amount of surfactant used and the size of the nanoparticles, with low concentration of surfactant generally yielding smaller particles. Unfortunately, surfactants may cause ocular irritation when administered at high concentrations and need to be removed from the formulation or used in concentrations that are compatible with the ocular surface. For ophthalmic products these approaches are combined with the aim of reducing the ammount of surfactants to concentrations that are tolerable by the eye without removing them completely as they usually act as penetration enhancers facilitating the entrance of compounds [157].

Clinical translation of nanomedicines for ophthalmic conditions has different requirements depending on the indication and route of administration. Topical ocular eye drops are the preferred option for treating the anterior segment of the eye whereas nanodrugs for the back of the eye are usually developed to release drug over an extended period of time to reduce the frequency of administration. Nanomedicines that include oligonucleotides should protect them against degradation by nucleases. Human biological fluids contain higher concentration of nuclease activity than most animal models, therefore stability studies of the nanomedicine need to be complemented to assess the stability of the drug in humans [158]. In addition, ophthalmic nanomedicines need to comply with the general requirements for drugs administered by the ocular route. For topical forms parameters such as sterility, osmolality,

antimicrobial agents, buffering, viscosity, pH, particulate matter and compatibility with packaging have to be considered.

Finally, it should be mentioned that development of complex delivery systems for ophthalmic drugs is highly costly. As previously mentioned, the processes for developing nanomedicines may require additional data not generally required for small molecules. This, added to the insufficient regulatory framework and the potential complexity of the scale-up processes may further increase the associated costs of developing nanomedicines. As such, it seems difficult for the industry to design developmental plans where the size of the ophthalmic market outweighs the efforts and financial expenses associated to the development.

Currently there are several advanced preclinical programs developing nanomedicines with small drugs for different ocular conditions. Most of these drugs are already commercialized and are being reformulated in order to improve their efficacy as well as to find new indications for them. Overall, the more advanced technologies include the production of nanoemulsions and liposomes. As these compounds reach the market it is expected that nanoformulations are also incorporated into the pipelines of more complex molecules such as oligonucleotides or peptides.

5. Conclusions and future perspectives

Although therapies involving polynucleotides for treating ocular diseases are in an early development stage, preliminary human clinical trials begin to show promising results. Currently, there are two FDA approved nucleic acid-based drugs for eye conditions: Vitravene®, an AS-ODN for cytomegalovirus-induced retinitis treatment in immunocompromised patients, and Macugen® an aptamer designed to treat wet AMD. Curiously, this aptamer is PEGylated and, as such, maybe considered as a nanomedicine. In principle polynucleotide-based drugs do not comply with the best physicochemical properties to be used as effective drugs, however, their chemical modification and the development of easy to produce nanocarriers offer a great window of opportunity for the exploitation of these new therapies. Currently, several ocular diseases of the anterior and posterior segment of the eye such as glaucoma, AMD, ocular pain associated to dry eye and CNV are under gene-based

clinical trials evaluation. Development of effective future ocular treatments will be a combination of understanding the diseases genetic basis as well as improving and developing long-term and nontoxic ocular drug delivery systems for both segments of the eye. It is believed that AS-ODN and RNA-based therapeutics (specially siRNA-based as it is a potent inhibitor of protein expression) will continue further development reaching the market in a reasonable time frame being the major task to achieve their nanosystems delivery along with investigating alternative routes of administration.

If nanotechnology is successfully combined with polynucleotides, their delivery, the greatest hurdle holding these drugs from the clinic, could be overcome. The following years will tell if these combined approaches can be used in the treatment of severe ocular diseases that nowadays relay on painful, inconvenient and inefficacious treatments. Much effort will have to be put into place for the efficient delivery of the genetic material to targeted cells/tissues being one of the most challenging tasks to be accomplished by multidisciplinary research teams composed of researchers, clinicians and pharmaceutical companies.

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Background, Hypothesis and Objectives

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Background

Ocular pathologies can dramatically influence one's quality of life, especially when the outcome might be blindness. According to the most recent predictions, the number of people suffering moderate to severe visual impairment, is expected to raise from 217 (in 2015) to 237 million individuals by 2020 [1, 2]. Therefore, there is an unmet medical need that justifies the search for more innovative and effective treatments, such as siRNA-based therapies which specifically knockdown a target gene.

The efficient administration of siRNA-based therapies needs to face several challenges, namely, (i) to avoid premature degradation in biological fluids and be safely delivered to the target tissue; (ii) to translocate the plasma cell membrane of the target cell; (iii) to avoid endosomal entrapment and degradation and (iv) to be recognized by the RNA-induced silencing complex (RISC) machinery [3-6]. In the case of ocular pathologies, and despite the accessibility of the eye as a target organ, there are additional problems to circumvent, such as, the limited drug volume that is retained in the ocular mucosa after topical administration [7].

Nanotechnology offers the possibility to overcome these barriers and deliver the nucleotide to the site of action. In this work we selected polymeric nanocapsules, as RNA delivery vehicles, based on the previous experience of our research group [8-10]. In particular, polyarginine and protamine nanocapsules have shown the capacity to improve cell's transfection [10], to protect nucleotides from degradation [8] and to interact with the ocular surface [11], thereby improving their ocular residence time and their bioavailability. Aside from these properties, protamine has also shown its capacity to enhance cell penetration [12]. Furthermore, the oily composition of the nanocapsules might act as a lubricant and positively influence the structure of the tear film lipid layer [13, 14].

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Hypothesis

1. Protamine nanocapsules can associate siRNA molecules and facilitate their penetration into the corneal epithelial cells.

2. The association of high concentrations of siRNA is necessary in order to achieve the penetration of a significant amount of siRNA into the eye surface for a therapeutic effect.

3. The association of the siRNA molecules to cationic oil cores (nanoemulsion) and their subsequent coating with a protamine external layer will protect them from degradation and improve their interaction with the corneal epithelium after topical instillation.

4. The use of different cationic surfactants (cetrimide, benzethonium chloride, ethyl lauroyl arginate or oleylamine) and different nanocapsules fabrication techniques (solvent displacement or self-emulsification) will lead to the formation of protamine nanocapsules with different physicochemical properties, siRNA association efficiencies as well as distinct silencing outcomes.

Objectives

Taking into consideration the background of the field and the outlined hypothesis, the main goal of this thesis was the development of protamine nanocapsules with a high capacity to associate siRNA molecules (final concentration of siRNA 1mg/mL, preferably 5 mg/mL) and to deliver them to the ocular anterior segment, following topical instillation. In order to accomplish this goal, two different technological approaches were adopted, the solvent displacement and the self-emulsification techniques, and the following experimental activities were undertaken:

A) Study the influence of the NC's core composition (oil and surfactants) and methodological parameters on the capacity to associate a model siRNA;
B) Determine the most adequate conditions for the formation of an external protamine layer while preserving the capacity to control the release of the siRNA molecules;
C) Determine the capacity of the nanocapsules to maintain their physicochemical properties and siRNA loading upon incubation with simulated lacrimal fluid and during storage (as a suspension or as a freeze-dried form);
D) Evaluate the effect on cell viability and the silencing efficiency of the protamine nanocapsules loaded with a therapeutic siRNA;
E) Evaluate the biodistribution profile of the protamine NCs after topical administration to rabbit's eyes.

Chapter 1

Multiple-layered siRNA/protamine nanocapsules as ocular delivery systems – The solvent displacement technique

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Chapter 1

Multiple-layered siRNA/protamine nanocapsules as ocular delivery systems – The solvent displacement technique

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Abstract

Polymeric nanocapsules (NCs), consisting of an oily core surrounded by a polymer shell, are versatile vehicles that enable the association of both, lipophilic drugs and polynucleotides. The main objective of the present work was to develop protamine NCs with adequate quality attributes with regard to their use as siRNA topical ocular delivery vehicles. These were (i) a high siRNA association efficiency, (ii) a reproducible size, (iii) and a capacity to control the release of siRNA in simulated lacrimal fluid (SLF). The NCs were produced by the solvent displacement technique. Initially, the influence of a cationic surfactant, i.e. cetrimide, benzethonium chloride or ethyl lauroyl arginate, on the capacity of a nanoemulsion (NE) to associate siRNA was investigated. Subsequently, a protamine shell was added in order to form the NCs. The association of siRNA to the protamine NCs reached the highest values (final concentration of 2.5 mg/mL) when cetrimide was used as a surfactant. This high amount of siRNA remained associated upon dilution in SLF and also during storage. The developed protamine NCs presented a low cytotoxicity in HeLa cells, although their performance in terms of silencing the expression of the target gene was lower than expected (19 ± 15 %). The limited performance of the said composition should not lead to a conclusion about the value of the protamine nanocapsules as an RNA delivery platform, but rather to the specific composition of the developed nanocapsules. A number of hypothesis have been formulated with regard to the limited *in vitro* performance of the formulation, which in any case would imply a lack of performance *in vivo*.

1. Introduction

Since 1990, when the first nucleotide-based therapy (plasmid DNA (pDNA), construct for an immunodeficiency disease) entered into clinical trials [1], an increasing number of gene and nucleotide therapies (pDNA, small interference RNA (siRNA), messenger RNA (mRNA), antisense-oligonucleotides (AS-ODN) and aptamers among others) have reached clinical trials for a wide array of diseases, including high impact diseases such as cancer or ocular pathologies as well as several unmet diseases such as polyneuropathy caused by hereditary transthyretin amyloidosis (hATTR), Duchenne muscular dystrophy (DMD) or spinal muscular atrophy (SMA). These developments have led to the marketing of a few drugs, namely in 2004, the aptamer Macugen® was approved for the treatment of Neovascular age-related macular degeneration, and in 2016, naked AS-ODN Exondys 51™ (Sarepta Therapeutics) and AS-ODN survival motor neuron 2 (SMN2) Spinraza® (Biogen) were approved by the Food and Drug Administration (FDA) for the treatment of DMD and SMA, respectively [2]. In 2017 Luxturna™ (Spark Therapeutics, Inc.), a therapy based on adeno-associated viral vector type 2 (AAV2) carrying RPE65 gene was approved by the FDA for the treatment of retinal dystrophy and, in 2018, by the European Medicines Agency (EMA). Also in 2018 the first iRNA therapy, Onpattro® (Alnylam Pharmaceuticals), consisting of lipid nanoparticles was approved by the FDA and EMA for the treatment of hATTR. More recently, Zolgensma (Novartis) a survival motor neuron 1 cDNA delivered by adeno-associated viral vector type 9 (AAV9), was approved by the FDA also for the treatment of SMA on children.

Different non-viral nanocarriers have been developed so far for improving the stability of polynucleotides and their delivery to the target site. Among them, polymeric nanocomplexes and nanoparticles containing polyethyleneimine (PEI) [3-5], chitosan [6-8], hyaluronic acid (HA) [5, 7, 8] and poly(lactic-co-glycolic acid) (PLGA) [9-11] have received a great deal of attention. Among them, our group pioneered in 2008 the development of chitosan/HA nanoparticles for the delivery of pDNA [7, 8]. Other types of nanocarriers include nanoparticles composed of cationized proteins [12, 13] and albumin [14, 15], liposomes containing cationic lipids, i.e. 1,2-dioleoyl-3- trimethylammonium propane (DOTAP) [16-19], nanoemulsions [20, 21], micelles [22, 23], niosomes [24, 25] and dendrimers [26, 27].

Recently, researchers have explored the utility of combinations of lipids and polymers for improving the delivery capacity of the carriers. For example, liposomes and solid lipid nanoparticles have been combined with polymers, such as protamine, HA and dextran in order to improve their biocompatibility and cargo protection from degradation [16, 17, 28, 29]. On the other hand, our group was the first showing the potential of polyester nanocapsules for topical ocular drug delivery [30-32]. More recently, Reimondez et al. showed the efficacy of protamine NCs for corneal wound healing [33].

Based on this background information, in the present work we selected protamine NCs, as a carrier for the transport of siRNA across the cornea. This selection was based on their observed biocompatibility and capacity to interact with the corneal/conjunctival epithelium [33]. In addition, protamine molecules rich in arginine groups, were expected to enhance cell penetration [34]. On the other hand, it was presumed that the oil contained in the core of the NCs would act as lubricant and influence the structure of the tear film lipid layer [35, 36]. In fact, the use of oils in tear substitutes has improved the symptoms of dry eye syndrome [37]. DL-α-tocopherol (Vitamin E), was selected for the oil core of the NCs because of its antioxidant properties and, hence, its capacity to protect cells from oxidative stress damage [38-41]. It is also present in eye drop formulations for the treatment of dry eye symptoms. Other components selected for the formation of the NCs were non-ionic surfactants, i.e. polysorbate 80, widely used in ocular products [42] and the cationic surfactants, cetrimide, benzethonium chloride (Bz Cl) and ethyl lauroyl arginate HCl (LAE). This composition differs from the single layer protamine NCs previously disclosed by Reimondez et al. [33, 43] and González-Aramundiz et al. [44], which did not contain cationic surfactants. In these previously developed NCs, nucleotides [43] or antigens [44] were adsorbed on the protamine surface layer and, hence, exposed to the surrounding environment.

Overall, the main objective of this work was to produce multiple layer protamine NCs, where siRNA molecules were adsorbed onto a cationic surfactant layer and protected by an external protamine layer. These NCs were developed with the objective to achieve specific quality attributes, namely (i) a high siRNA association efficiency, (ii) a reproducible size (iii) and a capacity to control the release of siRNA in SLF, (iv) and a low toxicity.

2. Materials and Methods

2.1. Materials

The oils Miglyol® 812N and DL-α-tocopherol were bought from Cremer Oleo Division (Witten, Germany) and Merck (Darmstadt, Germany), respectively. The surfactants Polysorbate 80 and Cetrimide were acquired from Acofarma (Barcelone, Spain), whilst Benzethonium chloride and Ethyl lauroyl arginate HCl (Mirenat P100) were bought from Spectrum Chemical (Gardena, CA, USA) and Vedeqsa (Barcelone, Spain), respectively. Protamine sulfate (Mw 5 KDa) was purchased from Yuki Gosei Kogyo, Ltd., (Tokyo, Japan). Analytical grade ethanol was obtained from Thermo Fisher Scientific (Madrid, Spain). RNAse free water was acquired from Invitrogen (Paisley, UK). siRNA and siRNA* were kindly provided by Sylentis (PharmaMar group, Madrid, Spain). HeLa cell line (ATCC®, CCL-2™), Eagle's Minimum Essential Medium (EMEM) and Keratinocyte cell culture medium (CCM) were purchased from ATCC® (Manassas, VA, USA). Heat-inactivated fetal bovine serum (FBS) was bought from EMD Millipore (Berlin, Germany). Penicillin-streptomycin-glutamine and Dulbecco's Phosphate-Buffered Saline (DPBS) were obtained from Life Technologies (Grand Island, NY, USA). Trypsin–EDTA was acquired from Invitrogen (Paisley, UK). CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was obtained from Promega (Madison, USA). DharmaFECT™ 3 (Dharmacon™) was purchased from Horizon Discovery (Lithuania). RNA extraction kit, RNeasy® Plus Mini Kit was acquired from QIAGEN (Hilden, Germany). Block-it-Alexa fluor red fluorescent oligonucleotide was obtained from Invitrogen (Maryland, USA). High Capacity cDNA Reverse Transcription Kit was purchased from Thermo Fisher Scientific (Vilnius, Lithuania) and TaqMan® Universal PCR Master Mix was obtained from Applied Biosystems (Warrington, UK).

2.2. Preparation of protamine multiple-layered nanocapsules

2.2.1. Selection of the NC core composition

The NCs' core is based on a cationic NE prepared by the solvent displacement technique, as previously described by our research group [31, 33, 45]. In brief, 60 mg of oil (DL-α-tocopherol or Miglyol® 812N), 30 or 10 mg of non-ionic surfactant polysorbate 80 and 10 mg of cationic

surfactant (cetrimide, Bz Cl or LAE) were dissolved in 5 mL of ethanol, by vortex. This clear organic phase was immediately injected into 10 mL of RNAse free water, under vigorous magnetic stirring. After 10 to 30 min of stirring at room temperature (RT), the ethanol was removed and the NEs concentrated to 5 mL, by evaporation under vacuum (Rotavapor Heidolph Hei−VAP Advantage, Schwabach, Germany). Finally, the NEs containing Miglyol® were isolated and concentrated by ultracentrifugation (Optima™ L-90K Ultracentrifuge Beckman Coulter, Rotor type 70.1 Ti) at 30,000 rpm for 1 h at 15 °C whereas DL-α-tocopherol formulations were isolated and concentrated by ultrafiltration using Amicon® ultra centrifugal filter devices, 100 KDa pore size (Merck Millipore, Germany) at 5000 x g for 30 min at 15 °C. Both carriers were resuspended for a final volume of 1 mL.

2.2.2. Single-layered NCs (siRNA layer)

siRNA was associated to the performed cationic nanoemulsions by electrostatic forces. Different concentrations were obtained by adding dropwise a fixed volume of siRNA (model siRNA - siRNA or therapeutic siRNA – siRNA*), at different concentrations, to a known volume of cationic NE, under stirring at 500 rpm and left for 30 min at room temperature. These carriers will be referred as siRNA-loaded NEs or NE-siRNA.

2.3. Protamine multiple-layered NCs

After siRNA association to the surface of the cationic NEs, they were further coated with a protamine layer. For that 0.05 mL of a protamine solution, at different concentrations, were added dropwise to 0.25 mL of the NE-siRNA suspension, under stirring at 500 rpm. These multiple-layered NCs were left stirring at RT for 30 min. Different weight ratios of siRNA:protamine were studied (1:0.1 up to 1:0.7). These formulations will be referred as protamine NCs (prot. NC 0.1 – prot. NC 0.7) or protamine multiple-layered NCs.

2.4. Physicochemical and morphological characterization of nanocarriers

Particle mean size and polydispersity index (PDI) of the nanocarriers (cationic NE, siRNA-loaded NEs and protamine NCs) were measured by dynamic light scattering (DLS) using a Zetasizer® (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) after the nanocarriers dilution in ultrapure water. The zeta potentials (ζ-potential) were measured by laser-Doppler anemometry (LDA) after diluting the nanocarriers in 1 mM KCl (Zetasizer®, NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK).

Morphological analysis was carried out by transmission electron microscopy (TEM). For this purpose, samples were diluted with ultrapure water placed on copper grids and stained with a 2 % (w/v) phosphotungstic acid solution, allowed to dry and subsequently observed under the microscope (CM12 Philips; Eindhoven, The Netherlands).

2.5. Evaluation of siRNA association

The efficiency of siRNA association onto the surface of the cationic NEs and stability after protamine coating was qualitatively determined by gel retardation assay. Furthermore, siRNA displacement was performed by incubating samples with an excess of heparin (25:1 w/w heparin:RNA) for 30 min at 37 °C and horizontal shaking 300 rpm (Heidolph Titramax 1000; Schwabach, Germany). The samples were loaded in agarose gels (2 % w/v in Tris Acetate-EDTA buffer) in order to have 1 µg of siRNA per lane. A control lane containing only siRNA was also included. Loading buffer contained 1x SYBR® Gold nucleic acid stain (Life Technologies, Carlsbad, CA, USA) for sample visualization. Gels were run for 30 min at 90 V, in a Sub -Cell GT cell 96/192 (Bio-Rad, Hercules, CA, USA). Gels were imaged with a Molecular Imager® Gel Doc™ XR System (UV light 302 nm; Bio-Rad, Madrid; Spain).

2.6. Colloidal stability

2.6.1. Stability in simulated lacrimal fluid

The colloidal stability of the multiple-layered protamine NCs in SLF was evaluated by diluting the nanocarriers 1:1 (v/v) in SLF pH 7.4 (0.18 % KCl, 0.63 % NaCl, 0.006 % $CaCl_2H_2O$ and

$MgCl_26H_2O$ in RNAse free water) [46] during 30 min at 37 °C and horizontal shaking at 300 rpm (Heidolph Titramax 1000; Schwabach, Germany). After, they were further diluted in ultrapure water and its particle size analyzed by DLS (Zetasizer® NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK). Moreover, after the incubation with SLF, the siRNA association/release was also assessed by gel retardation assay, performed as previously described (section 2.5).

2.6.2. Stability in cell culture medium (CCM)

The stability of the multiple-layered protamine NCs, containing siRNA* (therapeutic siRNA), in EMEM CCM (supplemented with 10 % FBS) at 1 mg/mL was studied by determining the particle size evolution after 1 h incubation at 37 °C and horizontal shaking at 300 rpm (Heidolph Titramax 1000; Schwabach, Germany).

2.7. Cell Culture

HeLa cells were cultured in flasks containing complete EMEM (supplemented with 10 % (v/v) heat-inactivated FBS and 1 % (v/v) penicillin-streptomycin-glutamine). Cells were maintained in complete EMEM in an incubator at 37 °C with 95 % of relative humidity and a 5 % CO_2 atmosphere.

2.8. *In vitro* cytotoxicity

Cell viability was determined by using MTS assays, a Non-Radioactive Cell Proliferation assay, in which viable cells are able to convert tetrazolium to formazan by mitochondrial enzyme succinate dehydrogenase. The amount of this medium-soluble product is directly proportional to cell viability.

HeLa cells were seeded in complete medium (EMEM supplemented with FBS 10 % and 1 % of antibiotics-glutamine) at a density of 80.000 cells/cm^2. Twenty-four hours after the seeding, the medium was replaced by EMEM containing increasing concentrations (7.5 to 120 µg/mL) of the protamine NCs loading siRNA*. In table 1 the concentrations of siRNA correspondent

to the tested NCs' concentrations, are presented. One hour after the treatment, NCs were replaced by fresh medium (complete EMEM) and cells were allowed to grow for 24 h in normal culture conditions.

Twenty-four hours after the treatment, the medium of the plates was replaced by EMEM/MTS mixture and plates were incubated for 30 min at 37 °C and 5 % CO_2, protected from light. One hundred microleters of the reaction of each well were collected into a 96-well plate and the absorbance was measured at 490 nm in a POLARstar® Omega plate reader (BMG Labtech). Non-treated cells were used as control. The percentage of cell viability was calculated according to the following equation. All MTS experiments were performed in duplicate and repeated in three independent experiments.

$$Cell\ viability\ (\%) = \frac{\overline{X} - \overline{B}}{\overline{C} - \overline{B}} \times 100$$

Where $\overline{X}$ refers to the absorbance mean of NCs-treated cells, $\overline{C}$ corresponds to the absorbance mean of untreated cells (control) and $\overline{B}$ to the absorbance mean of blank (EMEM/MTS mixture).

Table 1 - siRNA* concentrations (nM) correspondent to the *in vitro* tested concentrations of the protamine NCs containing cetrimide, Bz Cl or LAE.

Protamine NCs (μg/mL)	siRNA* (nM)		
	NCs (Cetrimide)	NCs (Bz Cl)	NCs (LAE)
7.5	46	29	24
15	92	58	48
30	185	117	95
60	369	233	191
120	738	466	381

2.9. Gene silencing study

HeLa cells (ATCC®). previously grown to 90 % confluence, to maintain the cell growth in exponential phase, were seeded in 24-well plates at a density of 80.000 cells/cm^2, 24 h prior treatment with 100 nM of siRNA per well. Naked siRNA was complexed with the commercially available reagent, DharmaFECT™ 3 (DHF3), following the manufactures instructions. One hour after the exposure to siRNA*-loaded protamine NCs or DHF3-siRNA* complexes, cells were washed with DPBS and left incubating at 37 °C in fresh medium for 24, 48 and 72 h. Block-it-Alexa fluor red fluorescent oligonucleotide treated cells (non-active siRNA-DHF3) were used as control of transfection efficiency 24 h post treatment.

2.10. Quantitative real-time PCR assay

Gene expression levels were determined by gene specific primers and probes as Taqman gene expression assays specific for the target of interest and the internal control (Polr2A (Hs00172187)). 24, 48 and 72 h after NCs exposure cells were collected and RNA was extracted using RNEasy RNA extraction kit (Qiagen) in the automatic extractor QIAcube (Qiagen), according to manufacturer's instructions. Total RNA was quantified and analysed for signs of degradation using Agilent 2100 Bioanalizer following standard procedures. RIN (RNA integrity number) values ≥ 9 were obtained indicating that RNA degradation did not occur. Total RNA (4 µg) was reversely transcribed in 80 µL reactions using High-Capacity cDNA Archive Kit (Applied Biosystems), following the manufactures instructions. Real time PCR was performed using StepOnePlus detection systems (Applied Biosystems). Then, 100 ng of cDNA were amplified in a Taqman Universal master mix, under the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All experiments were performed in triplicate and repeated in three independent experiments, always including reverse transcription and template controls. In order to quantify the results obtained by real-time RT-PCR; the Comparative Threshold (Ct) method, also known as 2–ΔΔCt method was used as described by Livak *et al* [47]. Gene expression data was normalized to the control gene Polr2A.

2.11. Statistical analysis

The statistical analysis was carried out using with GraphPad Prism (software version 7.0). The results are presented as a mean ± standard deviation (SD). Unless indicated, at least 3 different replicates of each experiment were performed. Data were compared using two-way ANOVA and p-values less than 0.05 were considered to be statistically significant.

3. Results and discussion

The main objective of this work was to develop a nanocarrier able to associate a high amount of siRNA. Nanocapsules were selected as an adequate delivery system due to their capacity to associate a variety of drugs, including polynucleotides [48] small molecules [32, 33, 49] and proteins and peptides [48, 50-53]. For example, our group has developed NCs involving the co-association of pDNA and docetaxel [45], miRNA and curcumin [43] or miRNA and a chemokine [48]. In this last work, we showed that RNA adsorbed onto the surface of polyarginine NCs had to be further coated with polyarginine and hyaluronic acid layers in order to protect RNA from degradation [48]. Moreover, we verified that the addition of an external layer of protamine to miRNA-loaded protamine NCs improved by 100-fold micro RNA expression on SW480 cells [43].

Based on this background information, our approach in this work was to associate the siRNA molecules on the surface of a cationic NE and to form a protamine shell around the oily cores (see Fig 1). This multiple-layer (siRNA/protamine) was formed thanks to the electrostatic forces between the cationic surfactant of NE, siRNA and protamine. The protamine outer shell was expected to protect the siRNA molecules from premature release and degradation and to facilitate the interaction with the corneal epithelium [33].

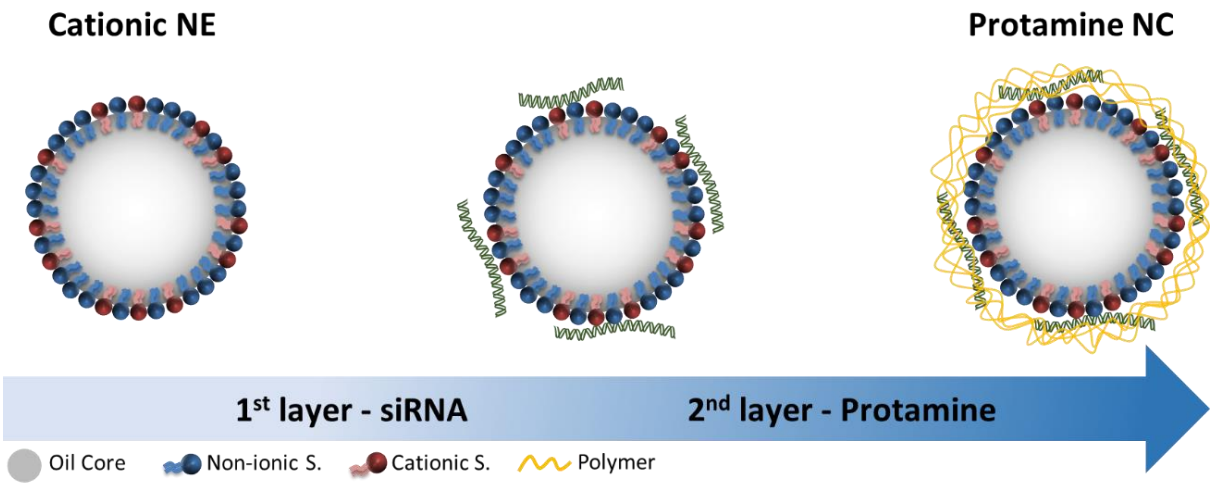

Fig. 1 - Graphical representation of the multiple-layered protamine nanocapsules (S., surfactants).

3.1. Formulation of the core nanoemulsion

Crecente at al. y Abellan-Pose et al., previously showed that apart from formulation factors, such as the type/concentration of the materials, the type/amount of organic phase solvent, the mixing process, which could be done dropwise, pouring or injection influenced the size of the NCs [54, 55]. In order to produce small particles with a large surface area for RNA association, the organic/oily phase was injected into the aqueous phase [55].

After selecting the most appropriate mixing procedure, we studied the influence of the oil, as well as that of the non-ionic and cationic surfactants, on the physiochemical properties of the NE for siRNA association and later for the polymeric coating.

3.1.1. Effect of the amount of non-ionic surfactant on NE properties

Non-ionic surfactants, i.e. poloxamers and polysorbates, are broadly used in ophthalmic formulations, not only as solubilizers, but also as ocular permeability enhancers [42]. Polysorbate 80, mainly composed of oleic acid, palmitic acid and linoleic acid, is considered as a GRAS (generally recognized as safe) material in Europe and FDA-approved as an inactive ingredient [56]. This polysorbate is present in several marketed ophthalmic formulations, such as Restasis® and Refresh Optive® (Allergan), Avitears® (Pinnacle Science, LLc), Soothe XP®

(Bausch & Lomb), Durezol® (Alcon Laboratories), among others. Our research group previously observed that protamine NCs containing polysorbate 80 presented a higher DNA association than those prepared with polyethylene glycol derivatives, such as stearate (PEG-st) and D-α-tocopheryl polyethyleneglycol 1000 succinate (TPGS) [43]. This was attributed to the repulsive effect of free PEG molecules towards DNA attachment.

Initially, we tested different amounts of polysorbate 80 (10 and 30 mg), maintaining fixed the amounts of oil DL-α-tocopherol (or vitamin E, 60 mg) and cationic surfactant cetrimide (10 mg). As expected, by increasing the amount of the non-ionic surfactant the globule size of the nanoemulsion decreased significantly (Fig. 2). On the other hand, the increase of the amount of polysorbate 80 did not have an effect on the surface charge of the particles, which remained highly positive. Therefore, as a smaller size could be translated into a larger surface area for siRNA association, we selected the amount of 30 mg of polysorbate 80 for further studies.

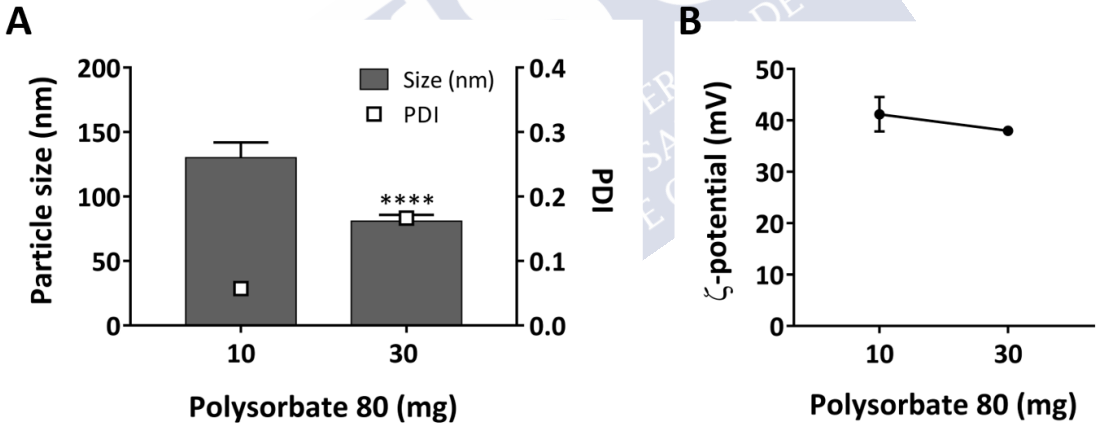

Fig. 2 - Influence of the amount of polysorbate 80 on the nanoemulsions' size/PDI (**A**) and ζ-potential (**B**). Numerical values represent the mean ± SD (n=3). Two-way ANOVA was applied for the statistical analysis of size, PDI and ζ-potential (**** $p < 0.0001$, * refers to size).

3.1.2. Effect of the type of oil on the NE properties

Vitamin E and medium-chain triglycerides (MCT) have been used in different ophthalmic marketed products such as Navi®Lipo (Vidal Vademecum), Boots dry eyes® (Boots), Tears again® (Optima), Artelac® Lipids (Bausch & Lomb) and Ikervis® (Santen). Vitamin E is known

for its capacity to protect cells from oxidative damage due to its antioxidant properties. Because of this, it has been described to have a protective role in the progression of cataracts [39, 40], retinal photodegeneration [39] and age-related macular degeneration [38]. Moreover, it presents antiproliferative properties that can be helpful in glaucoma surgery recovery [57, 58]. Our group has shown the beneficial effect of protamine NCs with vitamin E/polysorbate oily core loading curcumin for corneal wound healing [33].

On the other hand, MCTs, mainly composed of glycerol, caprylic and capric fatty acids are known to improve tear's stability [59]. In this work, the MCT, Miglyol® 812N was selected. The influence of the type of oil (vitamin E and Miglyol®) was studied by fixing the amount of oil (60 mg), polysorbate 80 (30 mg) and cetrimide (10 mg).

The results presented in Fig. 3 indicate that the nanoemulsions containing Miglyol® exhibit a significantly larger size than the ones containing vitamin E. The same tendency was observed in other prototypes developed in our group [44, 55]. Therefore, the type of oil influences the physicochemical properties of the carrier but its effect is also dependent on the other components of the formulation. Due to their appropriate physiochemical properties (small size and highly positive ζ-potential) further studies were performed using vitamin E-based NEs.

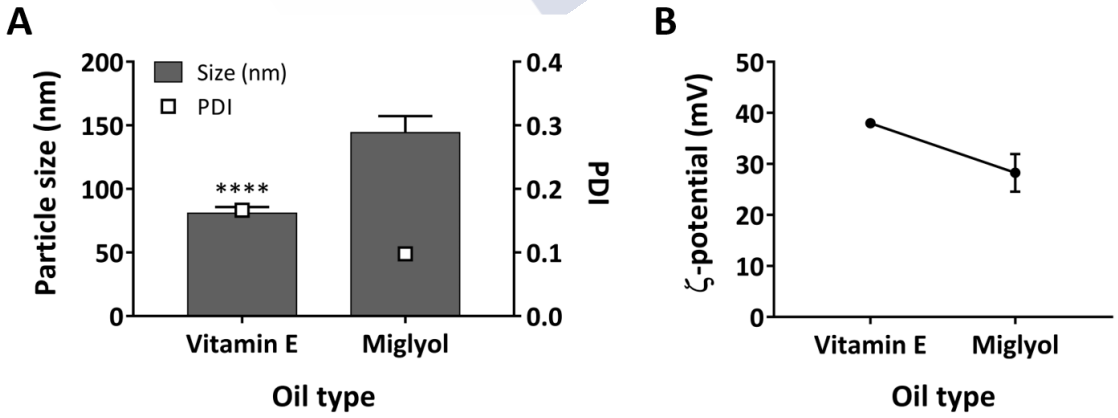

Fig. 3 - Influence of the amount of oil type (DL-α-tocopherol or vitamin E and Miglyol®) on the NEs' physiochemical properties: size/PDI (**A**) and ζ-potential (**B**). Numerical values represent the mean ± SD (n=3). Two-way ANOVA was applied for the statistical analysis of size, PDI and ζ-potential (**** $p < 0.0001$. * refers to size).

3.1.3. Effect of the type of cationic surfactant on the NE properties

Cationic surfactants have been used as preservatives in ocular formulations in order to avoid or limit microbial contamination. Benzalkonium chloride (BAK), a surfactant containing quaternary ammonium groups, was first used in the ophthalmic field in 1940, in solutions to preserve contact lenses. It is the most commonly used ophthalmic preservative due its broad antimicrobial properties, being present mainly in eye drops for glaucoma treatment due to their chronical use (Xalatan, Alphagan and Lumigan, among others). BAK has been reported to break tight-junctions of the corneal epithelium and, hence, to improve drugs' permeability [60, 61]. However, this effect is controversial [62-64], as the chronical exposure to BAK might cause deleterious effects including irritation and dryness of the ocular surface. Fortunately, these deleterious effects can be reduced by the incorporation of the surfactant into oily droplets [61, 65, 66]. Indeed, based on Santen's Novasorb technology (cationic nanoemulsions containing cetalkonium chloride) different formulations have been marketed, like Cationorm® (drug-free) for dry eye syndrome, Ikervis® and Vekacia® (loading ciclosporin) for dry eye syndrome with severe keratitis and vernal keratoconjunctivitis, respectively. These positively charged nanoemulsions are able to interact with the negatively-charged mucins and corneal/conjunctival epithelia. These electrostatic interactions with the eye surface were described as responsible for their increased ocular spreading and residence time upon instillation [67, 68].

In the present study, three different cationic surfactants (cetrimide, Bz Cl and LAE) were selected to determine their capacity to associate siRNA. These surfactants present different head groups (quaternary ammonium or guanidine groups) and hydrophobic chain lengths (Fig. 4).

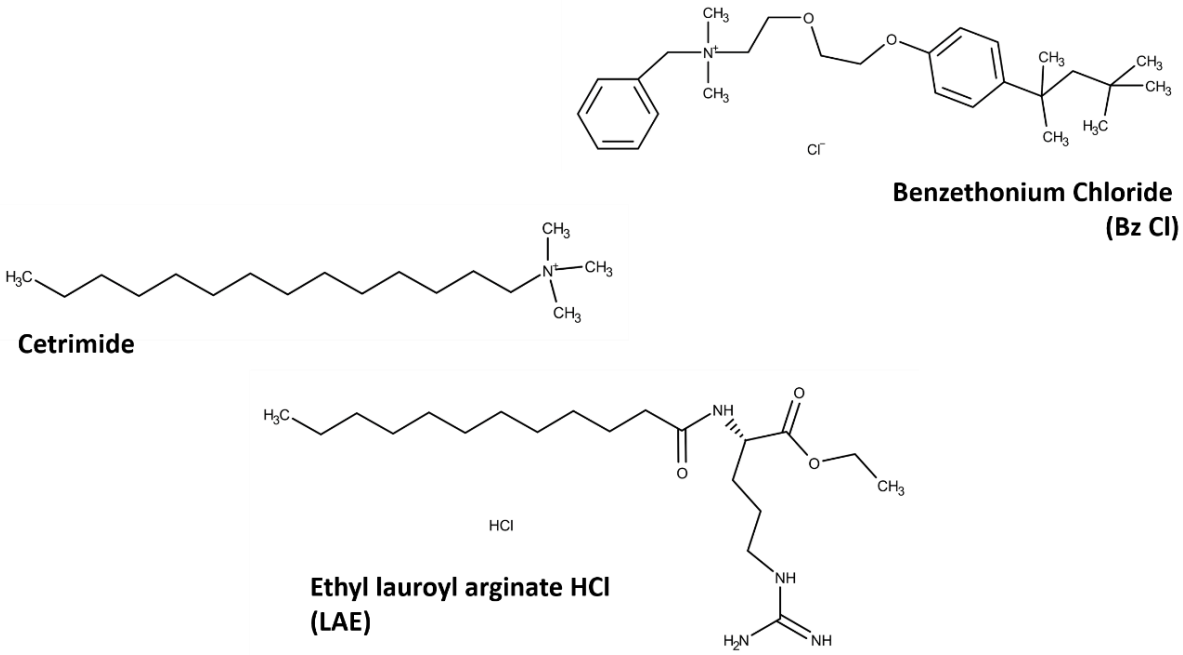

Fig. 4 - Chemical structure of the cationic surfactants used in the present work: Cetrimide, Benzethonium Chloride (Bz Cl) and Ethyl lauroyl arginate HCl (LAE).

The cationic surfactant cetrimide, composed of different alkyltrimethylammonium bromides, mainly tetradecyltrimethylammonium bromide (C14), small amounts of dodecyltrimethylammonium bromide (C12) and hexadecyltrimethylammonium bromide (C6), is being currently used as a preservative in ophthalmic formulations such as Liposic® and Artelac® Lipids (Bausch & Lomb). Bz Cl is also a cationic surfactant composed by a quaternary amine group.

On the other hand, the surfactant LAE composed of a short aliphatic chain (C11) and a positively charged L-arginine group, is classified as GRAS and approved as a novel food and cosmetics preservative. Its toxicity profile led us to consider it as a promising alternative to the other surfactants containing quaternary ammonium groups.

The influence of the type of cationic surfactant on the physiochemical properties of the vitamin E nanoemulsions was studied. As previously described, vitamin E, polysorbate 80 and the cationic surfactants were dissolved in ethanol and this mixture was injected over water under stirring. Despite the different characteristics of the surfactants, the nanoemulsions

presented identical properties, like small size (~ 80 nm), PDI (< 0.2) and positive ζ-potential (~ +35 to +40 mV) (see Fig. 5).

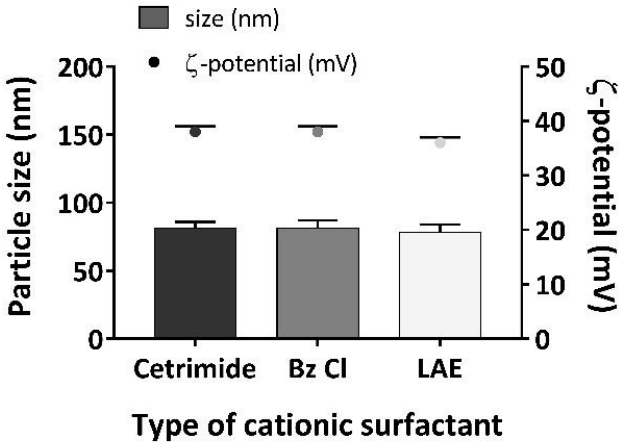

Fig. 5 - Physicochemical properties of cationic nanoemulsions containing as cationic surfactant cetrimide, Bz Cl or LAE. Numerical values represent the mean ± SD (n=3).

3.2. Single-layered NCs (siRNA layer)

The three vitamin E-based NEs containing different cationic surfactants (cetrimide, Bz Cl or LAE) were investigated for their capacity to associate siRNA. Aqueous solutions of siRNA (1.5 to 4.5 mg/mL of siRNA) were added to the cationic nanoemulsions (containing cetrimide, Bz Cl or LAE as cationic surfactants) at a volume ratio of 1:2 (NE:siRNA, corresponding to a theoretical initial concentration of 110 mg/mL of NE) so that the siRNA-loaded NEs presented a final siRNA concentration of 1 to 3 mg/mL.

The tested siRNA concentrations lead to a significant increase of NEs' size, being the cetrimide ones slightly bigger than the ones of Bz Cl and LAE (Fig. 6). The increase of siRNA concentration did not lead to changes in particle size or PDI. However, it led to major differences on the ζ-potential of the NEs, whose value went from highly positive in the absence of siRNA (+40 mV) to -20 mV. The surface neutralization process due to the siRNA attachment was gradual for LAE nanoemulsions, but it occurred drastically in the case of Bz Cl and cetrimide NEs when a certain concentration of siRNA was added to the NEs (2 mg/mL in the case of Bz Cl and 3 mg/mL in the case of cetrimide).

The total reversion of the ζ-potential clearly indicated that siRNA was associated to the NEs. In order to determine the potential presence of free siRNA we performed a gel retardation assay. The results showed that the addition of 2 mg/mL of siRNA to Bz Cl and LAE NEs resulted in the presence of free siRNA, whereas in the case of the cetrimide NE the whole amount of siRNA (2 mg/mL) was associated. We have speculated that the greater capacity of the cetrimide NE for the association of siRNA might be related to the more adequate surface exposure of the quaternary ammonium group of this surfactant. The results are in agreement with the study by Hagigit et al. study where the lipid containing a quaternary ammonium group also presented a higher association efficiency that the one with a single primary amine [69].

In order to check the release and integrity of the RNA molecules, the NEs were incubated with an excess of heparin [45, 48]. This negatively charged polysaccharide acts as a competitor of siRNA for positively charged components. In accordance to the electrophoresis gel, the cetrimide- (1, 2 mg/mL siRNA), and the Bz Cl-based NEs (1 mg/mL of siRNA) present a high resistance to heparin displacement, maintaining siRNA associated, as we can see by the lack of a band on the gel (Fig. 7). We speculated that the negatively charged heparin molecules acted as a coating instead of a displacing agent. In accordance to our hypothesis, this attachment did not happen in the case of LAE NE-siRNA (1 mg/ml of siRNA), which presented a neutral ζ-potential.

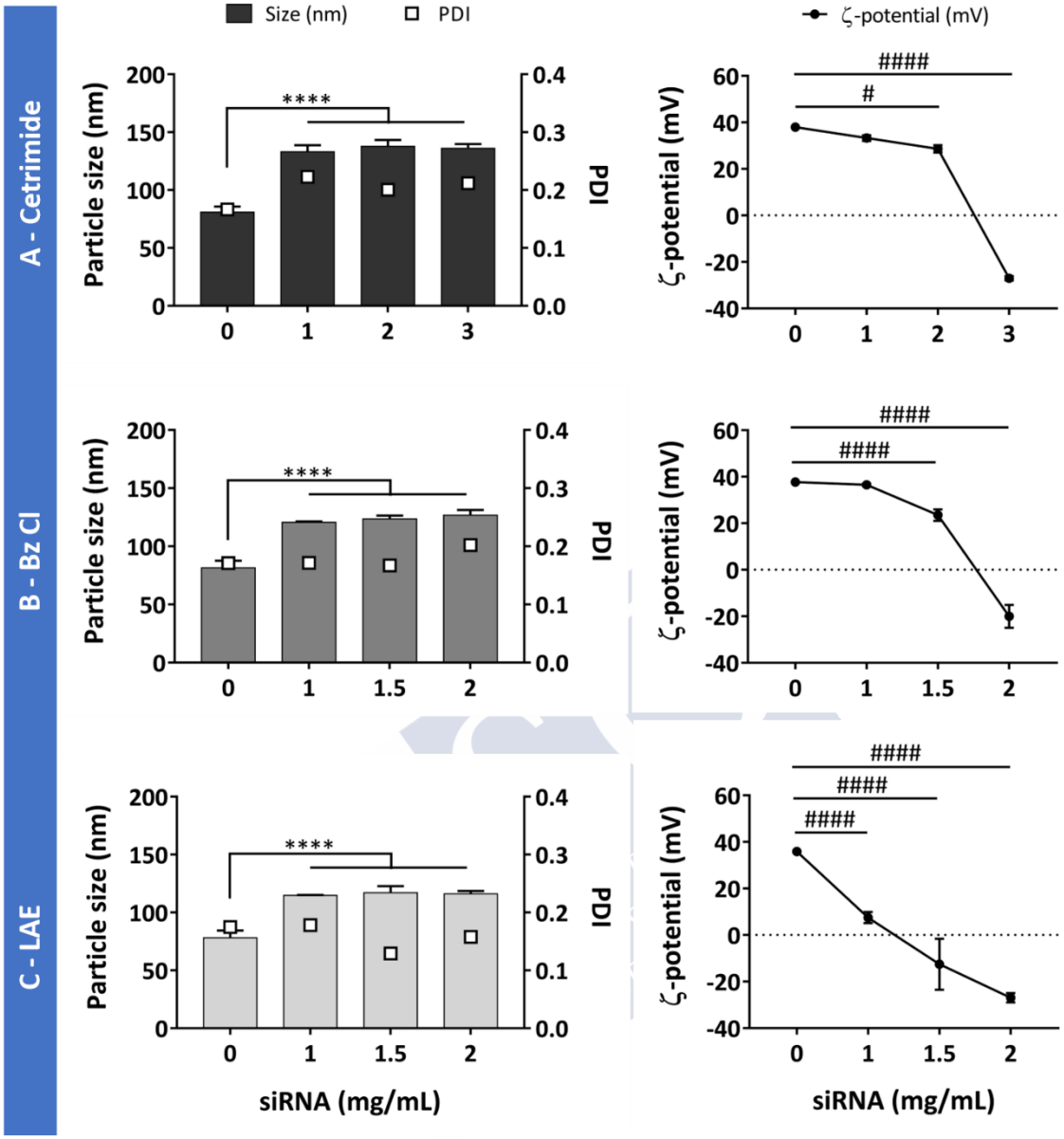

Fig. 6 - Physiochemical properties of vitamin E NEs containing cetrimide (A), Bz Cl (B) or LAE (C) after the association of siRNA at increasing concentrations: particle size/PDI (left hand) and ζ-potential (right hand) of blank-NEs and siRNA-NEs. The siRNA concentration refers to the final concentration in the formulation of NE-siRNA. Values represent the mean ± SD (n=3). Data was analyzed using a two-way ANOVA (**** $p < 0.0001$; # $p < 0.05$; #### $p < 0.0001$. * and # refer to particle size and ζ-potential, respectively).

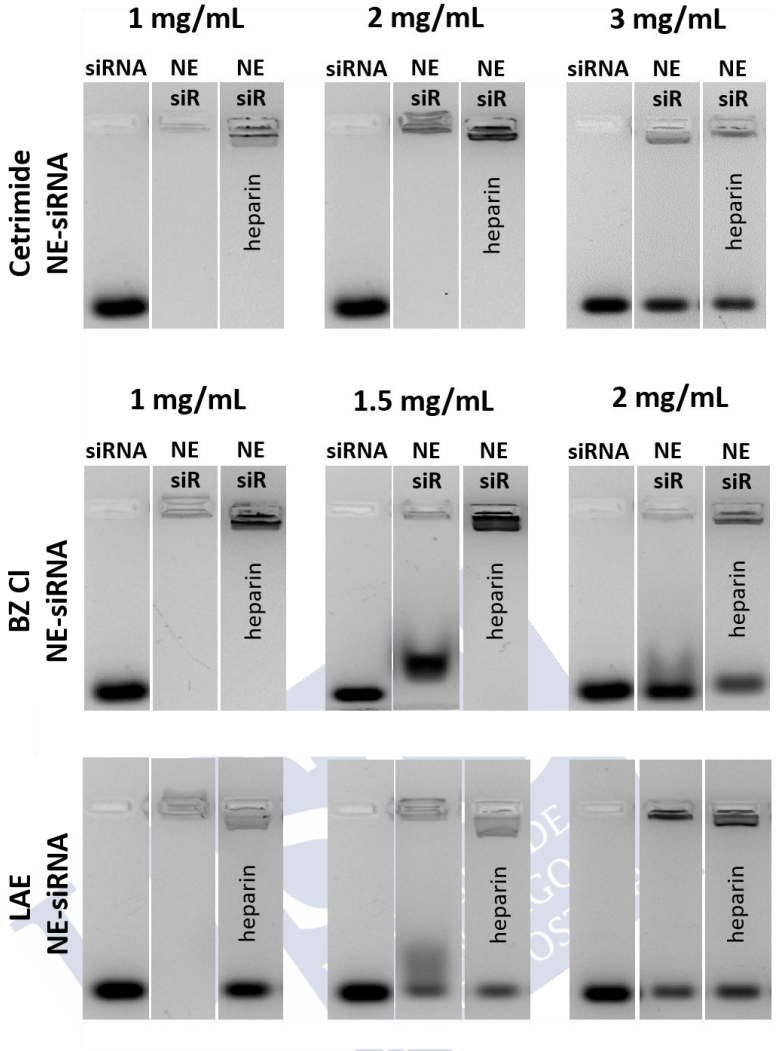

Fig. 7 - Effect of the association of increasing siRNA concentrations (1 to 3 mg/mL) to vitamin E nanoemulsions containing different cationic surfactants (cetrimide, Bz Cl and LAE). The siRNA concentration refers to the its final concentration in the formulation of NE-siRNA. For each siRNA concentration (columns) and each NE (lines), the 1st lanes correspond to siRNA control, 2nd and 3rd lanes correspond to the siRNA-loaded NEs before and after incubation with heparin.

Overall, the conclusion from this study was that the highest siRNA association was observed for the cetrimide-based NE (2 mg/mL), however the LAE-based NE (1mg/mL) might be more appropriate from the siRNA delivery point of view.

3.3. Protamine multiple-layered NCs

As indicated, the capacity of protamine NCs to increase drug residence time on the ocular surface by interacting with the negatively charged mucins on the tear film [33], persuaded us to add a protamine coating around the siRNA-loaded NEs. The best condition for the formation of the protamine multiple-layer NCs was determined by incubating the NE-siRNA with increasing concentrations of protamine, obtaining different siRNA:protamine weight ratios (1:0.1 to 1:0.7).

For both types of siRNA-loaded NEs, containing cetrimide or Bz Cl, the ratios above 1:0.3 led to a saturation of the NC surface since no further increase of the ζ-potential was observed (Fig. 8). Using this threshold, a sharp inversion of the ζ-potential from negative to positive was observed for the cetrimide and LAE NEs. In the case of the positively charged Bz Cl NEs, there was also an increase in the ζ-potential, becoming more positive.

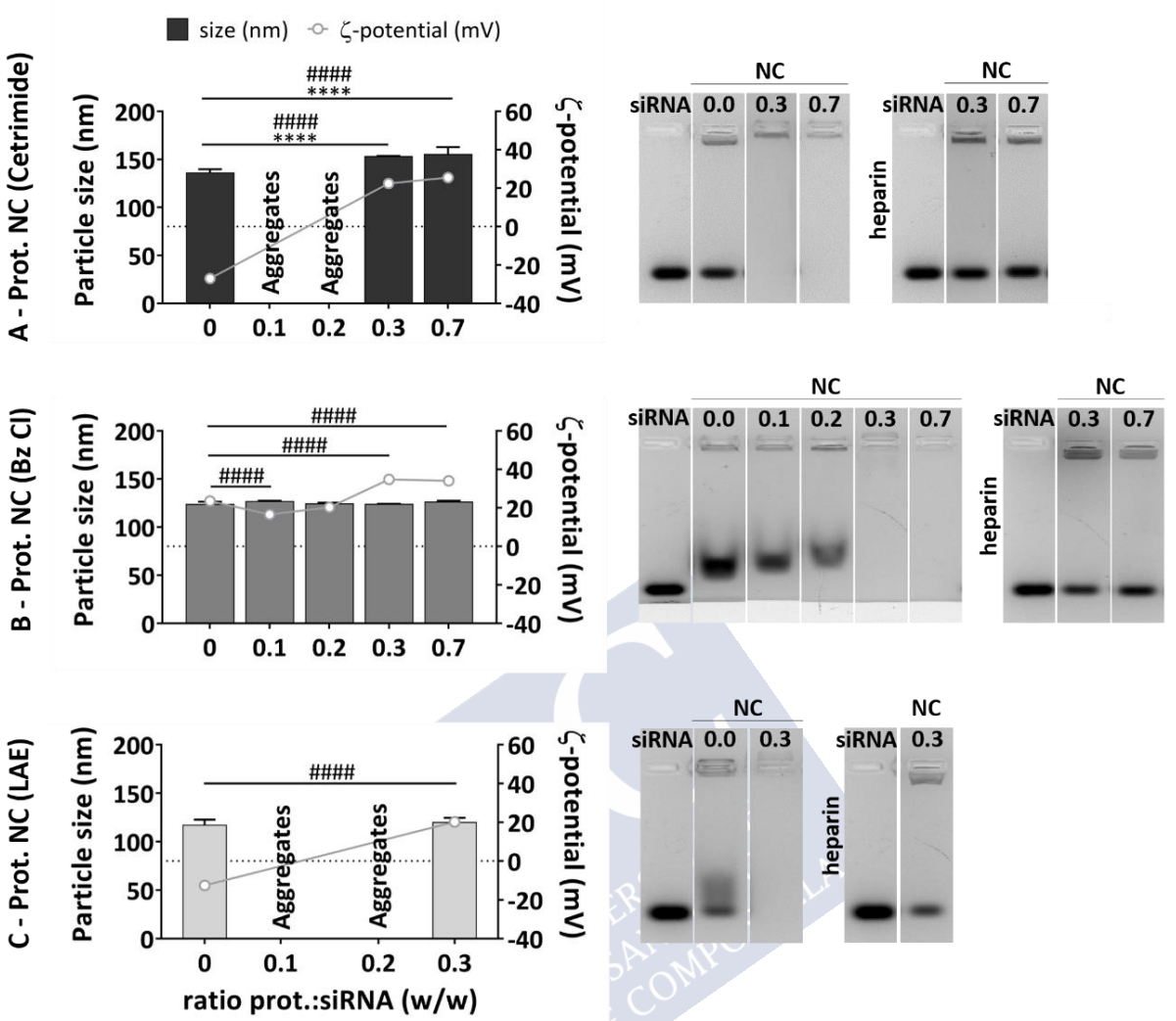

Fig. 8 - Characterization of multiple-layered protamine NCs loading siRNA. Effect of protamine coating of siRNA-loaded NEs (containing Cetrimide (**A**), Bz Cl (**B**) and LAE (**C**)) at different siRNA:protamine weight ratios (1:0.1 up to 1:0.7) on particle size and ζ-potential. siRNA association assay by gel electrophoresis (right hand), of the siRNA-loaded NEs coated with protamine at different weight ratios (1:0.1 to 1:0.7) and siRNA displacement by incubation with heparin. NC 0.0 to 0.7 refer to the NCs which coating was performed at the weight ratio siRNA:protamine of 1:0.1 to 1:0.7. Numerical values represent the mean ± SD (n=3). Data was analyzed using a two-way ANOVA (**** $p < 0.0001$; #### $p < 0.0001$; * and # refer to particle size and ζ-potential, respectively).

It is also possible to observe that the 1:0.3 ratio (NC 0.3) led to the full entrapment of siRNA and its improved release upon incubation with heparin, when compared with the siRNA-loaded NEs (NC 0.0). Following our previous hypothesis, in the case of the NCs, heparin competes with siRNA molecules in their interaction with both, protamine and cationic surfactant molecules, leading to the destabilization of the electrostatic equilibrium established between the oppositely charged cationic NE-siRNA-protamine.

The developed cetrimide-, Bz Cl- and LAE-based protamine NCs present a final siRNA concentration of 2.5, 1.25 and 0.83, which correspond to a loading of 8.0, 4.2 and 2.9 %, respectively.

3.3.1. Morphological analysis of nanocarriers

According to the results obtained from transmission electron microscopy analysis (Fig. 9), irrespective of their surface composition, the NEs and NCs presented a spherical shape, as typically observed for this type of carriers [44]. Moreover, it is also possible to observe an increase in particle size from blank NE to siRNA and protamine layered ones.

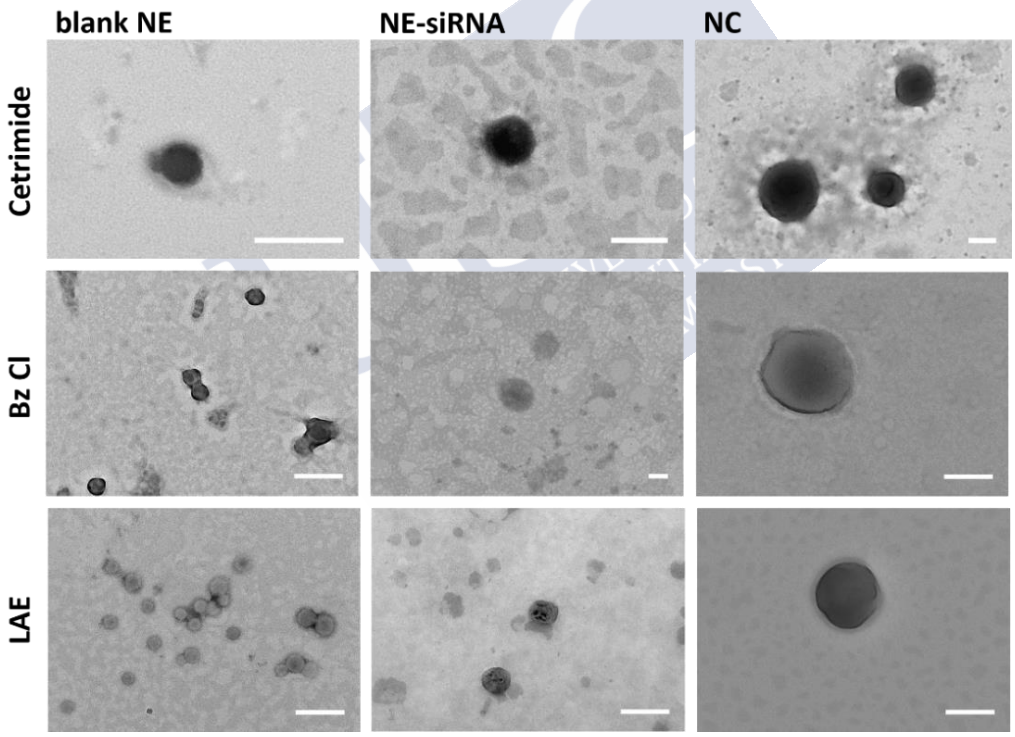

Fig. 9 - Transmission electron microscopy images of nanocarriers containing cetrimide, Bz Cl and LAE: blank NEs, siRNA-loaded NEs (NE-siRNA) and multiple-layered protamine NCs (Prot. NCs). All scale bars correspond to 200 nm.

3.3.2. Colloidal stability

3.3.2.1. Stability in simulated lacrimal fluid

Both particle size and cargo stability are of great importance in order to maintain the nanocarrier capacity to interact with ocular surface and to deliver the drug at the target site (cytoplasm). Keeping in mind that the developed formulations are aimed for topical administration, the stability of protamine NCs in terms of both, particle size and siRNA release, were studied upon their incubation in SLF. Usually, topically administered drugs are cleared from the ocular surface within a couple of minutes due to blinking reflex and fast rate of tear film renewal. Thus, we considered that an incubation period of 30 min would be enough in order to determine the utility of these nanocarriers for such application. As we can observe in Fig. 10, protamine NCs containing either cetrimide or LAE maintained their particle size and no siRNA was released to the SLF incubation medium, on the other hand, NCs containing Bz Cl suffered a little increase in particle size.

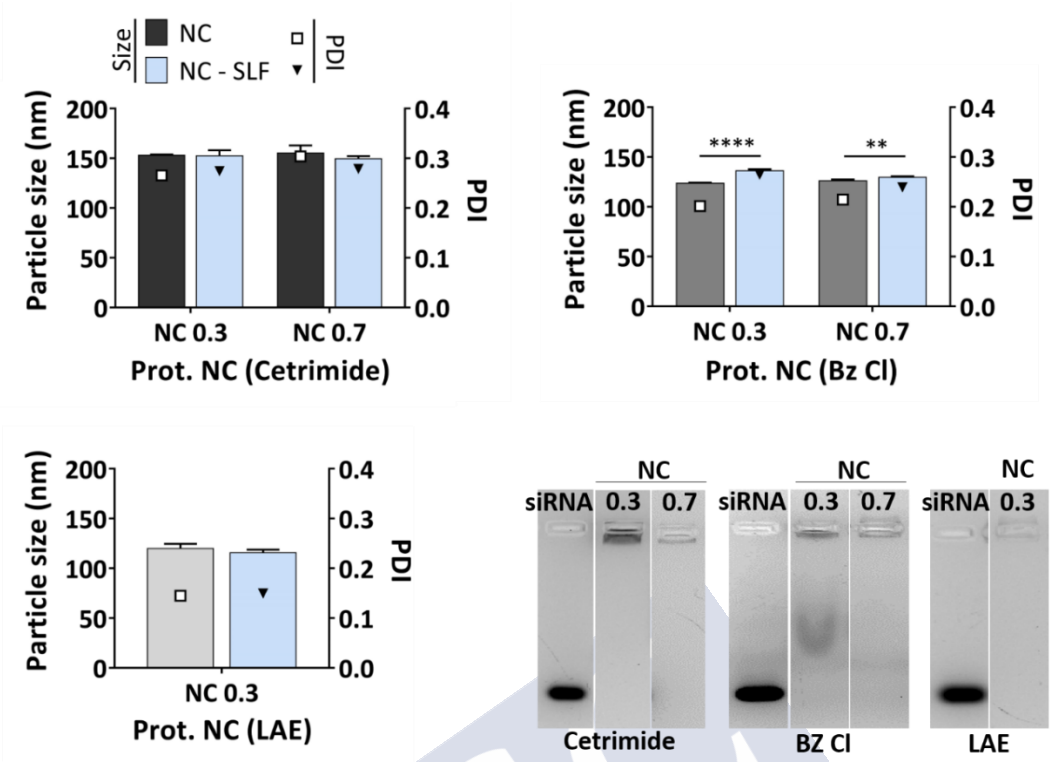

Fig. 10 - Colloidal stability of protamine NCs (containing cetrimide, Bz Cl and LAE) upon incubation with SLF for 30 min at 37 °C. Grey and black graphic bars refer to the protamine NCs before incubation with SLF while the blue bars refer to the NCs after incubation with SLF. Gel electrophoresis of protamine NCs after incubation with SLF for 30 min at 37 °C. NC 0.3 or NC 0.7 refer to the protamine NCs with a siRNA:protamine weight ratio of 1:0.3 and 1:0.7, respectively. Numerical values represent the mean ± SD (n=3). Data was analyzed using a two-way ANOVA (** $p < 0.01$; **** $p < 0.0001$. * refers to particle size).

3.4. Multiple-layered protamine NCs containing therapeutic siRNA (siRNA*)

After determining the most appropriate conditions for the preparation of protamine NCs in the presence of siRNA, we explored the possibility to associate the therapeutic siRNA*. Thus, we started by using the previously determined higher associated concentrations of siRNA, i.e. 3 and 1.5 mg/mL to the cetrimide, Bz Cl or LAE NEs, respectively, and adding the protamine shell at the selected ratio 1:0.3 (siRNA:protamine w/w).

The siRNA*-loaded NE presented similar physiochemical properties to the siRNA-loaded NE, with some differences on siRNA association efficiency (data not shown). Nonetheless, the addition of protamine coating led to particle aggregation. Therefore, we assessed the best conditions for siRNA association to the NEs containing cetrimide, Bz Cl or LAE in order to further coat the carriers with a protamine layer at the previously determined ratio of 1:0.3.

In the case of cetrimide NEs, the slight reduction on siRNA* concentration associated to these NEs (3 to 2.75 mg/mL) enabled the formation of the subsequent protamine layer at 1:0.3 weight ratio (siRNA:protamine), which resulted in a reversion of the ζ-potential values from about -18 to +24 mV, maintaining a similar particle size (Fig. 11).

NE:siRNA* (v/v)	siRNA* mg/mL (NE-siRNA)	NC siRNA*:Prot (w/w)	Size (nm)	PDI	ζ-potential (mV)
-	-	-	79 ± 7	0.2	+39 ± 4
1:2	2	-	129 ± 7	0.2	+27 ± 1
	2.5	-	130 ± 7	0.2	+14 ± 3
	2.75	-	131 ± 6	0.2	-18 ± 5
		1:0.3	**137 ± 8**	**0.3**	**+24 ± 0**
	3	-	129 ± 3	0.2	-27 ± 1
		1:0.3	**Agg**		

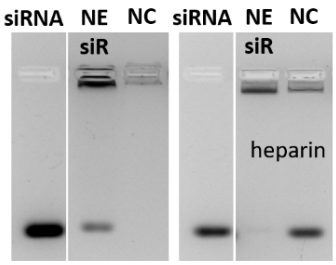

Fig. 11 - Properties of siRNA*-loaded protamine NCs containing cetrimide as cationic surfactant (left hand). The NCs highlighted in grey were selected for further studies due to their full siRNA association and adequate physicochemical properties. Gel electrophoresis (right side) of both siRNA*-loaded NE (2.75 mg/mL) and protamine NC (1:0.3, siRNA:protamine weight ratio) before (left gel) and after (right gel) incubation with heparin. The first lane of each gel corresponds to naked siRNA control. Numerical values represent the mean ± SD (n=3).

Concerning Bz Cl and LAE NEs, different approaches were pursued so that we could maintain the final loading of the nanocarriers. In this sense, siRNA* was associated to Bz Cl- and LAE-based NEs at a volume ratio of 1:4 and 1:1 (NE:siRNA*), respectively, at different concentrations (Fig. 12 and 13).

In the case of Bz Cl-based formulations (NE:siRNA volume ratio of 1:2; 1.5 mg/mL of siRNA and siRNA:protamine weight ratio of 1:0.3), despite their high ζ-potential, they showed free siRNA and the formation of aggregates. This led us not only to decrease the volume of NE added to the siRNA solution (NE:siRNA, v/v) from 1:1 to 1:4 but also of the siRNA added (1 mg/mL on NE-siRNA). The coating with protamine at 1:0.3 (siRNA:protamine, w/w) led to a full siRNA association, increasing ζ-potential while maintaining a similar particle size.

On the other hand, in the case of the LAE-containing NEs (Fig 13), at 1:1 ratio (NE:siRNA v/v) it was possible to achieve a full association for a 2 mg/mL concentration of siRNA*. This formulation could be efficiently coated with protamine at 1:0.1 ratio (siRNA:protamine w/w), leading to a more positive ζ-potential.

NE:siRNA* (v/v)	siRNA* mg/mL (NE-siRNA)	NC siRNA*:Prot (w/w)	Size (nm)	PDI	ζ-potential (mV)
-	-	-	69 ± 8	0.2	+36 ± 3
1:4	1	-	112 ± 1	0.2	+9 ± 1
		1:0.3	**118 ± 0**	**0.2**	**+29 ± 0**
	1.5	-	125 ± 1	0.2	-26 ± 0
1:2	1.5	-	119 ± 5	0.2	+16 ± 7
		1:0.3	**122 ± 1**	**0.2**	**+31 ± 1**

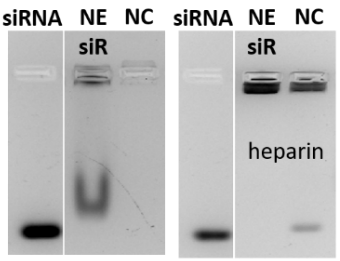

Fig. 12 - Properties of siRNA*-loaded protamine NCs containing Bz Cl as cationic surfactant (left hand). The NCs highlighted in grey were selected for further studies, due to their adequate physicochemical properties and complete siRNA association. Gel electrophoresis (right side) of both siRNA-loaded NE (1 mg/mL siRNA*, using 1:4 ratio (NE:siRNA* v/v) and protamine NC (1:0.3, siRNA:protamine weight ratio) before (left gel) and after (right gel) incubation with heparin. The first lane of each gel corresponds to naked siRNA control. Numerical values represent the mean ± SD (n=3).

The release of siRNA* from the NEs and NCs was determined by incubating the systems with an excess of heparin. Unexpectedly, in the case of Bz Cl and LAE formulations only a tenuous or no siRNA band was observed after incubation with heparin. This might be an indication of the strong affinity of these surfactants towards the siRNA molecules.

NE:siRNA* (v/v)	siRNA* mg/mL (NE-siRNA)	NC siRNA*:Prot (w/w)	Size (nm)	PDI	ζ-potential (mV)
-	-	-	67 ± 2	0.2	+38 ± 9
1:2	1.5	-	115 ± 4	0.1	-9 ± 16
		1:0.3	**Agg**		
1:1	1.5	-	137 ± 3	0.3	+23 ± 2
	2	-	115 ± 7	0.1	+5 ± 5
		1:0.1	**125 ± 2**	**0.2**	**+16 ± 0**
		1:0.2	**Agg**		
		1:0.3	**Agg**		
	2.25	-	140 ± 32	0.2	-14 ± 6
		1:0.3	**Agg**		

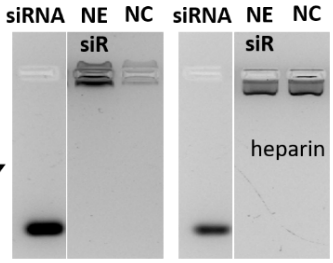

Fig. 13 - Properties of protamine NCs (containing LAE as cationic surfactant) loaded with siRNA*. Considering the adequate physicochemical properties and complete siRNA association the NCs highlighted in grey, these NCs were selected for further studies. Gel electrophoresis (right hand) of both siRNA*-loaded NE (2 mg/mL) and protamine NC (1:0.3, siRNA:protamine weight ratio) before (left gel) and after (right gel) incubation with heparin. The first lane of each gel corresponds to siRNA control. Numerical values represent the mean ± SD (n=3).

The selected formulations of protamine NCs (marked as grey in the former tables of Fig 11 to 13) containing cetrimide, Bz Cl or LAE presented a final siRNA*concentration of 2.3, 0.8 and 1.7 mg/mL, corresponding to a theoretical loading of 7.4, 4.7 and 3.8 %, respectively.

3.4.1. Colloidal stability

3.4.1.1. Stability in simulated lacrimal fluid

The suitability of these protamine NCs to be topically applied was also determined by incubating them with SLF for 30 min at 37 °C (Fig. 14). All formulations resulted to be stable in these conditions. Additionally, according to the results obtained by gel retardation assay, all formulations were able to prevent the siRNA premature release during the incubation period.

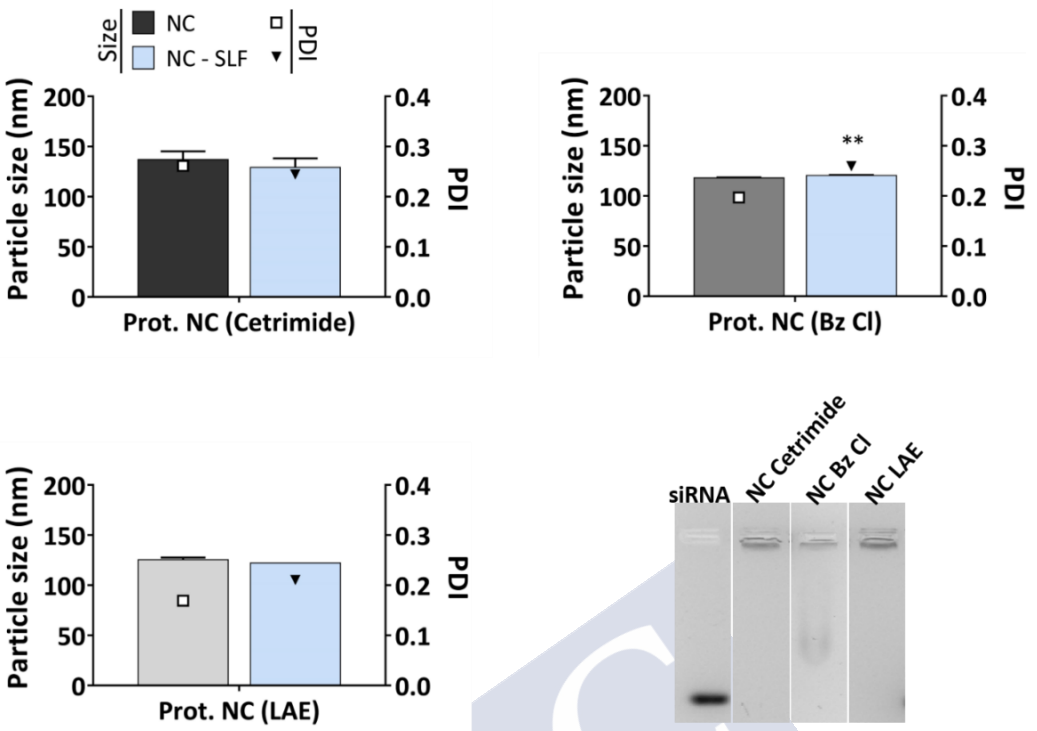

Fig. 14 - Colloidal stability of siRNA*-loaded protamine NCs (containing cetrimide, Bz Cl or LAE) when incubated with SLF for 30 min at 37 °C. Grey and blue bars refer to NCs before and after incubation with SLF. Gel electrophoresis presents siRNA release from protamine NCs after 30 min incubation with SLF at 37 °C. The first lane corresponds to siRNA control. Numerical values represent the mean ± SD (n=3). Data was analyzed by two-way ANOVA (** $p < 0.01$, * refers to particle size).

3.4.1.2. Stability in cell culture medium

Prior to the analysis of protamine NCs' cytotoxicity profile, their stability in CCM was tested. The NCs were incubated with CCM at a theoretical concentration of 1 mg/mL. After 1 h at 37 °C the samples were loaded on a 2 % (w/v) agarose gel. As shown in Fig. 15, protamine NCs containing cetrimide maintained its size after incubation with CCM while the ones containing Bz Cl and LAE suffered slight changes on particle size. Nevertheless, the absence of RNA bands on the gel retardation assay indicates that the tested conditions did not lead to siRNA premature release.

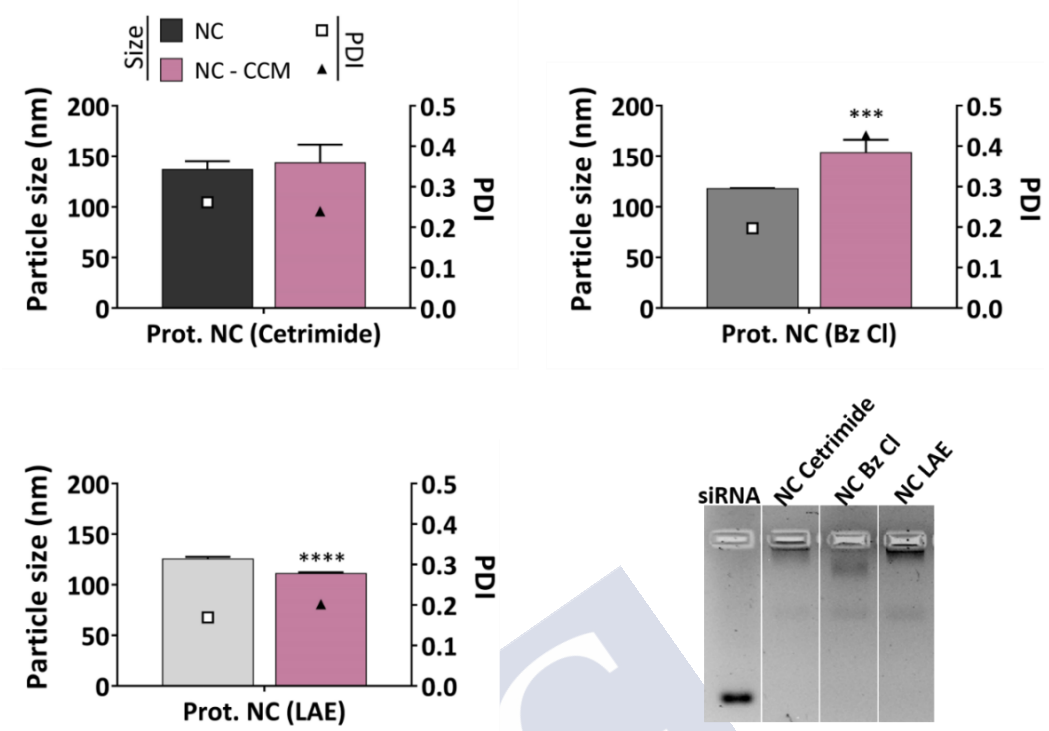

Fig. 15 - Colloidal stability of siRNA*-loaded protamine NCs (containing cetrimide, Bz Cl or LAE) when incubated with EMEM CCM for 1 h at 37 °C. Grey and pink bars refer to NCs before and after incubation with CCM. Gel electrophoresis presents siRNA release of protamine NCs after 1 h incubation with CCM. The first lane corresponds to naked siRNA control. Numerical values represent the mean ± SD (n=3). Data was analyzed by two-way ANOVA (*** $p < 0.001$, **** $p<0.0001$, * refers to particle size).

3.4.2. Cell viability

The cytotoxic effects of cationic surfactants used as preservatives in several ocular formulations have raised major concerns in the development of such products. As reported by Debbasch et al., the exposition of human conjunctival cells to low and high concentrations of surfactants like BAK and cetrimide, which contain quaternary ammonium groups, leads to cell death by apoptosis and necrosis, respectively. Moreover, cells treated with 0.005 % for 15 min led to a 75 % decrease on cell membrane integrity [70]. These deleterious effects can be circumvented by including these compounds in nanoemulsions [61, 65, 66]. In the case of the nanocarriers here reported, the cationic surfactants were enclosed in multiple-layered NCs.

Prior to determine the silencing efficiency of the developed protamine NCs (containing cetrimide, Bz Cl or LAE), their effect of the on HeLa cells viability was assessed. Considering that the silencing efficiency would be determined by using 100 nM of siRNA as reference, and

that this concentration of siRNA corresponds to 16, 26 and 31 µg/mL of NCs containing cetrimide, Bz Cl or LAE, respectively, the NCs' biocompatibility was determined up to 120 µg/mL. The increasing concentrations of the nanocarriers were incubated with the cells for 1 h. After that period, cells were maintained in culture with fresh medium for 24 h and then their metabolic activity was measured through MTS assay. The results presented in Fig. 16 show that, under the range of concentrations tested, protamine NCs did not compromise cell viability. The high biocompatibility of the developed NCs is in agreement to previously reported data on protamine NCs by our group [44]. Thus, these results confirm that the incorporation of cationic surfactants (cetrimide, Bz Cl or LAE) on NCs allowed the association of high amounts of siRNA without affecting cell viability.

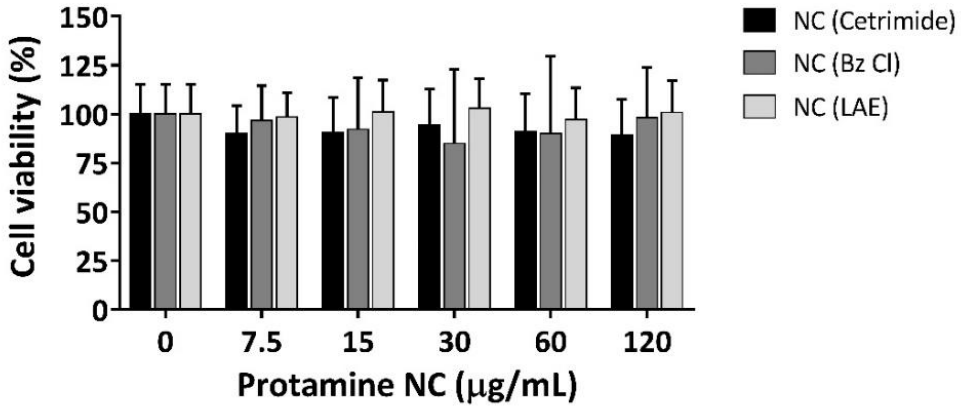

Fig. 16 - Viability of HeLa cells upon treatment with increasing concentrations (µg/mL) of siRNA*-loaded protamine NCs (containing cetrimide, Bz Cl or LAE) for 1 h. Cell viability was determined by MTS 24 h post-treatment. Values represent the mean ± SD (n=3).

3.4.3. *In vitro* silencing effect

Multiple-layered protamine NCs were developed considering that arginine-rich protamine would improve cellular uptake not only for its characteristic cell penetrating capacity but also by conferring a positive surface charge to the NCs.

The cell viability assay indicated that at the concentration of 120 µg/mL of protamine nanocapsules, which corresponds to siRNA* concentrations of 738, 466 and 381 nM for the

formulations containing cetrimide, Bz Cl and LAE (see table 1), cells were fully viable. Next, cells were treated with the developed formulations at 100 nM siRNA*, and with a transfection lipid (DHF3) for 1 h and gene expression was quantified 24, 48 and 72 h post-treatment. Naked siRNA was not included since in previous studies it led to no gene silencing, which is due to the known inability of nucleotides to cross cell membranes. Moreover, DHF3 was selected as a transfection reagent for siRNA*, due to its higher transfection capacity on HeLa cells when compared to other typically used *in vitro* transfection agents (data not shown).

The results presented in Fig. 17 show that siRNA*-DHF3 complexes were able to lead to a gene expression level of about 52 % after 72 h post-treatment (gene silencing of about 48 %). However, the protamine NCs containing cetrimide, Bz CL or LAE lead to high gene expression levels, 81, 90 and 89 %, corresponding to a gene silencing efficiency of about 19, 10 and 11%, respectively, in the same period.

The limited *in vitro* performance of the developed nanocapsules could be explained taking into account the cellular barriers that need to be overcome. First, the carrier must be able to maintain its physicochemical properties and protect cargo from premature release and degradation. Then, the carrier must be able not only to enter the cells but also to escape endosomal degradation and be released into the cytosol. Our group has previously reported that different nanocarriers containing cationic polymers, such as protamine nanocapsules [44] and nanoparticles [71], chitosan nanoparticles [72] and liposome–chitosan nanoparticle complexes [46], were efficiently taken-up by different cell types within 30 min. Hence, we presumed that this incubation period would be enough to allow cell internalization. However, we cannot discard the possibility that longer incubation times would result more effective in terms of silencing efficiency. Another possible explanation of the limited silencing effect could be that protamine NCs were not able to escape the endosomal compartment. There is also the possibility that the siRNA remained associated to the protamine NCs, due to strong interactions between cationic surfactant-siRNA-protamine. This hypothesis would be in agreement with the obtained agarose gels in which upon incubation of protamine NCs with heparin the siRNA stays tightly associated to the NCs not being displaced (protamine NCs containing LAE) or being only partially displaced (protamine NCs containing Bz Cl or cetrimide).

Considering the obtained results, other formulation composition namely other cationic surfactants should be taken into consideration so that the NC is able to associate the required high siRNA concentration (1-5 mg/mL), capable of releasing the loaded siRNA and efficiently silence the gene of interest.

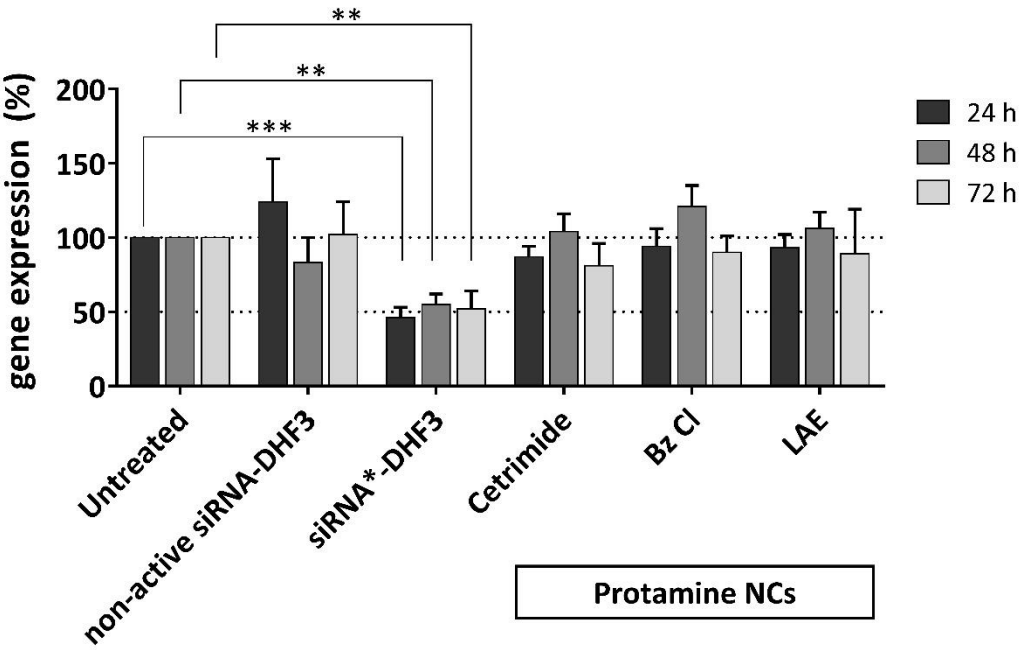

Fig. 17 - Quantitative analysis of gene expression by real-time PCR 24, 48 and 72 h post-treatment. HeLa cells were incubated with 100 nM siRNA* delivered by transfection lipid DHF3 (non-active siRNA-DHF3 and siRNA*-DHF3) and protamine NCs (entitled as cetrimide, Bz Cl and LAE). Values represent the mean ± SD (n=3). Data was analyzed by two-way ANOVA (** $p < 0.01$, *** $p<0.001$).

4. Conclusions

Here we report a new composition of protamine NCs, which exhibits a very high siRNA loading (up to 2.5 mg/mL). The protamine NCs maintained both, particle size as well as cargo loading, during incubation with SLF and demonstrated an adequate cell biocompatibility at high siRNA concentrations. Nonetheless, these formulations were not able to act as efficiently as the control transfection agent reducing gene expression. Further studies are necessary in order to understand the mechanistic behavior of the NCs *in vitro*.

Acknowledgments

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Supporting information

Supplementary methods

Protamine NCs (cetrimide) stability and siRNA release in cell culture medium

The stability of the protamine NCs containing cetrimide as cationic surfactant and loading siRNA (model siRNA), were used as formulation model to study the NCs' stability and siRNA release in CCM. Protamine NCs were incubated with Keratinocyte CCM (supplemented with 0.05 mg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone and 0.005 mg/mL insulin) at 1 mg/mL at 37 °C and horizontal shaking at 300 rpm (Heidolph Titramax 1000; Schwabach, Germany) and their stability and siRNA release were monitored at different time points (1 to 72 h) by particle size measurement and gel retardation assay, as previously described.

Supporting figures

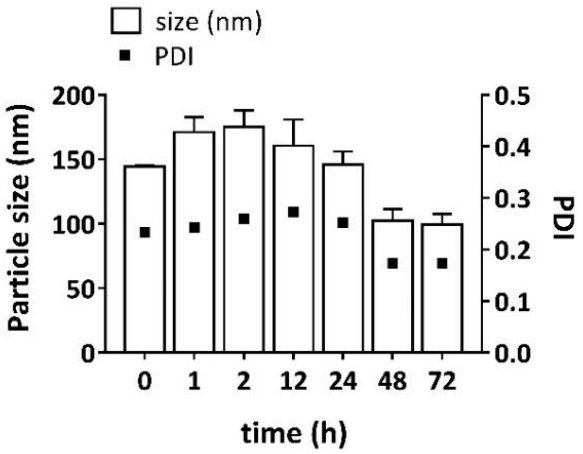

Fig. S1 - Colloidal stability of protamine NCs, containing cetrimide and loading siRNA, in supplemented keratinocyte CCM.

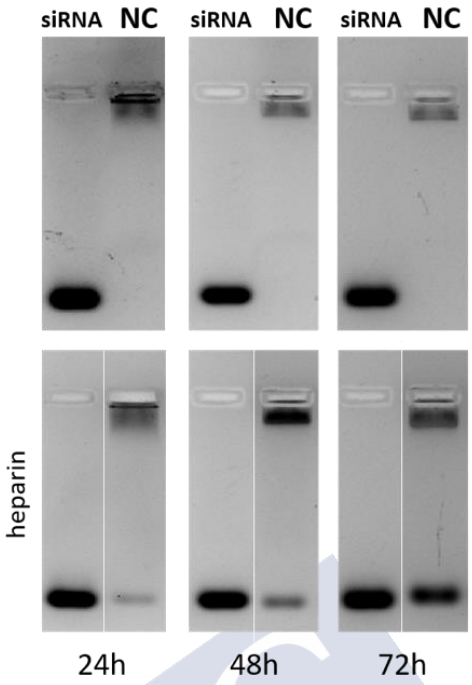

Fig. S2 - siRNA release from Protamine NCs (containing Cetrimide) after 24, 48 and 72 h of incubation with supplemented keratinocyte CCM at 37 °C. After 24, 48 and 72 h of incubation with CCM the samples were further incubated 30 min with heparin excess for siRNA displacement.

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Chapter 2

Development of a formulation of multilayer protamine nanocapsules with a high siRNA concentration for ocular application

-The self-emulsifying technique

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Chapter 2

Development of a formulation of multilayer protamine nanocapsules with a high siRNA concentration for ocular application - The self-emulsifying technique

This work was performed in collaboration with Vanessa Castro-López[1], Ignacio Alcalde[2], Tamara Martinez[3]

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Abstract

The aim of this study was to investigate the potential of protamine nanocapsules (NCs) as a carrier for the topical ocular delivery of siRNA. The final goal would be to use such formulation for increasing the penetration of the polynucleotide across the eye. A requirement established for this formulation was to achieve a final concentration of about 5 mg/mL. For the loading of siRNA into the carrier, siRNA molecules were first associated to the positively charged oily droplets of a nanoemulsion (NE) prepared by a self-emulsifying technique. Next a protamine layer was assembled onto the surface of the siRNA-loaded NE. Protamine was expected to protect siRNA molecules from degradation and facilitate their cell internalization. A siRNA model was used for the formulation development while a therapeutic siRNA was used for *in vitro* assays. The developed nanocarriers exhibited adequate physicochemical properties (size of 130-150 nm, low PDI and loading of a high siRNA concentration), which were preserved after their incubation in simulated lacrimal fluid (SLF), and the capacity to prevent the premature release of the cargo. The siRNA-loaded NCs were also stable during storage at 4 °C for 15 days and could be freeze-dried and resuspended without altering their original properties. Protamine NCs (30 µg/mL) loaded with 100 nM of therapeutic siRNA were found

to be non-cytotoxic after 1 h in contact with cells and resulted in a significant gene silencing effect at 72 h post-treatment. Finally, following topical ocular administration to rabbits, it was found that protamine nanocapsules have a favorable interaction with the cornea. Overall, these highly siRNA loaded nanocapsules might have a potential for topical ocular instillation.

1. Introduction

Ocular diseases may dramatically affect people's quality of life, especially when the outcome might be blindness. According to recent analysis by the international Vision loss expert group (VLEG), from 2015 to 2020 the number of people suffering from blindness is expected to raise by 3 millions [1, 2], being leading diseases cataracts, glaucoma, age-related macular degeneration (AMD) and diabetic retinopathy. Because of their severity, these and other diseases affecting the anterior and posterior segments of the eye are being addressed with the development of innovative polynucleotide-based therapies [3]. According to the clinical trials database (clinicaltrials.gov), up to date 62 clinical trials making use of siRNAs have been registered, 13 of which are indicated for the treatment of eye disorders, such as glaucoma, AMD, choroid neovascularization, among others.

The discovery of interference RNA (iRNA) in the late 90s [4] established a new landmark in the medicinal field due to its high therapeutic potential. Specifically, small interference RNA (siRNA), a 21-23 base pairs double-stranded RNA, has shown the capacity to specifically silence disease-causing genes. Despite the promises, in the last two decades researchers and companies have struggled with significant challenges associated to the multiple barriers that siRNA molecules need to confront before reaching their targets. Namely, in order to attain their therapeutic potential siRNA molecules, need to (i) be safely delivered to the target tissue; (ii) enter the target cell; (iii) avoid endosomal entrapment and degradation and (iv) be recognized by the RNA-induced silencing complex (RISC) machinery in order to play its role [5-8].

A variety of chemical modifications of the siRNA molecules have been investigated so far, specially intended to increase their stability in biological fluids, improve their efficacy and reduce side-effects [9-11]. In addition, different approaches, including viral and synthetic vehicles have been developed for the protection and efficient delivery of their cargo. Despite the recent advances in terms of reducing their mutagenic and immunogenic effects, there still some safety concerns and limitations related to their cargo capacity and complex scale-up production [12-14], have prompted the attention to the synthetic nanocarriers. These

nanocarriers can be produced by simple and scalable methods and be engineered according to the cargo and target organ's needs [5, 8, 11, 15].

iRNA has come a long way with many step backs but finally last year marked a milestone for this type of therapies. The Food and Drug Administration (FDA) approved Onpattro® (Alnylam Pharmaceuticals) for the treatment of polyneuropathy caused by hereditary transthyretin amyloidosis. This is not only the first approved iRNA therapeutic, but also the only among the group of polynucleotide and gene therapies whose cargo is delivered by a non-viral vehicle, more specifically by lipid nanoparticles. Apart from Onpattro® the so far available polynucleotide therapies are usually used in the naked form or encapsulated in viral vectors, however the tendency to use synthetic carriers, is increasingly present in clinical trials [16].

Taking into account this background information, the general aim of our work was to develop a synthetic nanocarrier for the delivery of siRNA to the anterior segment tissues of the eye. It was expected that such a vehicle would be of interest for the treatment of pathologies such as dry eye syndrome, corneal neovascularization and glaucoma, among others. From the practical stand point, the goal was the design of a formulation that has the capacity to load high concentrations of siRNA (about 5 mg/mL). It was hypothesized that this concentration would be necessary in order to achieve a significant amount of siRNA into the eye and, hence, to obtain a therapeutic effect. Additionally, our objective was to produce the formulation using an easy and scalable method.

To this end and based on previous information from our laboratory showing the improved ocular retention and corneal wound healing capacity of protamine NCs [17], we sought to adapt this delivery carrier for the association of siRNA. Hence, we developed a cationic nanoemulsion that was coated by a layer of siRNA and an additional protamine shell intended to protect siRNA from the external environment. Protamine, an arginine-rich (70 %) peptide, was selected based on a number of facts. First, its safety record, which is justified by the presence in the market of the parenteral formulation, Neutral protamine hagedorn (NPH) for insulin sustained release and also by the clinical development of an RNA-based cancer immunotherapy (RNActive® vaccine by CureVac AG). Second, protamine has the capacity to translocate through the cell membrane. These facts justify the previous use of protamine in combination with different lipidic nanocarriers such as solid lipid nanoparticles [18, 19],

liposomes [20-22] for the delivery of polynucleotides and, also, in our group, for the formulation of drug- and RNA-loaded protamine nanocapsules [17, 23].

2. Materials and Methods

2.1. Materials

Labrafac™ lipophile WL 1349 was kindly provided by Gattefossé (Saint-Priest, France). DL-α-tocopherol (vitamin E) was bought from Merck (Darmstadt, Germany). Castor oil, polysorbate 80 and cetrimide were obtained from Acofarma (Barcelona, Spain). Analytical grade ethanol was obtained from Thermo Fisher Scientific (Madrid, Spain). RNAse free water was acquired from Invitrogen (Paisley, UK). Protamine sulfate (Mw 5 KDa) was bought from Yuki Gosei Kogyo, Ltd., (Tokyo, Japan). 5-Carboxy tetramethyl rhodamine succinimidyl ester (5-TAMRA-SE) was acquired in EMP Biotech (Berlin, Germany). SYBR® Gold nucleic acid stain was obtained from Life Technologies (Carlsbad, CA, USA). HeLA cell line (ATCC®, CCL-2™), Eagle's Minimum Essential Medium (EMEM) were purchased from ATCC® (Manassas, VA, USA). Heat-inactivated fetal bovine serum (FBS) was obtained from EMD Millipore (Berlin, Germany). Penicillin-streptomycin and Dulbecco's Phosphate-Buffered Saline (DPBS) were acquired from Life Technologies (Grand Island, NY, USA). Trypsin–EDTA was purchased from Invitrogen (Paisley, UK). CellTiter 96® AQueous non-radioactive Cell proliferation assay (MTS) was obtained from Promega (Madison, USA). DharmaFECT™ 3 (Dharmacon™) was acquired from Horizon Discovery (Lithuania). RNA extraction kit, RNeasy® Plus Mini Kit was purchased from QIAGEN (Hilden, Germany). Block-it-Alexa fluor red fluorescent oligonucleotide was acquired from Invitrogen (Maryland, USA). High Capacity cDNA Reverse Transcription Kit was obtained from Thermo Fisher Scientific (Vilnius, Lithuania) and TaqMan® Universal PCR Master Mix was purchased from Applied Biosystems (Warrington, UK). siRNAs were kindly provided by Sylentis (PharmaMar group, Madrid, Spain).

2.2. Preparation of cationic nanoemulsions

The cationic nanoemulsions were prepared by the simple self-emulsifying technique. For that, an aqueous phase was added to an oil phase at room temperature (RT) under vigorous stirring (1200 rpm) and the mixture was left stirring for 30 min at 500 rpm. The composition of both phases was changed according to three different approaches:

i) The aqueous phase (RNAse free ultrapure water) was added into the oil phase containing the oil (labrafac™), non-ionic surfactant (polysorbate 80) and the cationic surfactant (oleylamine or cetrimide) for a final concentration of about 80 mg/mL of oil and non-ionic surfactant and 30 and 40 mg/mL of oleylamine and cetrimide, respectively. Both cationic surfactants are present at equal molar concentrations;

ii) The aqueous phase composed by cetrimide was added over the oil phase containing oil (labrafac™, castor oil or vitamin E) and polysorbate 80. The final concentration of cetrimide was maintained at 40 mg/mL while the oils and non-ionic surfactant were used for both 80 and 40 mg/mL;

iii) The aqueous phase (RNAse free ultrapure water) was added to the oil phase containing oil (labrafac™, castor oil or vitamin E), polysorbate 80 and 200 µL of an ethanolic solution of cetrimide. The formulations were placed in a centrifugal vacuum concentrator (miVac Duo concentrator, Genevac, England) for 30 min at 37 °C, for ethanol removal. The formulations contained a final concentration of 80, 40 or 10 mg/mL of oil, and 40 mg/mL of polysorbate 80 and cetrimide.

2.3. Preparation of nanoemulsions loading siRNA

The previously prepared NEs presenting a mean particle size lower than 150 nm were loaded with siRNA by a single or two steps procedure:

Single-step siRNA loading:

By using approach (i) the aqueous phase was replaced by a siRNA solution at increasing concentrations and added to the oil phase (labrafac, polysorbate and oleylamine) at high speed (1200 rpm) and left stirring for 30 min at 500 rpm at RT.

Two-step siRNA loading:

The formerly prepared blank NEs (approaches i, ii, iii) were incubated with a high concentrated siRNA solution (22.5 mg/mL) while stirring at high speed, in order to obtain the desired siRNA concentrations (1 up to 6 mg/mL) in the NE-siRNA formulations. The formulations were left stirring at RT for 30 min at 500 rpm.

2.4. Preparation of protamine nanocapsules

Protamine NCs were prepared using an oleylamine oil core. Initially oleylamine NEs were produced by approach (i) where the aqueous phase (RNAse free ultrapure water) was added to the oil phase (labrafac™, polysorbate 80 and oleylamine) at high speed and then left stirring at 500 rpm for 30 min at RT. Afterwards, in a second step, the blank NEs were incubated with high concentrated siRNA solution, first at high speed for 30 s and then 30 min at 500 rpm at RT. Finally, the siRNA-loaded NE (6 mg/mL of siRNA) were further coated with a protamine layer by incubating 0.25 mL of the NE-siRNA suspension with a 0.05 mL protamine solution (1.5, 3 and 5 mg/mL). Two different siRNAs were used, siRNA (model siRNA) for the optimization of the protamine NCs and siRNA* (therapeutic siRNA) for the *in vitro* cell studies.

For the biodistribution assay, fluorescent NCs were prepared with TAMRA-labeled protamine (Prot-T). The labeling was performed according to manufacturer's instructions. In brief, protamine was dissolved in 0.1 M sodium bicarbonate buffer (pH 9.0) while TAMRA was dissolved in dimethyl sulfoxide (DMSO), both at 10 mg/mL. One hundred microliters of TAMRA solution were slowly added to 1 mL of protamine solution and left stirring at RT for 1 h. Afterwards, the solution of the labeled protamine was dialyzed against ultrapure water for 48 h (SnakeSkin, cellulose membrane Mw 3.5 KDa, Thermo Fisher, Spain). The solution was freeze-dried and stored at -20 °C until further use. The fluorescent NCs were prepared as previously described, by adding the Prot-T (1.5 mg/mL) to the NE-siRNA at a volume ratio of 1:0.2.

2.5. Nanocarriers' physicochemical characterization

The hydrodynamic diameter and polydispersity index (PDI) of blank NE, NE-siRNA, protamine NCs and Prot. T NCs were measured by dynamic light scattering (DLS) The nanocarriers were diluted in ultrapure water and measurements were performed at 25 °C with a detection angle of 173 °. Zeta potentials (ζ-potential) were measured by laser-doppler anemometry (LDA) at 25 °C, after diluting the nanocarriers in 1 mM KCl. The measurements were performed using a Zetasizer® (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK).

2.6. Morphological analysis of nanocarriers

Morphological analysis of the blank NE, NE-siRNA and protamine NCs was conducted by transmission electron microscopy (TEM). Nanocarriers' samples were diluted in ultrapure water, placed on copper grids. The excess was removed with filter paper and the samples were stained with a 2 % (w/v) phosphotungstic acid solution. Finally, the grid was washed with ultrapure water and the excess of water was removed with a filter paper. Once dried, the samples were observed under the microscope (CM12 Philips; Eindhoven, The Netherlands).

2.7. Evaluation of siRNA association

NEs and protamine NCs' siRNA association efficiency was qualitatively determined by gel retardation assay. Moreover, in order to confirm siRNA integrity and determine the capacity of the carrier to release siRNA, the formulations were incubated with an excess of heparin (25:1 w/w heparin:RNA) for 30 min at 37 °C and horizontal shaking 300 rpm (Heidolph Titramax 1000; Schwabach, Germany). The samples were loaded at 1 µg of siRNA per lane in 2 % (w/v) agarose gels (in Tris Acetate-EDTA buffer). Naked siRNA was also included as control. Loading buffer contained 1x SYBR® Gold nucleic acid stain for sample visualization. Gels were run for 30 min at 90 V, in a Sub -Cell GT cell 96/192 (Bio-Rad, Hercules, CA, USA). Finally, gels were imaged with a Molecular Imager® Gel Doc™ XR System (UV light 302 nm; Bio-Rad, Madrid; Spain).

2.8. Freeze-drying of NE-siRNA and protamine NCs

A powder form of siRNA-loaded NEs and protamine NCs was obtained by freeze-drying the nanocarriers suspension in the presence of trehalose as cryoprotectant for a final concentration of 10 % (w/v) upon resuspension. The nanocarriers were diluted in a trehalose solution for a concentration of 20 mg/mL, quickly frozen at -80 °C and then lyophilized in a freeze-drier Genesis 25 ES, VirTis Model-Wizard 2.0 (SP Industries, USA). The first drying step lasted for 35 h and the temperature was gradually increased from -40 to -20 °C. During the second step, of about 11 h, the temperature continued to increase gradually up to +20 °C. Samples were reconstituted to the initial particle concentration in RNAse free ultrapure water under vortex agitation for 30 s. The physicochemical characterization of the freeze-dried (FD) nanocarriers was performed as described in section 2.5. siRNA association efficiency as well as colloidal stability in SLF were also determined as described in section 2.7 and 2.9.2, respectively.

2.9. Colloidal stability

2.9.1. Stability under storage conditions

Suspensions of both siRNA-loaded NEs and protamine NCs before and after FD were stored at 4 °C. The FD NE-siRNA and FD-NCs in powder form were stored at 4 °C and also at RT. During 1 month their physicochemical properties were determined, at predetermined time points, as described in section 2.5. Moreover, their siRNA association efficiency was determined by gel retardation assay (section 2.7).

2.9.2. Stability in simulated lacrimal fluid

The colloidal stability in SLF of both siRNA-loaded NEs and protamine NCs, before and after FD was studied. The nanocarriers were incubated with SLF pH 7.4 (0.18 % KCl, 0.63 % NaCl, 0.006 % $CaCl_2H_2O$ and $MgCl_26H_2O$ in RNAse free water) [24] at 1:1 (v/v) during 30 min to 60 min at 37 °C and horizontal shaking at 300 rpm (Heidolph Titramax 1000; Schwabach, Germany). Particle size and PDI were measured through DLS (Zetasizer® NanoZS, ZEN 3600, Malvern

Instruments, Worcestershire, UK) by diluting the nanocarrier-SLF mixture in ultrapure water. The effect of particles incubation with SLF on siRNA association/release was assessed by gel retardation assay (section 2.7).

2.9.3. Stability in cell culture medium

Protamine NCs (loading 5 mg/mL of therapeutic siRNA*) were incubated with cell culture medium (non-supplemented EMEM) for 1 h at 37 °C and horizontal shaking at 300 rpm (Heidolph Titramax 1000; Schwabach, Germany) at a final NC concentration of 1 mg/mL. Particle size and PDI were determined by DLS using a Zetasizer® (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) at 25 °C with a detection angle of 173 °.

2.10. Cell Culture

In vitro assays were performed using HeLa cells, a cell line typically used in transfection assays. The cells were cultured in EMEM supplemented with 10 % (v/v) of heat-inactivated FBS and 1 % (v/v) penicillin-streptomycin. HeLa cells were maintained in culture for a confluence of 90 % in an incubator at 37 °C with 95 % of relative humidity and a 5 % CO_2 atmosphere.

2.11. *In vitro* cytotoxicity

The effect of siRNA*-loaded protamine NCs on cell viability was evaluated by MTS assays. HeLa cells were seeded at a density of 80.000 cells/cm^2 in 24 well plate for a 90 % confluence and incubated with supplemented EMEM for 24 h before transfection. Increasing concentrations (7.5 to 60 µg/mL) of protamine NCs (siRNA*) in non-supplemented EMEM were incubated with cells for 1 h. Afterwards, it was substituted by fresh medium and cells were incubated for another 24 h in normal culture conditions. Medium was replaced by an EMEM/MTS mixture and plates were incubated for 30 min at 37 °C and 5 % CO_2, protected from light. One hundred µL of the reaction mixture of each well were collected into a 96-well plate and the absorbance of formazan was measured at 490 nm in a POLARstar® Omega plate reader (BMG Labtech). All MTS experiments were performed in duplicate and repeated in three independent

experiments. The percentage of cell viability compared to control cells (untreated cells) was calculated according to the following equation.

$$Cell\ viability\ (\%) = \frac{\overline{X} - \overline{B}}{\overline{C} - \overline{B}} \times 100$$

Where $\overline{X}$ refers to the absorbance mean of NCs-treated cells, $\overline{C}$ corresponds to the absorbance mean of untreated cells (control) and $\overline{B}$ to the absorbance mean of the blank (EMEM/MTS mixture).

2.12. Gene silencing study

HeLa cells were seeded in 24-well plates at a density of 80.000 cells/cm^2, 24 h prior treatment with 100 nM of siRNA per well. siRNA was complexed with DHF3 following the manufacture instructions. One hour after transfection with siRNA*-loaded protamine NCs or DHF3-siRNA* complexes, cells were washed with DPBS and left incubating at 37 °C in fresh medium for 24, 48 and 72 h. HeLA cells transfected with Block-it-Alexa fluor red fluorescent oligonucleotide also complexed with DHF3 (named as non-active siRNA-DHF3) for 1 h and left incubating for more 24 h post treatment were used as transfection efficiency control.

2.13. Quantitative real-time PCR assay

At different time points (24, 48 and 72 h) cells were lysed with RLT buffer/mercaptoethanol to determine gene expression levels. RNA was extracted using RNEasy RNA extraction kit (Qiagen) in the automatic extractor QIAcube (Qiagen), according to manufacturer instructions and after RNA was analyzed to ensure its integrity and quantified using Agilent 2100 Bioanalyzer. Reverse transcription of RNA into cDNA was performed using High-Capacity cDNA Archive Kit (Applied Biosystems), following the manufacture instructions. Real time PCR was performed using StepOnePlus detection systems (Applied Biosystems). Gene-specific primers and probes available as Taqman Gene Expression assays were used for the target gene and

internal control. Polr2a (Hs00172187_m1) was selected as internal control regarding previous expression analysis showing that it presented the most stable expression across samples for the target gene and for this cell line, and because the amplification had a comparable efficiency to that of the target gene. One hundred nanograms of cDNA were amplified using a Taqman Universal master mix at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All experiments were performed in triplicate and repeated in three independent experiments. Data was analyzed by Comparative threshold (Ct) method, also known as 2–ΔΔCt method [25]. Ct values of the samples (cells treated with protamine NCs) were compared to the control (untreated cells). Ct values of both samples and control were previously normalized to the endogenous housekeeping gene (Polr2a).

2.14. Biodistribution assay

A total of 6 New Zealand rabbits were used for this experiment divided in two separated groups. One group (n=3 rabbits) received one unique drop of 100 µL Prot-T NCs formulation in the conjunctival sac while the other group received the same volume of Prot-T free solution. Prior to administration a trehalose solution was added to the Prot-T NCs for a final concentration of 2.5 % (w/v), in order to improve NCs formulation isotonicity. Lids were maintained closed carefully for 30 seconds to avoid the elimination of the formulation by blinking. Only right eyes were instilled and left eyes were used as negative controls. Formulations were allowed to remain in the ocular surface for 6 hours before euthanasia with 120 mg/Kg i/v sodium pentobarbital (Dolethal, Vetoquinol, Lure, France) and eye globe evisceration. All procedures from this point were carried out protecting eye globes from light to avoid fluorescence signal fading.

Animals were handled and housed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the applicable guidelines of the EU (2010/63/EU) and the Spanish Government (RD 53/2013). The ethics committee of the University of Oviedo approved all procedures (PROAE 32/2018).

2.15. Histopathological analysis

Eyes were immediately fixed by immersion in 4 % buffered paraformaldehyde for 2 h at RT, washed thoroughly in PBS and cryoprotected overnight with 30 % buffered sucrose solution. Then, eyes were snap frozen in liquid nitrogen and embedded in OCT compound (Optimum Cutting Temperature; Tissue Teck, Sakura, Tokyo, Japan). Transversal sections (10 µm) were obtained with a Microm HM550 cryostat (Microm International GmbH, Walldorf, Germany) through the central region of the cornea. In order to locate TAMRA fluorescence emitting particles in the tissue, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) at 2 µg/mL (Molecular Probes, Eugene, OR, USA). TAMRA fluorescence was visualized without any additional processing under a Leica TCS-SP8X confocal scanning microscope (Leica Microsystems, Wetzlar, Germany) using a 542 nm laser wavelength. DAPI was visualized by applying a 359 nm laser wavelength. Stacks of confocal images spaced 1.5 µm were obtained and automatically superposed with LAS X software (Leica Microsystems). Colocalization analysis was performed with FIJI (ImageJ 1.51n, NIH, Bethesda, MD, USA).

To study the intracellular location of TAMRA fluorescent particles in the epithelium cells of the cornea, 3D surface plots were performed on stack image collections using the Interactive 3D Surface Plot v2.4.1 utility of FIJI.

To calculate the depth at which TAMRA fluorescent particles were observed at the epithelium, the Straight Line tool of FIJI was used on calibrated images, starting at the uppermost external surface of the epithelium and ending at the top of the clusters of fluorescent dots. Length was automatically calculated by the FIJI software.

2.16. Statistical analysis

GraphPad Prism (software version 7.0) was used for statistical analysis. The results are presented as a mean ± standard deviation (SD) of at least 3 different experiments. Data were compared using two-way ANOVA and p-values less than 0.05 were considered to be statistically significant.

3. Results and discussion

Our research group, amongst others, has pioneered the development of synthetic nanocarriers for the topical ocular delivery of drugs [17] and polynucleotides [26, 27]. In particular, our group was the first one reporting the potential of polymeric nanocapsules for the topical ocular administration in the early 90's [28-30]. Since then, nanocapsules have been seen as attractive vehicles allowing the encapsulation of both, hydrophilic [31-35] and lipophilic drugs [17, 28, 35-38] and the development of combination therapies within a single delivery vehicle [23, 34, 35]. Furthermore, nanocapsules were shown to interact with the corneal epithelium [17], thereby improving the transport of drugs across the ocular mucosa.

Recently we showed that RNA can be efficiently adsorbed on the surface of polyarginine nanocapsules and, then, protected by subsequent polymeric layers (polyarginine and hyaluronic acid) [34]. We have also shown that topically applied polyarginine and protamine NCs improve ocular retention and corneal wound healing [17]. Moreover, we reported that the coating of protamine-miRNA NCs with an additional protamine layer improved the transfection efficiency of NCs in colorectal cancer cells [23].

Considering this information, we selected protamine nanocapsules as an appropriate carrier for siRNA topical delivery to the eye. In order to produce and easily scalable formulation containing a high siRNA concentration (5 mg/mL), our approach was to develop a cationic nanoemulsion by the self-emulsifying technique and to assemble a protamine layer around the siRNA-loaded nanoemulsion. The resulting prototypes were fully characterized *in vitro* and one of them was selected for the *in vivo* evaluation of its biodistribution in the rabbit model.

3.1. Formation of the nanocapsules lipid core (Nanoemulsion, NE)

For industrial purposes, easy and scalable production methods are a must. Therefore, we chose a mild self-emulsifying technique for the production of the NE. This method leads to NE production by mixing the oil and aqueous phases without the need of organic solvents nor heat [37, 39, 40]. Medium-chain triglycerides (MCT) and non-ionic surfactants, such as labrafac™ lipophile WL 1349 and polysorbate 80, were selected for the formation of the NE as they are present in different ophthalmic formulations. In addition, polysorbate has shown the

capacity to stabilize DNA-loaded nanoparticles and improve their transfection capacity [41, 42]. On the other hand, we selected the cationic surfactants cetrimide and oleylamine in order to produce cationic oil cores able to associate siRNA. Fig. 1 shows the chemical structure of both surfactants. Cetrimide was chosen due to its higher avidity for siRNA when compared to benzethonium chloride and ethyl lauroyl arginate, as reported in the previous study (chapter 1). It presents a quaternary ammonium group and a long carbon chain (C14) while oleylamine is composed by a primary amine group and a longer carbon chain (C18). The later was selected due to its higher lipophilicity, which was expected to improve the surfactant entrapment in the oil core. In addition, the primary amine group of oleylamine has been reported to favor the association of polynucleotides to NEs [43-45], while the unsaturated carbon chain has been found to enhance the transfection efficiency.

Fig. 1 - Chemical structure of the cationic surfactants cetrimide and oleylamine.

Initially we studied the preparation of cationic NEs by mixing an oil phase, containing labrafac™, polysorbate 80 and oleylamine, with ultrapure water (approach i). Cetrimide could not be completely dissolved in the oil phase even at high temperature and, hence, was discarded in this approach. The resulting NE had a nanometric size of 148 ± 25 nm, low polydispersity (PDI 0.1) and a positive ζ-potential (+27 ± 2 mV) appropriate for siRNA association.

The development of NEs containing cetrimide was further explored by using approach ii) where cetrimide was dissolved in water and mixed it with the oil phase (labrafac™ and polysorbate 80) and approach iii) where cetrimide was dissolved in a small amount of ethanol (200 µL), then added to the oil phase and finally mixed with water. Aside from labrafac™, castor oil and vitamin E were also explored for the development of cetrimide NEs (Table S1 and S2). As expected, the different approaches to incorporate cetrimide into the NE led to different results. In particular, when using approach (ii) the decrease in the concentration of polysorbate 80 as well as the oils labrafac™ and vitamin E from 80 to 40 mg/mL (in the final formulation) led to the formation of NEs with a mean particle size of 127 and 193 nm, for labrafac™ and vitamin E respectively, and a high positive charge (Table S1). However, the use of castor oil at the same concentrations resulted in phase separation, indicating poor miscibility of this oil with the surfactants used. On the other hand, the use of EtOH in approach iii), improved cetrimide solubility and miscibility with the other oil phase components, leading to the formation of NEs of 150-110 nm, low PDI (≤0.2) and highly positive ζ-potential (~ +60 mV) at 10 mg/mL of labrafac or castor oil and 40 mg/mL of vitamin E. Higher concentrations of oils led to phase separation or the formation of a gel, respectively (Table S2).

In conclusion, the different chemical structures of the tested cationic surfactants (oleylamine and cetrimide) strongly influenced the formation of NEs. Moreover, in the case of cetrimide we observed that the solvent used (ethanol or water) as well as the amount and type of oil also had a great influence. Once determined the best conditions for cationic NE preparation, the next step was to assess their capacity to associate siRNA.

3.2. siRNA association to the oily cores of the NE

3.2.1. Oleylamine-containing NEs

First, for the preparation of the NE we adopted approach i), where siRNA was added to the aqueous phase and the oil phase was composed of labrafac™, polysorbate 80 and oleylamine. The increase of siRNA concentration (up to 6 mg/mL, in the final formulation) led to a gradual decrease of the ζ-potential from +27 to -19 mV (Fig. S3 A) and a reduction of NE size from 154 to 141 nm. The gel retardation assay (Fig. S3 B) shows that oleylamine NEs present a high

affinity towards siRNA molecules, leading to a full association and no displacement upon incubation with heparin. We hypothesized that siRNA interacted with the oleylamine molecules during NE preparation, causing a partial entrapment in the oily core.

siRNA was also added to a preformed oleylamine NE (Fig. 2A and C) in the adequate amount in order to reach a final concentration of siRNA of 6 mg/mL. This association resulted in an inversion of the ζ-potential (+27 to -18 mV) and NE shrinkage (148 to 134 nm). Using this approach, siRNA was efficiently associated to the NE and displaced, upon heparin incubation, as intended. Additionally, when incubated with SLF (Fig. 2B and C) the siRNA-loaded NEs were able to maintain their physicochemical properties as well as siRNA associated and again to release it upon incubation with heparin.

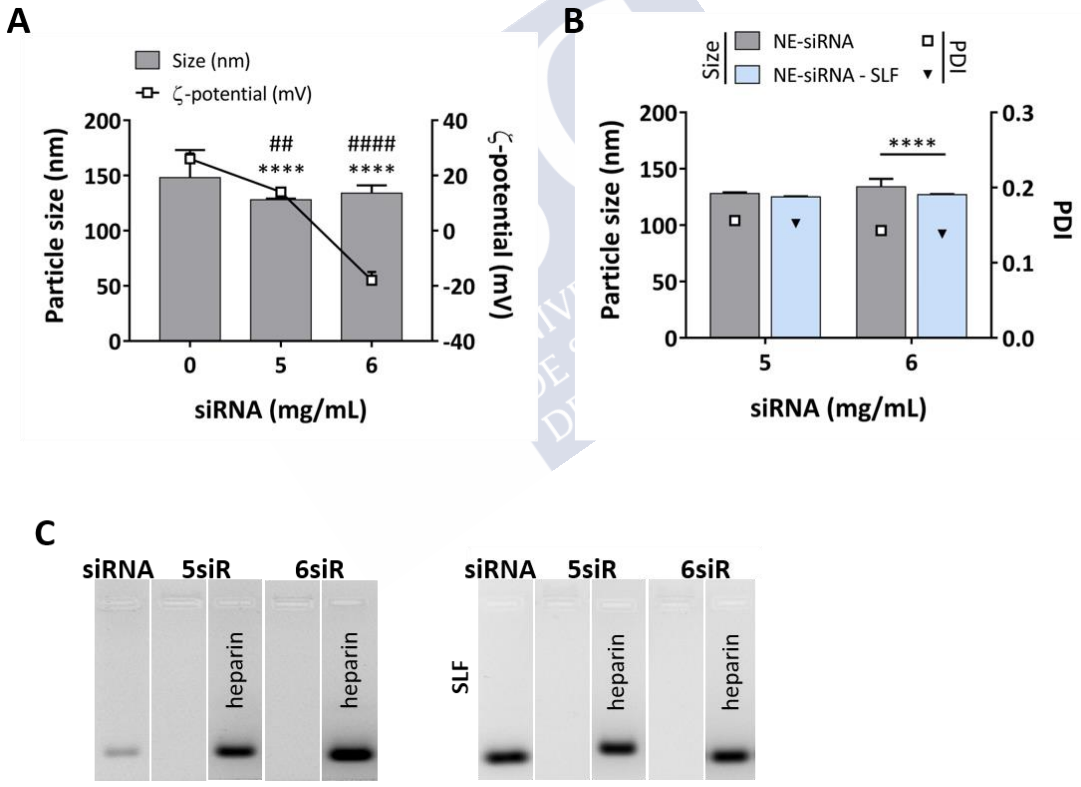

Fig. 2 - Physicochemical properties of oleylamine NEs associating 5 to 6 mg/mL of siRNA. Effect of siRNA association on NE mean particle size and ζ-potential (**A**). Colloidal stability of siRNA-loaded NE in SLF for 1 h at 37 °C. Grey and blue bars correspond to the siRNA-loaded NE before and after incubation with SLF (**B**). siRNA association assay by gel electrophoresis, of the siRNA-loaded NE (5 mg/mL and 6 mg/mL coded a 5siR and 6siR) before and after incubation with SLF or heparin (**C**). siRNA corresponds to the control of naked siRNA. Numerical values represent the mean ± SD (n ≥ 3). Data was analyzed using a two-way ANOVA (**** $p < 0.0001$; ## $p < 0.01$; #### $p < 0.0001$.* and # refer to particle size and to ζ-potential, respectively).

3.2.2. Cetrimide-containing NEs

We also studied the capacity for siRNA association of the cetrimide NEs produced by the two different approaches (ii and iii) and with a particle size of about 150 nm. The NEs prepared by including cetrimide in the aqueous phase (approach ii) led to the efficient association of 1 mg/mL of siRNA, that caused a great increase in particle size. However, the association of 2 mg/mL of siRNA led to particle aggregation (Table S3). In the case of the cetrimide-containing NEs, where cetrimide was dissolved in EtOH to its incorporation in the oil phase (approach iii), the addition of siRNA led to particle aggregation or a significant an increase of particle size when the oils used were castor oil or labrafac™, whereas in the case of vitamin E-containing NEs, siRNA could be efficiently associated reaching a final concentration of 1.6 mg/mL.

The high association efficiency of oleylamine (primary amine group) compared to cetrimide (quaternary ammonium group) NEs are in opposition to what Hagigit et al. and Martini et al., reported [43, 44]. According to these works, NEs containing N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium (DOTAP) that has a quaternary ammonium group presented a higher oligonucleotide association compared to the NEs containing oleylamine (single primary amine). The lowest association efficiency of cetrimide herein presented might be related to the lower lipophilic character of this surfactant in opposition to the high lipophilicity of oleylamine, which may favor its inclusion in the NE globules while exposing the cationic head group on NE surface, thus allowing an efficient nucleotide association.

Taking into account the high siRNA concentration achieved with the oleylamine NEs, this prototype was selected for further studies. As depicted in Fig. 3A, NE-siRNA presented only a small reduction of particle size after 15 days of storage at 4 °C. Moreover, a slight amount of siRNA appeared to be displaced in the gel (Fig. 3B). This NE formulation was subsequently coated with a protamine shell.

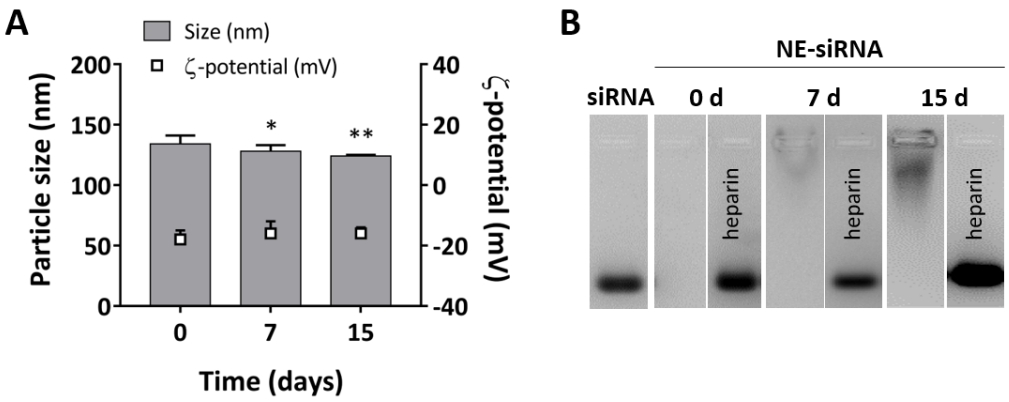

Fig. 3 - Colloidal stability of oleylamine siRNA-loaded NEs (6 mg/mL of siRNA) on storage conditions (4 °C). Particle size and ζ-potential evolution (**A**). siRNA association and heparin displacement assays by gel electrophoresis, during NE-siRNA storage (**B**). siRNA corresponds to the control of naked siRNA. Numerical values represent the mean ± SD (n ≥ 3). Data was analyzed using a two-way ANOVA (* < p 0.05; ** p<0.01.* refers to particle size).

3.3. Formation of the protamine shell

The next step was the shielding of siRNA-loaded NE with a protamine layer. The adequate conditions for the formation of this layer were empirically determined based on the changes produced in the ζ-potential of the NE [46, 47]. The formation of the protamine shell was achieved by mixing the siRNA-loaded NE with a solution of protamine (1.5, 3 and 5 mg/mL, initial concentrations of protamine solution) at a siRNA:protamine ratio of 1:0.2 (v/v). In this way we could obtain a NC formulation containing 5 mg/mL of siRNA (corresponding to a loading of about 3.9 % w/w). As can be noted in Fig 4A, the addition of protamine at the lowest concentration (1.5 mg/mL) led to a significant ($p < 0.0001$) reduction of the ζ-potential from about -18 to -11 mV, without causing changes in particle size. The increase of protamine concentration did not lead to a further reduction of the ζ-potential nor to an inversion to positive values, suggesting that the association of protamine to the surface of NE-siRNA may have reached the saturation [47]. According to the gel electrophoresis (Fig. 4C), the protamine coating did not either affect the siRNA association or hampered its displacement upon incubation with heparin. Moreover, the stability of the prepared NCs was assessed in SLF for 1 h at 37 °C and the results presented on Fig. 4B and C indicate that all prototypes remained stable without releasing the siRNA.

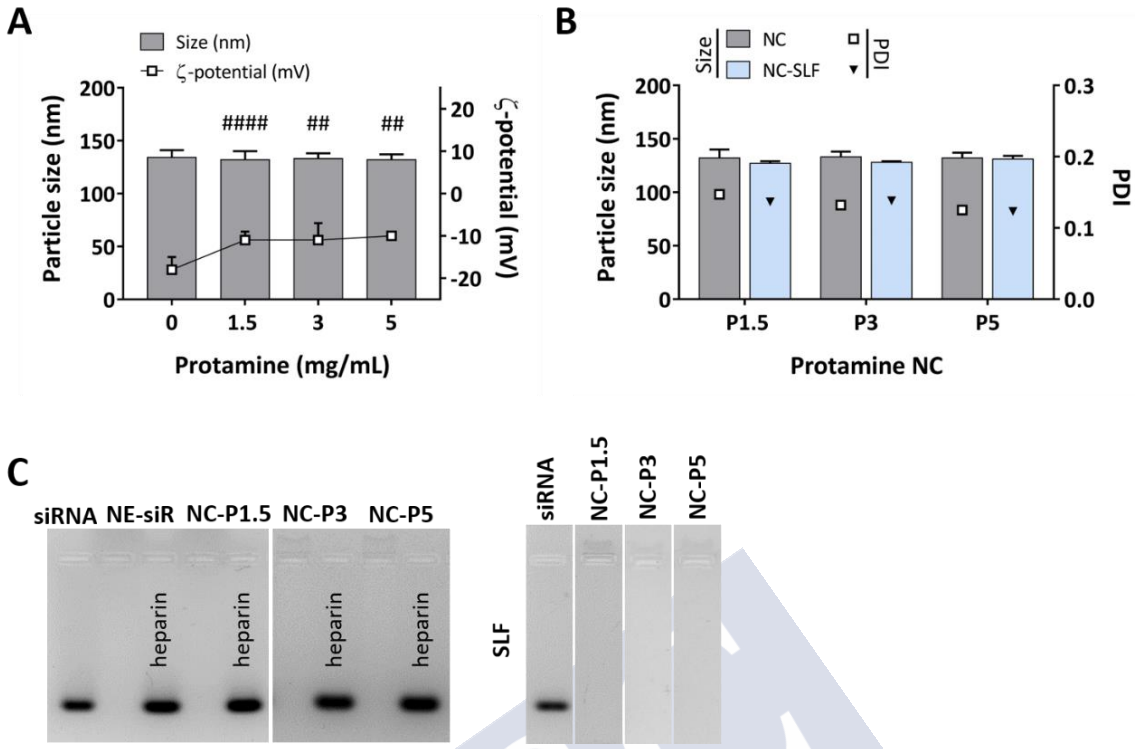

Fig. 4 - Characterization of protamine NCs (5 mg/mL siRNA), obtained by the addition of protamine to the siRNA-loaded NE at 1:0.2 volume ratio (siRNA-loaded NE:protamine). Effect of the protamine concentration on the particle size and ζ-potential (**A**). Colloidal stability of the protamine NCs (NC's coating performed with a protamine solution at an initial concentration of 1.5, 3 and 5 mg/mL, coded as NC P1.5, P3 and P5) in SLF for 1 h at 37 °C. Grey and blue bars correspond to the NCs before and after incubation with SLF (**B**). siRNA association assay by gel electrophoresis, of the NE-siRNA before (NE-siR) and after protamine coating (NC-P1.5, NC-P3 and NC-P5) and their capacity to release siRNA by incubation with heparin (**C**, left hand). Capacity to maintain siRNA associated after NC's incubation with SLF for 1 h (**C**, right hand). siRNA corresponds to the control of naked siRNA. Numerical values represent the mean ± SD (n ≥ 3). Data was analyzed using a two-way ANOVA (## $p < 0.01$; #### $p < 0.0001$. # refers to ζ-potential).

The colloidal stability of the protamine NCs loading 5 mg/mL of siRNA (NC's coating performed with a protamine solution at an initial concentration of 1.5 and 5 mg/mL, coded as NC P1.5 and NC P5) over the time was studied by storing them at 4 °C. As presented in Fig. 5A, after 15 days of storage the NC P1.5 remained stable. Similarly, the NCs with the highest protamine concentration (NC P5) maintained their size and the amount of siRNA associated remained constant (Fig.5B). Unfortunately, after 30 days of storage at 4 °C, the NCs aggregated independently of the amount of protamine.

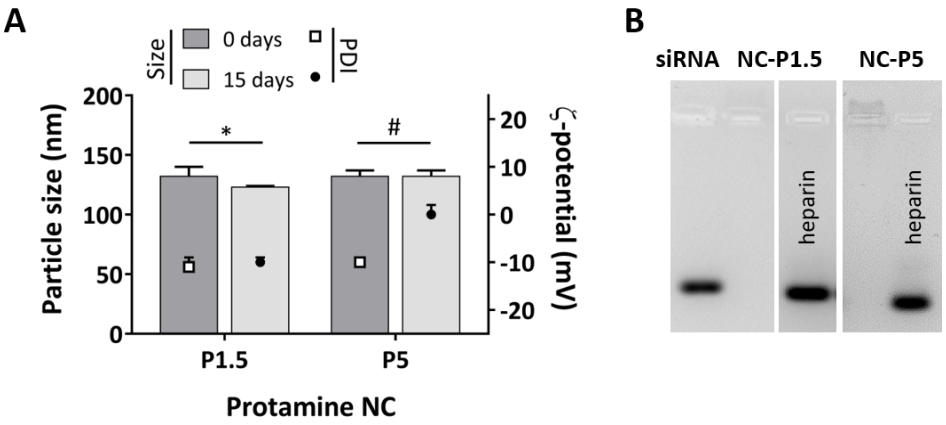

Fig. 5 - Storage stability of protamine NCs loading 5 mg/mL siRNA (NC's coating performed with a protamine solution at an initial concentration of 1.5 and 5 mg/mL, coded as NC P1.5 and NC P5) after 15 days at 4 °C. Physicochemical properties (particle mean size and ζ-potential) of protamine NCs (**A**). Protamine NCs' siRNA association by gel electrophoresis, before and after heparin incubation (**B**). siRNA corresponds to the control of naked siRNA. Numerical values represent the mean ± SD (n ≥ 3). Data was analyzed using a two-way ANOVA (* $p < 0.05$; # $p < 0.05$. * and # refer to particle size and to ζ-potential, respectively).

The morphology of the carriers before and after the addition of siRNA and protamine layers was analyzed by TEM (Fig. 6). Moreover, the effect of different protamine layer concentrations, 1.5 and 5 mg/mL (P1.5 and P5) on NCs' morphology was also analyzed. As typically observed for these types of carriers, they present a round shape structure [34, 37, 40]. Protamine NCs prepared with the highest protamine concentration (5 mg/mL, NC P5) seem to present a more compact structure.

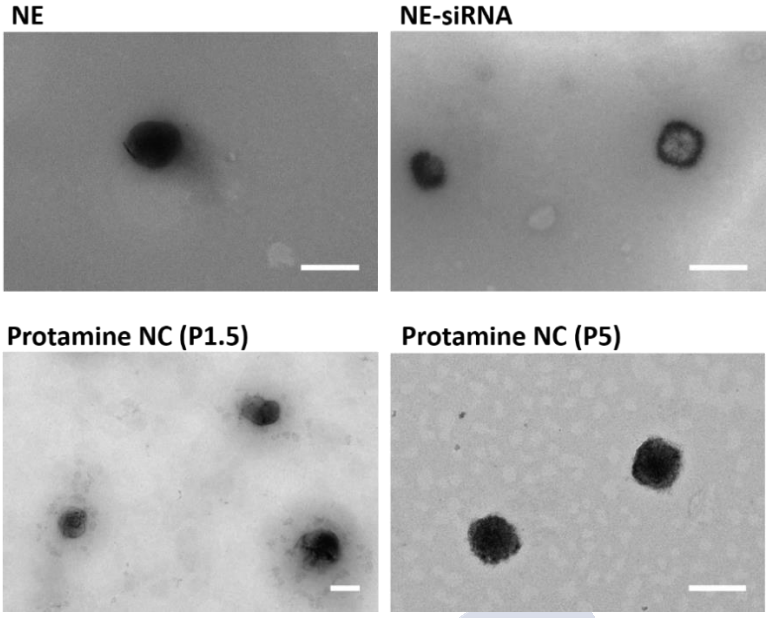

Fig. 6 - Transmission electron micrograph of oleylamine NEs, siRNA-loaded NEs (6 mg/mL siRNA) and protamine NCs (NC P1.5 and NC P5 loading 5 mg/mL siRNA). All scale bars correspond to 200 nm.

3.4. Protamine NCs loading therapeutic siRNA (siRNA*)

In order to study the biological activity of the developed protamine NCs, the model siRNA was replaced by a different one with a specific therapeutic activity (siRNA*). The oleylamine NEs and protamine NCs were prepared using the previously optimized parameters. Therefore, siRNA* was associated to formerly prepared oleylamine NEs (two-step siRNA loading) reaching a final concentration of 6 mg/mL. As previously observed, the association of this high siRNA concentration led to a decrease in particle mean size from 153 to 138 nm (Fig. 7A), indicating a compaction of the NE. This association also caused a sharp decrease in ζ-potential from +26 to -13 mV. The gel electrophoresis results (Fig. 7B) corroborate the efficient association of siRNA and its displacement upon incubation with heparin. For the preparation of protamine NCs, the siRNA-loaded NEs were incubated with a protamine solution of 1.5 mg/mL at a volume ratio of 1:0.2 (NE-siRNA:protamine), leading to a final siRNA concentration of 5 mg/mL. The addition of the protamine layer to the siRNA-loaded NE resulted in a reduction of the negative ζ-potential from -13 to -6 mV, indicating that protamine was associated onto the surface of siRNA-loaded NE. Protamine NCs preserved the capacity to retain the full amount of siRNA associated to the system and to allow the siRNA displacement

upon incubation with heparin. This capacity is considered to be an indicator of the possible release of RNA under biological conditions.

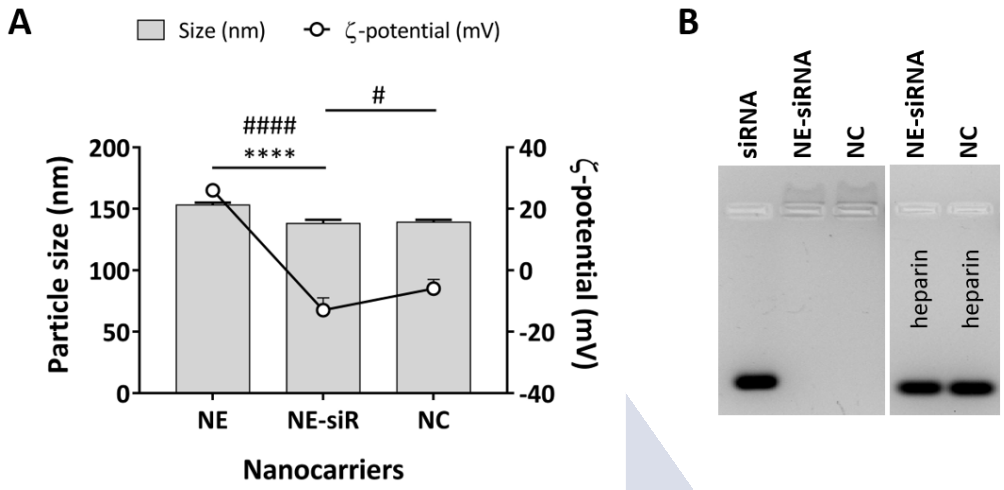

Fig. 7 - Characterization of the nanocarriers loading therapeutic siRNA*. Physicochemical properties (particle mean size and ζ-potential) of blank NEs, siRNA*-loaded NEs and protamine NCs (NC) (**A**). Gel electrophoresis of siRNA*-loaded NEs and protamine NCs (NC) before and after incubation with heparin (**B**). Numerical values represent the mean ± SD (n ≥ 3). Data was analyzed using a two-way ANOVA (**** $p < 0.0001$; # $p < 0.05$; #### $p < 0.0001$. * and # refer to particle size and ζ-potential, respectively).

As expected, siRNA*-loaded protamine NCs were stable when incubated with SLF for 30 min, maintaining their size (Fig. 8A) and avoiding premature siRNA release. In addition, the NCs were stable under storage at 4 °C up to 15 days (Fig. 8B and C).

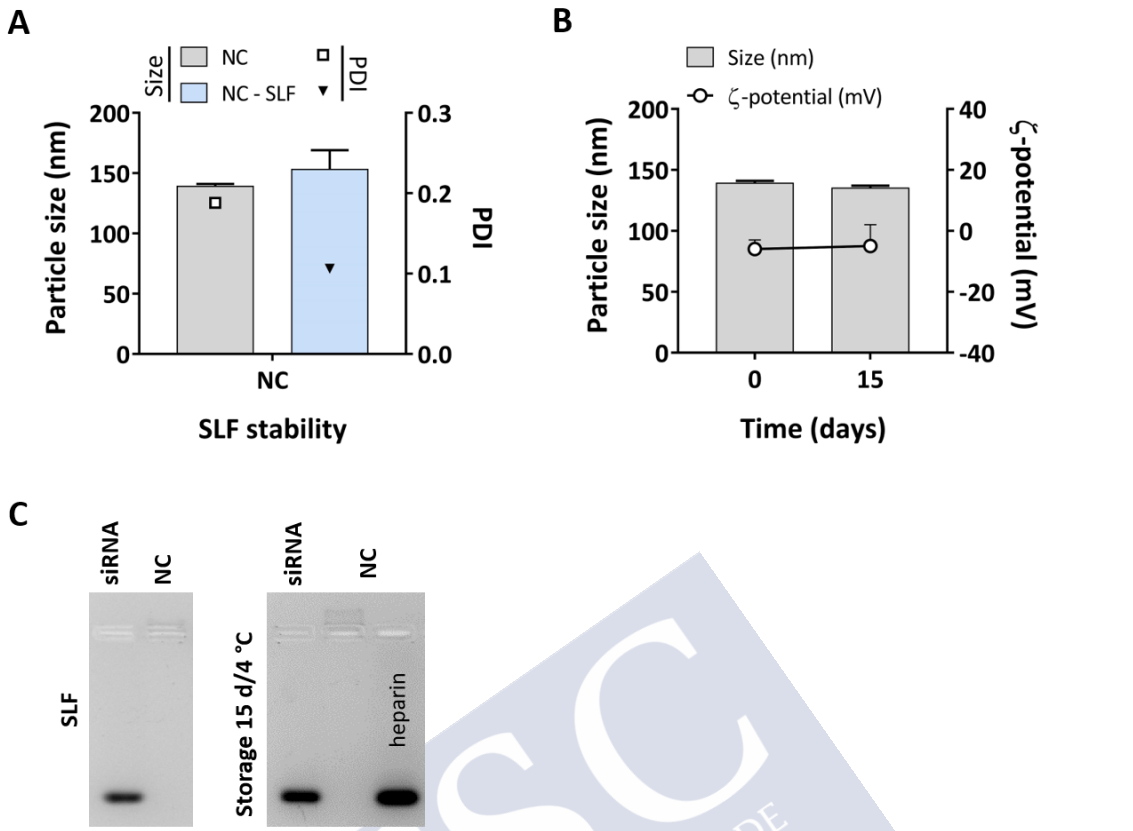

Fig. 8 - Colloidal stability of siRNA*-loaded protamine NCs (NC) containing a 5 mg/mL concentration of siRNA* upon incubation in SLF for 30 min at 37 °C. Grey and blue bars correspond to the NCs before and after incubation with SLF (**A**). Colloidal stability of siRNA*-loaded protamine NCs during storage at 4 °C for up to 15 days **(B**). Gel electrophoresis of protamine NCs after incubation with SLF for 30 min at 37 °C and after storage at 4 °C for 15 days (**C**). siRNA displacement was performed by incubation with heparin. siRNA corresponds to the control of naked siRNA*. Numerical values represent the mean ± SD (n = 3).

3.5. Freeze drying of nanosystems

In order to improve the long-term stability of the nanocarriers, they are commonly freeze-dried. This process generates stresses that can affect particle integrity and to avoid so, different excipients can be added to the formulation before freeze-drying. The most common are sugars such as glucose, sucrose and trehalose, which protect the formulation from damage related to freezing (cryoprotectant) and drying (lyoprotectant) stresses. Trehalose, among other excipients, has bulking and tonicity adjusting properties, conferring bulk to the formulation during the process and isotonicity upon resuspension. In addition, trehalose is usually preferred due to its low chemical interaction, aside from forming flexible hydrogen

bonds with the nanocarriers, low hygroscopicity and high glass transition temperature [16, 48, 49].

Previous results from a freeze-drying study performed with a similar prototype of a blank NE, where glucose, sucrose and trehalose were tested at different concentrations (2.5 up to 10%, w/v, in the final formulation) led us to select trehalose at 10 % for further studies (data not shown). siRNA-loaded NEs and protamine NCs were lyophilized at a concentration of 20 mg/mL and using trehalose for a final concentration of 10 % (w/v). In continuation, the storage stability of the freeze-dried formulations (powder) before and after restoring the powder was also studied.

3.5.1. Freeze drying of siRNA-loaded NE

The freeze-dried siRNA-loaded NE (powder form) was resuspended in the adequate volume of water in order to reach the original particle concentration. Fig. 9A shows the visual aspect of the formulation in the powder form and after resuspension. While maintaining a low polydispersity (PDI of 0.2), the resuspended NE-siRNA formulation presented a size reduction of 20 nm (Fig 8B), and a change in the ζ-potential from -9 to +2 mV, a result that may suggest a certain reorganization of the NEs due to the water replacement by trehalose during the freeze-drying process [16]. Similar changes during the freeze-drying process have been reported by other authors [50]. Despite these small changes, the siRNA molecules remained associated to the nanocarrier as observed by gel electrophoresis (Fig. 9D). In addition, the siRNA displacement by incubation with heparin shows that the siRNA integrity was not affected by the freeze-drying process.

The stability of the freeze-dried siRNA-loaded NEs in SLF was also studied. As shown in Fig 9E, the formulation remained stable upon incubation with SLF for 1 h, maintainin both size and PDI and without releasing the cargo. In addition, it was found that the NE maintainied its capacity to release siRNA upon incubation with heparin.

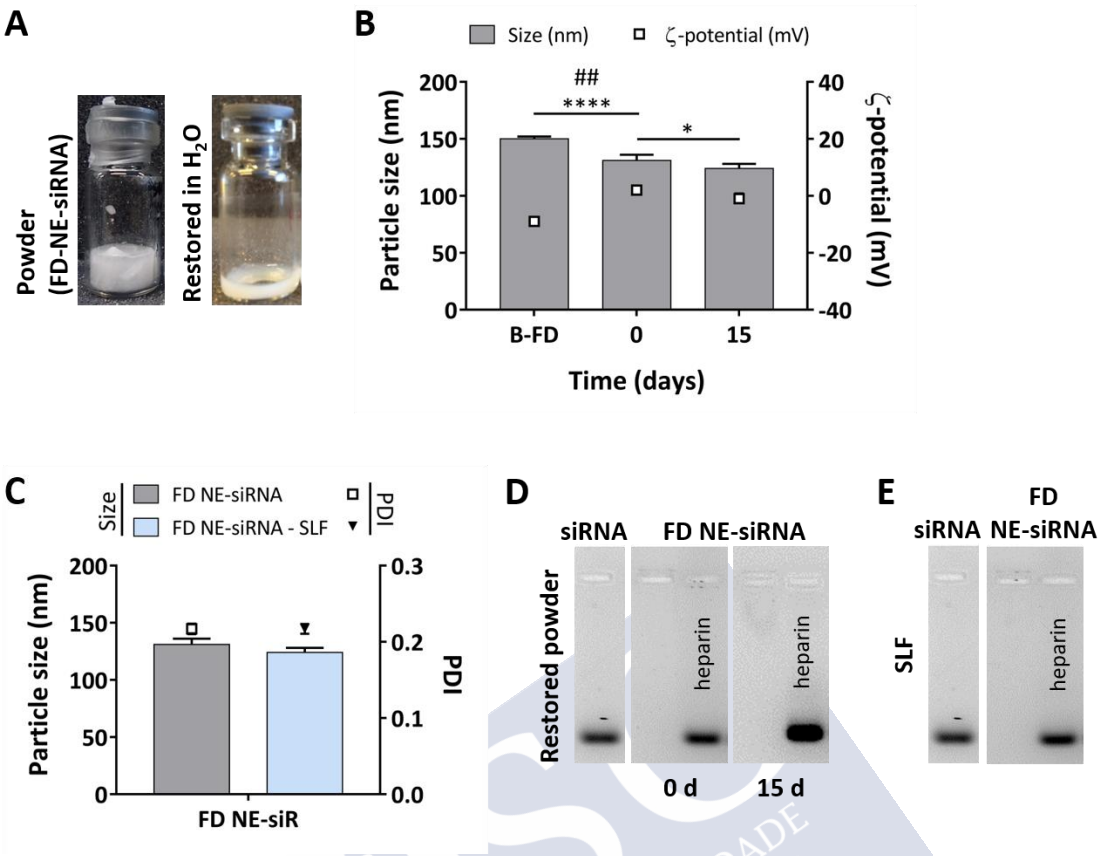

Fig. 9 - Characterization of freeze-dried siRNA-loaded NEs (FD NE-siRNA) upon resuspension in water. Visual aspect of FD NE-siRNA before and after being reconstituted in water (**A**). Particle size and zeta potential of the siRNA-loaded NEs before FD (B-FD) vs after resuspension (day 0) as well as after storage of the resuspended product for 15 days at 4 °C (**B**). Resuspended FD NE-siRNA (at day 0) stability in SLF for 1 h at 37 °C. Grey and blue bars correspond to the FD NE-siRNA before and after incubation with SLF, respectively (**C**). Gel electrophoresis of the reconstituted powder (restored FD NE-siRNA) at day 0 and after storage at 15 days at 4 °C (**D**) and of the freeze-dried siRNA-loaded NE after 1 h in SLF at 37 °C (**E**). siRNA displacement was performed by incubation with heparin. siRNA corresponds to the control of naked siRNA. Numerical values represent the mean ± SD (n = 3). Data was analyzed using a two-way ANOVA (* $p < 0.05$; **** $p < 0.0001$; ## $p < 0.01$. * and # refer to particle size and to ζ-potential, respectively).

We also studied the influence of temperature (4 °C and RT) on the storage stability of the freeze-dried siRNA-loaded NEs. As disclosed in Fig.10A, after storage for 15 days at both 4 °C and RT, the particle size remained similar. In addition, the particle surface charge decreased from +2 to -8 and -20 mV, respectively. This increase in the negative surface charge of the NE, especially when stored at RT, could be due to the disruption of the hydrogen bonds between trehalose and the surface components of the NE. The gel electrophoresis results, presented in Fig. 10B, corroborated this hypothesis. The NE stored at RT lost part of the associated siRNA

molecules, whereas the one stored at 4 °C was preserved of this premature release. After 30 days of storage at 4 °C, the particle size of the NE increased from 131 ± 5 to 183 ± 45 nm and PDI from 0.2 to 0.3, indicating some aggregation, however this process was not accompanied of siRNA release. On the contrary, when stored at RT, the particles became smaller and a significant amount of siRNA was released from the NE. In conclusion, the freeze-dried siRNA-loaded NE were stable upon storage at 4 °C for about 15 days, however, their stability was compromised at RT.

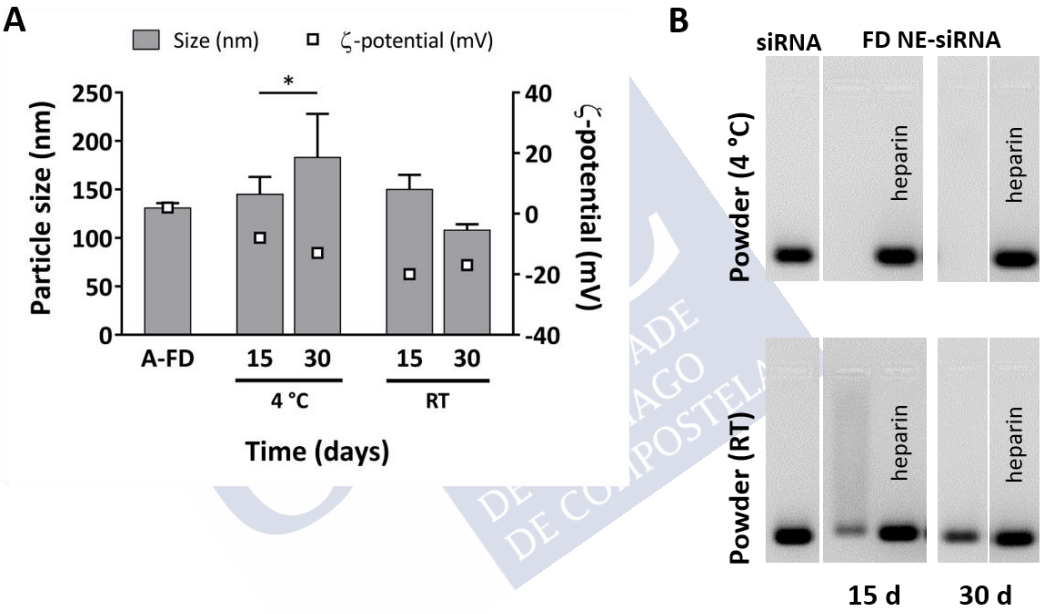

Fig. 10 - Particle size (nm) and ζ-potential (mV) of FD siRNA-loaded NE resuspended after FD (A-FD) and upon storage of the FD product at 4 °C and RT up to 30 days (**A**). siRNA release during FD NE-siRNA storage and displacement by incubation with an excess of heparin (**B**). siRNA corresponds to the control of naked siRNA. Numerical values represent the mean ± SD (n = 3). Data was analyzed using a two-way ANOVA (* $p < 0.05$. * refers to particle size).

3.5.2. Freeze drying of protamine NCs

Protamine NCs prepared with different concentrations of protamine (1.5, 3 and 5 mg/mL, initial concentration of protamine, see section 3.3) and a final concentration of 5 mg/mL of siRNA, were freeze-dried upon addition of trehalose (10 %, w/v, final concentration). The resuspension of the freeze-dried NCs, containing high amounts of protamine (3 and 5 mg/mL),

in water resulted in the aggregation of the formulation (Fig. 10A) whereas the ones prepared with 1.5 mg/mL of protamine could be efficiently rehydrated. As previously hypothesized, this could be due to the presence of an excess of protamine in solution that destabilized the system upon rehydration. The stability of the protamine NCs (1.5 mg/mL protamine) as a freeze-dried powder and also upon resuspension was monitored during storage at 4 °C (Fig. 11).

As shown in Fig. 11B (middle), after resuspension, the freeze dried NCs (FD NCs) maintained their size, however, their ζ-potential decreased from -11 to -2 mV. As hypothesized for siRNA-loaded NE, the trehalose molecules might interact with the surface of the NCs and reduce their negative surface charge. Importantly, as shown in the electrophoresis gel (Fig. 11B, left hand), the rehydration of freeze-dried NCs did not lead to a premature siRNA release and its displacement by heparin confirmed the maintenance of siRNA integrity. Similar properties were observed upon storage at 4 °C for 15 days (Fig. 11C, middle). However, after storage for 30 days the resuspended freeze-dried NCs presented some aggregates.

On the other hand, when stored at 4 °C in a freeze-dried form, the NCs were stable for up to 15 days, without releasing siRNA and maintaining its integrity (Fig. 11D, left hand and middle, respectively). However, as can be observed in Fig. 11E, the resuspension of the powder stored at RT, led to the formation of big aggregates.

It is important to note that, after freeze drying, the NCs maintain not only their physicochemical properties but also their colloidal stability in a biorelevant media (Fig. 11B, right hand). We also studied the stability of the resuspended FD NCs that were stored for 15 days at 4 °C, followed by their incubation in SLF for 1 h. The results presented in Fig. 11C (right hand) indicate that the NCs maintained their stability in terms of particle size and PDI. On the other hand, when the powder, stored for 15 days at 4 °C, was resuspended and incubated with SLF, the NCs suffered a small increase in their size with no apparent siRNA release (Fig. 11D, right hand).

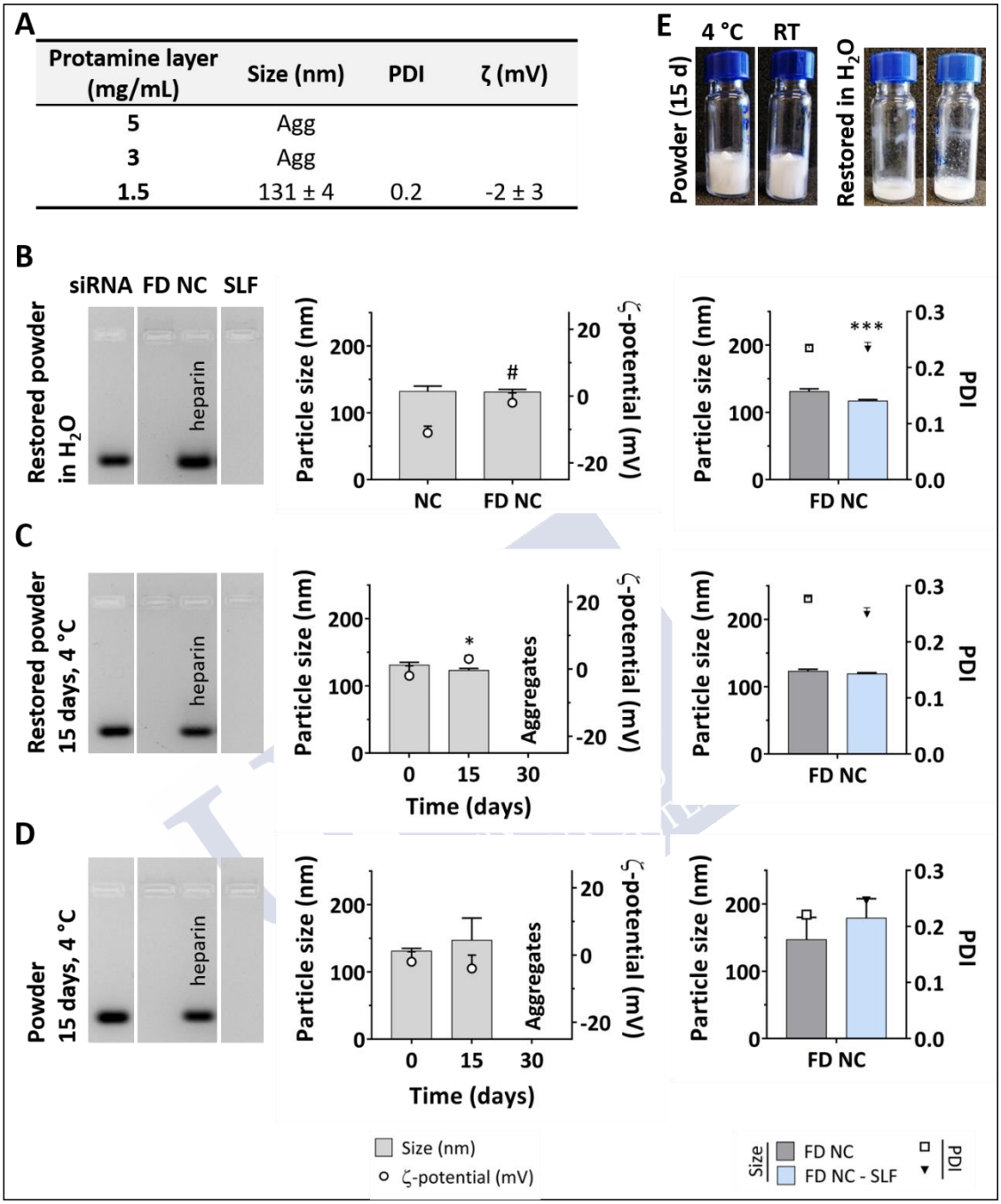

Fig. 11- Characterization of FD protamine NCs. Particle size and ζ-potential, of resuspended of FD protamine NCs prepared with different protamine concentrations (1.5, 3 and 5 mg/mL) (**A**). Gel electrophoresis before and after incubation with heparin or SLF (A, left hand), particle size/ζ-potential (A, middle) and colloidal stability upon incubation with SLF for 1 h at 37 °C (A, right hand) of FD protamine NCs (P1.5) (**B**). Storage stability at 4 °C of the resuspended FD NCs (**C**) and the FD NCs powder (**D**). Visual aspect of FD NCs (powder), after storage for 15 days at 4 °C and RT and upon resuspension (**E**, left and right hand, respectively). Numerical values represent the mean ± SD (n ≥ 3). Data was analyzed using a two-way ANOVA (* $p < 0.05$; *** $p < 0.001$; # $p < 0.05$. * and # refer to size and ζ-potential, respectively).

3.6. Cell viability

As described in section 3.1 protamine NCs (P1.5) containing a concentration of 5 mg/mL of siRNA were able to maintain their size and a low polydispersity (PDI 0.1-0.2) as well as their siRNA loading after incubation with SLF for 30 to 60 min. Similarly, prior to the *in vitro* evaluation of the cytotoxic profile of protamine NCs, their colloidal stability in cell culture media was determined. The results showed that protamine NCs remained stable upon incubation in non-supplemented CCM for 1 h (Fig. S4). Considering that the developed nanocarriers are envisaged for topical ocular administration and that the scarce drug contact time with the ocular surface is only of a few minutes [51], the incubation period of 1 h for *in vitro* assays was considered appropriate.

HeLa cells were treated for 1 h with protamine NCs at increasing concentrations (from 7.5 to 60 µg/mL) and 24 h post-treatment cell viability was determined by MTS assays. The results in Fig. 12 indicate that cell viability was not compromised for protamine NCs concentrations up to 30 µg/mL that correspond to about 100 nM siRNA*.

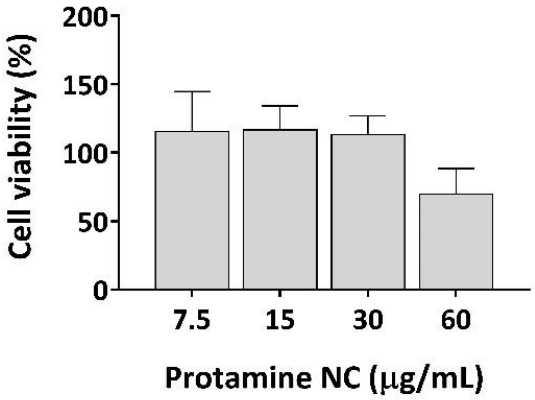

Fig. 12 - HeLa cells viability after 1 h treatment with increasing concentrations of siRNA*-loaded protamine NCs (7.5 to 60 µg/mL). MTS assay was performed 24 h post-treatment. Values represent the mean ± SD (n=3).

3.7. *In vitro* silencing effect

The silencing efficiency of siRNA*-loaded NCs (30 μg/mL) was studied on HeLa cells after incubation for 1 h. Gene expression was determined 24, 48 and 72 h post-treatment, as presented in Fig. 13. The cells that did not receive any treatment (untreated) were used as control. Naked siRNA is known to present poor cell transfection capacity, so both therapeutic siRNA (siRNA*) and non-active siRNA were combined with a transfection reagent (DHF3). The results indicate that siRNA*-DHF3 was able to significantly ($p < 0.0001$) reduce gene expression by 59 % after 24 h and this effect progressively decreased, leading to a 61 % level of gene expression at 72 h post-treatment. As expected, non-active siRNA had no effect on gene expression. On the contrary, in the case of protamine NCs, the opposite pattern was observed: while no silencing effect was observed at 48 h, a modest but significant ($p < 0.05$) reduction (24 %) of gene expression was observed at 72 h. At this time point there was no significant difference between the cells treated with siRNA* (siRNA*-DHF3) or protamine NCs (Prot NC). This might be indicative of a slow release of siRNA* from protamine NCs. According to the displacement tests described in section 3.3 the incubation of the protamine NCs with an excess of heparin led to the siRNA displacement. Nonetheless, once inside the cell, the siRNA release from the NCs might take longer, possibly due to endosomal entrapment, and therefore in this case start to cause gene silencing only after 72 h post-treatment. In order to confirm these hypothesis, further gene expression analysis should be performed for a longer period of time and also confocal analysis in order to understand to which extent the developed protamine NCs are able to enter cells and their co-localization at the tested time points and if the addition of a fusogenic lipid could avoid NCs endosomal degradation.

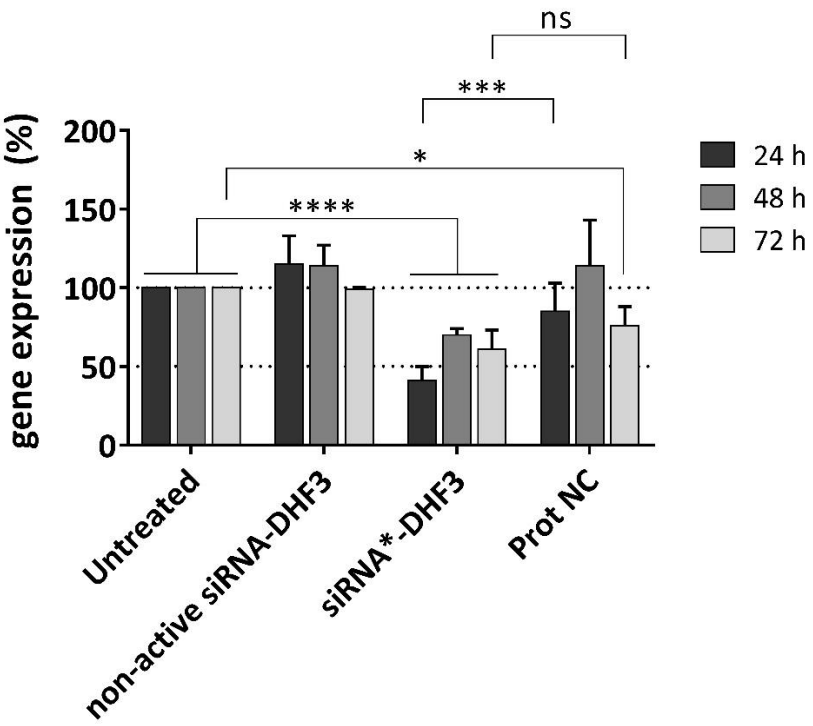

Fig. 13 - Expression of the gene of interest 24, 48 and 72 h post-treatment. HeLa cells were treated for 1 h with 100 nM siRNA, delivered by transfection lipid DHF3 (non-active siRNA-DHF3 and siRNA*-DHF3) and protamine NCs. Gene expression levels were quantified by real time-PCR and normalized with respect to an endogenous control (Polr2a). Data is expressed as % of gene expression, normalized to non-transfected (untreated) cells. Values represent the mean ± SD (n=3). Statistical significance was calculated by two-way ANOVA (ns, not-significant; * $p < 0.05$; *** $p < 0.001$, **** $p < 0.0001$).

3.8. Biodistribution

In order to determine if protamine NCs are able to interact with the corneal epithelium, siRNA-loaded NCs prepared using a TAMRA-labeled protamine were topically administered to healthy rabbit's eyes. These fluorescent NCs presented similar physicochemical properties (127 ± 2 nm, PDI 0.1 and -11 ± 4 mV) to the non-fluorescent protamine NCs and were also stable when incubated with lacrimal fluid. As shown in Fig. 14 and 15, at 6 h post-instillation, protamine NCs were able to enter the corneal epithelium superficial cell layer. The cells exhibit a homogeneous fluorescence distribution in cell cytoplasm. Protamine-TAMRA in solution was also able to enter the cells at the epithelium surface but only a low number of red stained cells were observed.

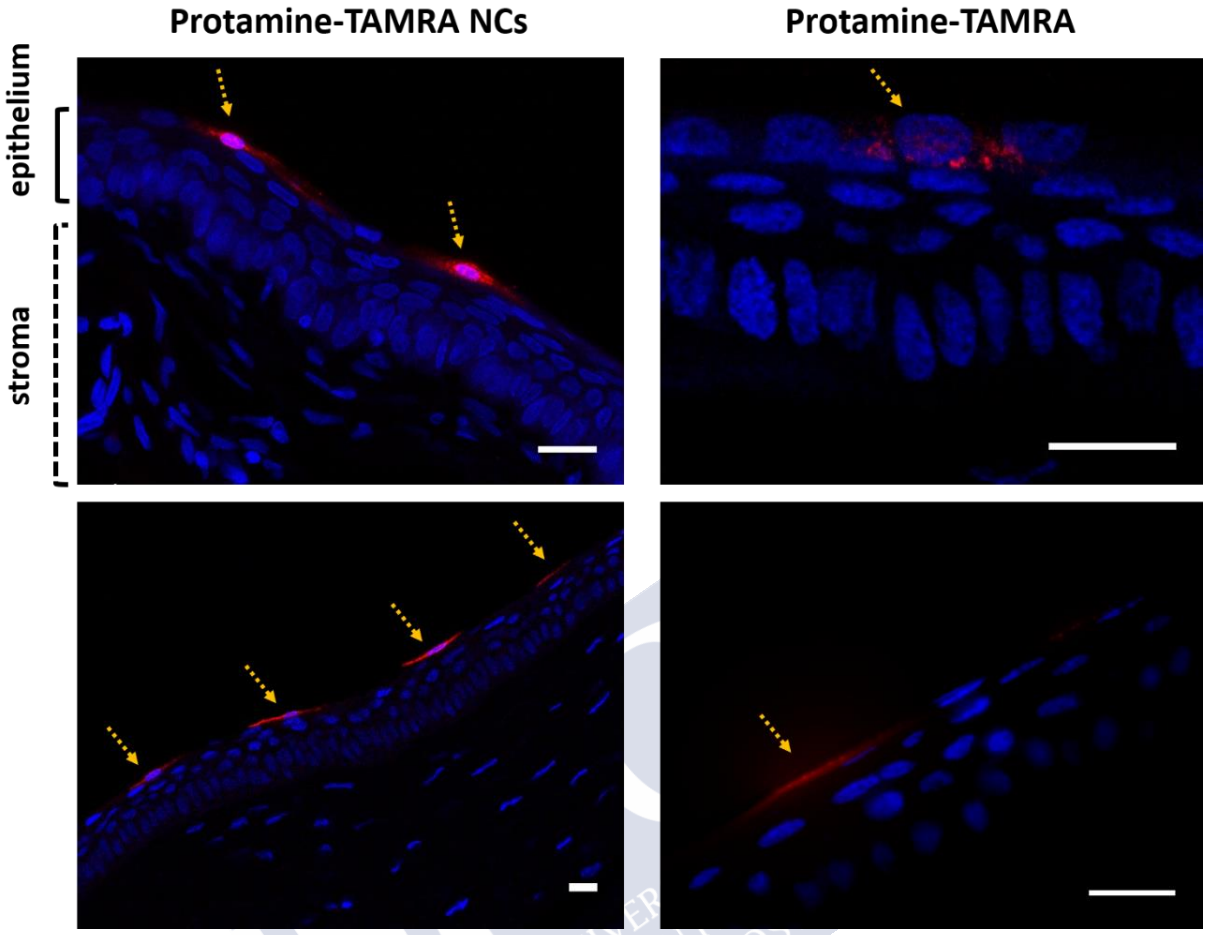

Fig. 14 - Confocal fluorescence images of excised rabbit corneal epithelium, 6 h after the topical administration of a solution of TAMRA-labeled protamine (protamine-TAMRA) and protamine-TAMRA nanocapsules (protamine-TAMRA NCs) loading siRNA. Blue channel corresponds to cell nuclei stained with DAPI while the red channel corresponds to TAMRA-labeled protamine in solution or coating NCs. Dot arrows refer to protamine-TAMRA NCs or protamine-TAMRA present in the superficial cell layer of the epithelium. Scale bars correspond to 20 µm.

Moreover, the NCs were also able to penetrate into the epithelium (Fig. 15, yellow arrows) being possible to observed their presence, though in lower amount, at the wing cell layer of the epithelium (11.72 ± 0.17 µm depth from the surface) and also at the apical side of columnar cells at the basal cell layer (27.59 ± 2.17 µm depth from the surface of a total of 41.44 ± 1.49 µm total thickness).

The internalization of protamine NCs in the first layer of the corneal epithelia is in agreement with previous results from our group showing the ability of protamine NCs to interact with the corneal epithelium [17]. As aforementioned, protamine is an arginine rich peptide that

enhances cell penetration [52], a characteristic that is attributed to the formation of hydrogen bonds between guanidine groups of arginines on protamine structure and cell membranes' sulphate/phosphate groups [53].

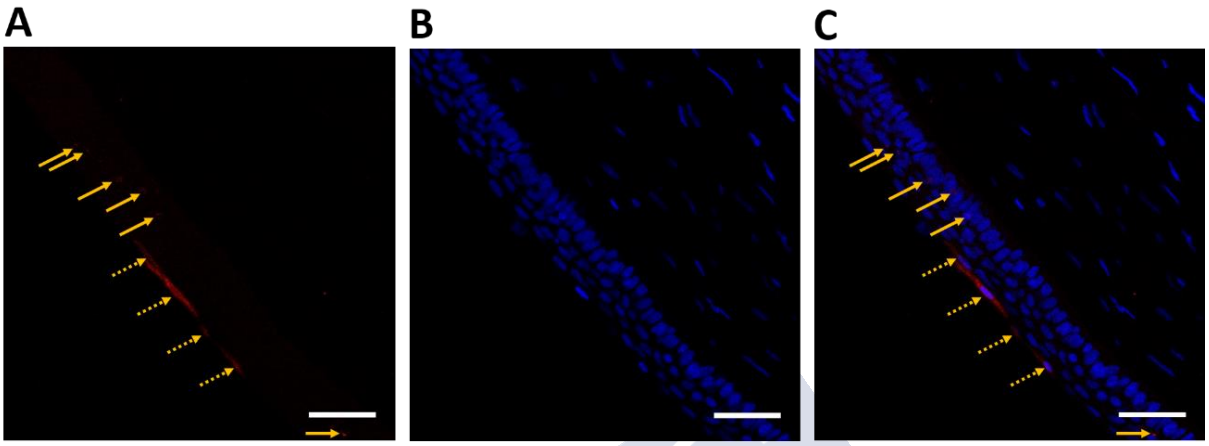

Fig. 15 - Confocal images of excised rabbit corneal epithelium representing the biodistribution of protamine-TAMRA NCs loading siRNA, 6h post topical administration. Blue channel corresponds to cell nuclei stained with DAPI while the red channel corresponds to protamine-TAMRA NCs. Dot arrows refer to NCs present in the external layer of the corneal epithelium and straight arrows refer to NCs present in the interior of the epithelium. Scale bars correspond to 50 µm.

4. Conclusions

Herein we report the development of protamine nanocapsules by the self-emulsifying technique for topical ocular administration. These nanocapsules entrapped the siRNA in sandwich-like manner. The composition of these NCs was adapted in order for them to fulfill a number of key properties, such as (i) a reproducible size (130-160 nm) and a very high siRNA concentration (5 mg/mL), (iii) an adequate stability upon dispersion in lacrimal fluid and (iv) the capacity to be freeze-dried. In addition, *in vitro* cell culture studies showed the capacity of these NCs to silence RNA expression *and in vivo* biodistribution studies showed their ability to enter the corneal epithelial cells. Overall, the developed protamine NCs exhibit a promising profile for their exploitation as ocular delivery carriers. Further *in vivo* proof-of-concept studies would be necessary to assess the value of these formulations.

Acknowledgments

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Supporting information

Supporting figures

Table S1 - Physicochemical characterization of cetrimide NEs containing different oils (Labrafac™, Castor oil or Vitamin E) at two different concentrations (80 and 40 mg/mL, meaning the final concentration). The aqueous phase containing cetrimide was added to the oil phase containing the oil and polysorbate 80 (40 mg/mL). Table indicates particle size, PDI and zeta potential. Values represent the mean ± SD (n=3).

Type of oil	Oil concentration (mg/mL)	
	80	40
Labrafac	na[1]	127 ± 2 nm; 0.2; +46 ± 1 mV
Castor Oil	na[1]	na[1]
Vitamin E	na[2]	193 ± 10 nm; 0.3 +56 ± 1 mV

na[1] – formulations presented phase separation; na[2] – formulation looked like a gel.

Table S2 - Physicochemical properties of cetrimide nanoemulsions containing different oils (Labrafac™, Castor oil or Vitamin E) at different concentrations (80, 40 or 10 mg/mL, meaning the final concentration). Cetrimide was dissolved in 200 uL of EtOH, mixed with the oil phase (oil and polysorbate 80 (40 mg/mL)) and subsequently with the aqueous phase. EtOH was removed by vacuum centrifugation Table indicates particle size, PDI and zeta potential. Values represent the mean ± SD (n=3).

Type of oil	Oil concentration (mg/mL)		
	80	40	10
Labrafac	na[1]	na[1]	153 ± 2 nm; 0.1; +56 ± 2 mV
Castor Oil	na[1]	na[1]	138 ± 3 nm; 0.2; +57 ± 1 mV
Vitamin E	na[2]	112 ± 2 nm; 0.2; +60 ± 5 mV	104 ± 43 nm; 0.6

na[1] – formulations presented phase separation; na[2] – formulation looked like a gel.

Composition (mg/mL)		Properties		
Oil	siRNA	Size (nm)	PDI	ζ (mV)
Labrafac [40]	0	127 ± 2	0.2	+46 ± 1
	1	201 ± 10	0.1	+35 ± 1
	2	Agg		

Fig. S3 - Characterization of siRNA-loaded cetrimide NEs containing Labrafac as oil (NE was formerly prepared with cetrimide dissolved in the aqueous phase): size, PDI and ζ-potential (left side). Gel electrophoresis before and after siRNA-loaded NEs incubation with heparin (right side). siRNA corresponds to the control of naked siRNA. Values represent the mean ± SD (n=3).

Composition (mg/mL)		Properties		
Oil	siRNA	Size (nm)	PDI	ζ (mV)
Labrafac [10]	0	153 ± 2	0.1	+56 ± 2
	1.6	226 ± 6	0.3	+36 ± 1
Castor oil [10]	0	138 ± 3	0.2	+57 ± 1
	1.6	Agg		
Vitamin E [40]	0	112 ± 2	0.2	+60 ± 5
	1.6	122 ± 7	0.3	+54 ± 0
	2.5	Agg		

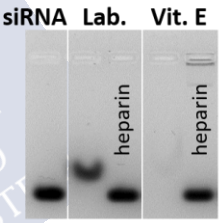

Fig. S4 - Physicochemical characterization of cetrimide NEs prepared using EtOH with and without siRNA: particle mean size, PDI and ζ-potential (left hand). Gel electrophoresis before and after siRNA-loaded NEs incubation with heparin (gel right side). siRNA corresponds to the control of naked siRNA. Values represent the mean ± SD (n=3 for blank NEs and n=2 for siRNA-loaded NEs).

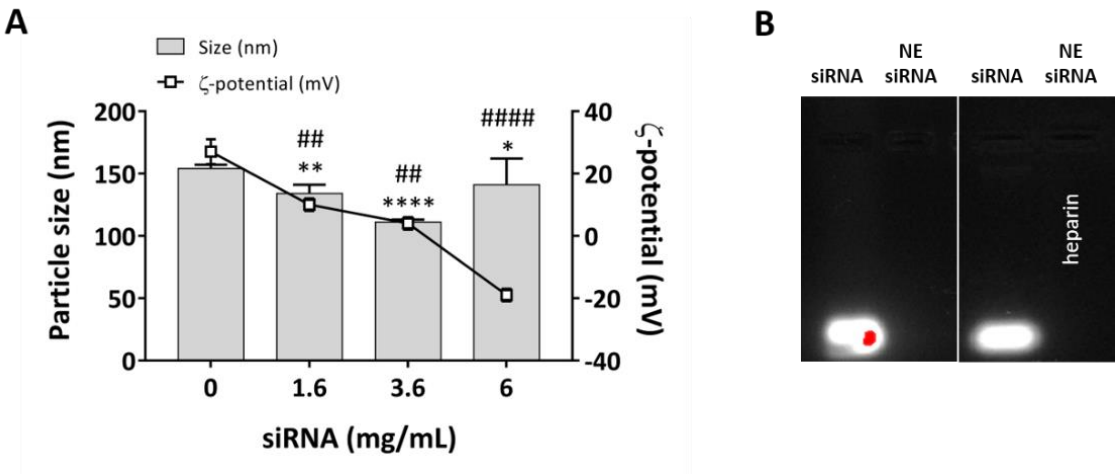

Fig. S5 - Characterization of oleylamine NEs loading siRNA - single step association. Effect of the increasing siRNA concentration on particle size and ζ-potential (**A**). Gel electrophoresis before and after siRNA-loaded NEs incubation with heparin (right side). siRNA corresponds to the control of naked siRNA (**B**). Numerical values represent the mean ± SD (n ≥ 3). Data was analyzed using a two-way ANOVA (* $p < 0.05$; ** $p < 0.01$, **** $p < 0.0001$; ## $p < 0.01$., #### $p < 0.0001$ * and # refer to size and ζ-potential, respectively).

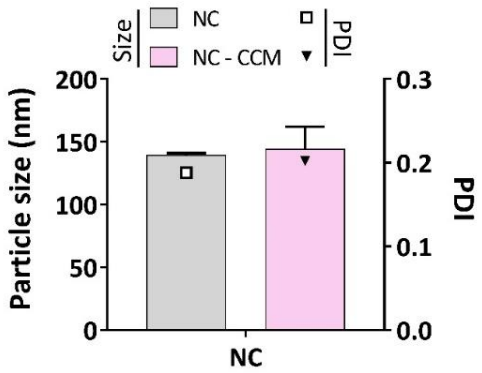

Fig. S6 - Colloidal stability of siRNA*-loaded protamine NCs, before and after incubation with non-supplemented cell culture media for 1 h at 37 °C. Numerical values represent the mean ± SD (n = 3)

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General discussion

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Polynucleotide-based therapies, as interference RNA, which has the capacity to modify or correct the expression of the gene(s) of interest, are gaining increasing attention due to their capacity to provide a treatment or even the cure for different heritable and acquired diseases that are so far untreatable by the available drugs. In the last years, different therapies (siRNA, As-ODN, cDNA, functional copy of a gene) have been approved by the FDA and the EMA for the treatment of diseases as spinal muscular atrophy, polyneuropathy caused by hereditary transthyretin, retinal dystrophy, among others. Furthermore, there are several therapies currently under clinical trials aiming the treatment of different cancers and ocular disorders, among other conditions.

Regarding ocular pathologies affecting the anterior and posterior segments of the eye, the increasing number of cases of people suffering from blindness mainly caused by cataracts, glaucoma, age-related macular degeneration and diabetic retinopathy [1, 2], evidences the need to develop disruptive and more effective treatments. As a target organ, the eye presents a significant advantage as compared to other organs and this is its accessibility. Nonetheless, the different barriers presented by both anterior and posterior segments of the eye are especially difficult to circumvent by hydrophilic molecules like nucleotides. In specific, siRNA molecules need to enter the target cell and avoid endosomal entrapment and degradation in order to be recognized by the RNA-induced silencing complex machinery and play its role [3-6].

Nanotechnology-based strategies (nanoparticles, liposomes, nanoemulsions, among others) have been developed in order to overcome the ocular barriers, protect the nucleotide from degradation and deliver it to the target tissue. Our group was among the pioneers in the development of nanoparticles and nanocapsules for ocular drug [7-9] and nucleotide delivery [10, 11]. Polymeric nanocapsules, consisting of an oil core surrounded by a polymer shell are versatile vehicles that enable the association of a variety of drugs, including small molecules [9, 12, 13], proteins and peptides [14-18] and polynucleotides [14, 19]. In addition, they have the capacity to protect RNA from degradation [14] and to interact with the ocular surface [12]. Within this context, the main goal of the work performed in this thesis was to develop protamine nanocapsules as siRNA delivery systems for ocular instillation. The foremost requirement of these NCs was to present an extraordinary siRNA loading capacity, so that a

concentration higher than 1 mg/mL, and preferable 5 mg/mL, was obtained. It was hypothesized that this concentration would be necessary in order to achieve a significant amount of siRNA into the eye surface for a therapeutic effect.

The design of the protamine NCs was based on the initial development of cationic NEs able to adsorb siRNA molecules on its surface and finally the addition of a protamine shell (Fig. 1), therefore protecting the siRNA from the external environment. In addition, we hypothesized that the protamine shell could interact with corneal epithelium [12] and improve cell penetration [20].

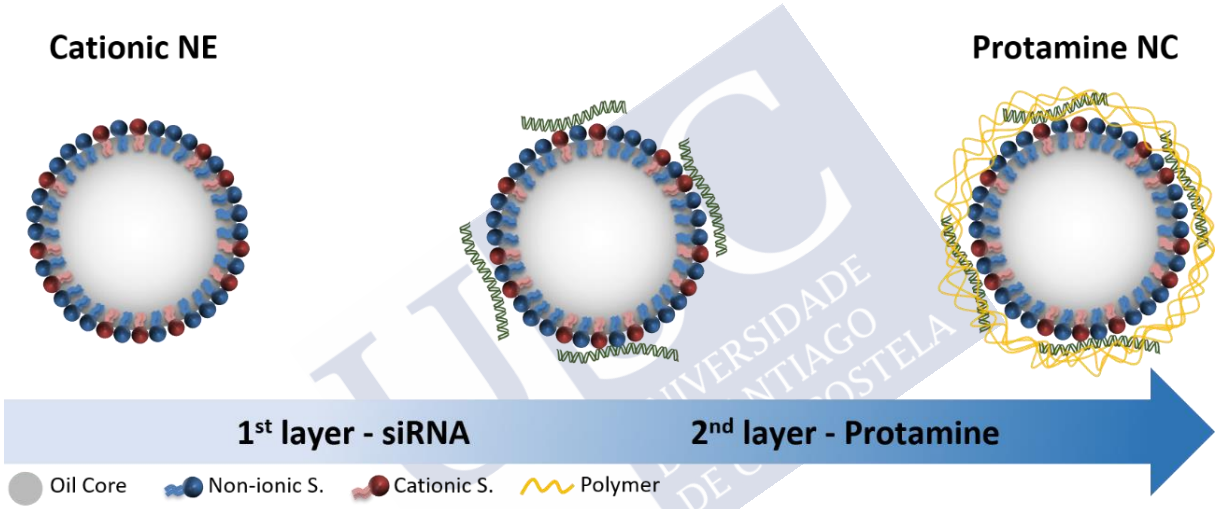

Fig. 1 - Graphical representation of the double-layered protamine nanocapsules (S., surfactant).

1. Protamine NCs as siRNA delivery systems prepared by the solvent displacement technique

For the preparation of the nanocapsules formulation, the first step was the preparation of a cationic NE able to associate siRNA. Taking into account the expertise of our research group on the development of NCs by the solvent displacement technique, we followed this method for the preparation of the multi-layered protamine NCs' core (oil core or NE). The composition of the NE was selected as follows. Polysorbate 80, a non-ionic surfactant present in ophthalmic formulations, was selected based on its capacity to improve polynucleotide-loaded nanoparticles stability and transfection capacity [19, 21, 22]. Vitamin E was selected due its

antioxidant properties, conferring the capacity to protect cells from oxidative stress involved in different pathologies [23-25], while Mygliol® was chosen because of its capacity to improve tear film stability [26]. The inclusion of cationic surfactants in oily droplets was intended to facilitate the association of the siRNA molecules to the carrier. Three different surfactants were selected: cetrimide, Bz Cl and LAE with different cationic groups (quaternary amine or guanidinium group) as well as hydrophobic chain length.

The following formulation parameters were investigated for their influence on the cationic oil cores (NEs) size, PDI and ζ-potential:

- Concentration of polysorbate 80 (10 and 30 mg in 5 mL of ethanol)
- Type of oil (vitamin E and Mygliol®)
- Type of cationic surfactant (cetrimide, Bz Cl and LAE)

As expected, the highest concentration of polysorbate 80 tested (30 mg) resulted in a smaller particle size without affecting the positive ζ-potential. With respect to the influence of the type of oil, NEs containing Miglyol® presented a larger particle size than those containing vitamin E. A similar trend was observed in other prototypes developed in our group [27, 28]. Considering that a smaller size could be translated into a larger surface area for siRNA association, the highest amount tested of polysorbate 80 and the oil vitamin E were selected for further studies. The positive ζ-potential of the NCs is conferred by cationic surfactants commonly used as formulation preservatives. Three different surfactants, cetrimide, Bz Cl and LAE were selected considering their different amine groups as well as carbon chain lengths. Despite the similar physicochemical properties of the NEs prepared with the different cationic surfactants, i.e. size, PDI and ζ-potential, they presented a different capacity to associate siRNA, being the cetrimide-based NEs the most efficient (2 mg/mL of siRNA, fully associated). The higher siRNA association efficiency of cetrimide might be due to its stronger cationic charge [29] and its more adequate availability on the nanodroplets surface, as compared to LAE or Bz Cl. Thereafter, the siRNA-loaded NEs were coated with increasing amounts of protamine (weight ratio of siRNA:protamine from 1:0.1 to 1:0.7). The results indicated that the weight ratio 1:0.3 led to the formation of stable NCs with a full siRNA association (2.5, 1.25 and 0.83 mg/mL of siRNA for NCs containing cetrimide and Bz Cl, LAE, respectively). The multi-

layered protamine NCs were able to maintain their physicochemical properties in simulated lacrimal fluid and to prevent the siRNA premature release. The effect of the different tested parameters on multi-layered protamine NCs' size, PDI, ζ-potential and siRNA association is summarized on table 1.

Table 1 – Effect of the factors under study on the size, PDI, ζ-potential (ζ-pot.) and siRNA association of the multi-layered protamine NCs.

Factors under study	Size	PDI	ζ-pot.	siRNA association
Amount of non-ionic surfactant: increasing the amount of polysorbate 80 (10 mg and 30 mg)	↓	≈	≈	-
Type of oil: change from vitamin E to Miglyol®	↑	≈	≈	-
Type of cationic surfactant: cetrimide, Bz Cl and LAE	≈	≈	≈	-
Layer of siRNA: increasing concentrations of siRNA (0 to 3 mg/mL)	↑	≈	↓	Cetrimide-based NEs presented the highest association (2 mg/mL)
Layer of protamine: increasing weight ratio of siRNA:protamine (1:0.1 to 1:0.7)	≈	≈	↑	Ratio of 0.3 led to a complete association of siRNA; Cetrimide-based NCs presented the highest association (3 mg/mL)

Protamine NCs were subsequently adapted for the loading of therapeutic siRNA*. The prototypes selected for the *in vitro* studies were composed by vitamin E, polysorbate 80 and the cationic surfactant cetrimide, Bz Cl or LAE (60, 30 and 10 mg in the initial organic phase, respectively). They presented a final siRNA concentration of 2.3, 0.8 and 1.7 (theoretical loading of 7.4, 4.7 and 3.8 %, respectively) and a protamine coating of 1:0.3 and 1:0.1 (siRNA:protamine w/w, for cetrimide-, Bz Cl- and LAE-based NCs). These multi-layered protamine NCs showed the capacity to maintain their physicochemical properties in simulated lacrimal fluid and cell culture media and to prevent the siRNA premature release.

The effect of siRNA*-loaded protamine NCs on cell viability as well as their silencing capacity was studied after 1 h of incubation with using HeLa cells. The MTS assays showed a good

cytotoxicity profile, confirming that the incorporation of the cationic surfactants in the oil core of the NCs allows the association of high siRNA concentrations without compromising cell viability. However, under the investigated conditions, the developed formulations were not able to induce gene silencing, leading to about 80 to 90 % of gene expression, in contrast to the 52 % of expression obtained when using a commercial transfection agent (DHF3).

Considering the capacity of protamine NCs to improve cell internalization and transfection [12, 19, 27], the unexpected limited silencing capacity of the herein developed protamine nanocapsules, could be justified by several hypotheses that do not question protamine's cell penetration capacity. First, there is the possibility that siRNA molecules are retained in the NCs, due to the strong electrostatic interactions between the cationic surfactant-siRNA-protamine, and not released into cytosol. Second, there is also the possibility of the NCs being entrapped into the endosomes, where the released siRNA molecules may be degraded.

Based on these hypotheses, the possibility to develop a protamine NC formulation presenting a high siRNA concentration and showing the capacity to release siRNA molecules and to escape endosomal degradation, may imply the selection of different surfactants. Hence, the results presented in this chapter led us to move to a different formulation approach, as described in point 2.

2. Protamine NCs loading a high concentration of siRNA prepared by the self-emulsifying technique

Considering the advantages of polymeric NCs and that a protamine surface layer could improve cell penetration and ocular retention, a layer of siRNA and another one of protamine were assembled onto the surface of the NE, thereby forming multi-layered protamine NCs. Furthermore, keeping in mind that the critical goal of this PhD work was the design of a siRNA formulation intended for ocular delivery and containing a high concentration of siRNA (about 5 mg/mL) we explored alternative technologies that would enable the fabrication of protamine nanocapsules using different surfactants and a more suitable method for industrial requests.

In this regard, instead of using the solvent displacement technique optimized by our group for the preparation of NCs, we selected an easier and more scalable production method [30-32], the self-emulsifying technique. Assuming that the cationic surfactant has a key role on the association and disassociation of the siRNA molecules as well as on the subsequent silencing effect of the nanocapsules, we compared the behaviour of cetrimide which presented the highest siRNA association efficiency in chapter 1, with that of oleylamine. This amphiphilic molecule was selected due to its primary amine group, unsaturated and long carbon chain, which was reported to be useful for the association of polynucleotides and to enhance the transfection efficiency [29, 33, 34]. Regarding the oil core composition, we selected Labrafac™ lipophile WL 1349 and polysorbate 80, an MCT and non-ionic surfactant present in different ophthalmic formulations. In summary, these main changes (cationic surfactant and production method) were expected to provide a scalable formulation loading the desired siRNA concentration.

Oleylamine was combined with the labrafac™ and polysorbate 80, forming NEs with an exceptional siRNA association capacity, reaching 6 mg/mL of siRNA. On the other hand, in the case of cetrimide different oils were tested in order to improve its inclusion in the system (labrafac™, vitamin E or castor oil), from which the ones containing vitamin E presented the highest siRNA association capacity, reaching 1.6 mg/mL of siRNA. The results obtained for the oleylamine NE do not fully agree with those reported by other authors, who indicated NEs containing DOTAP (quaternary amine group) present a higher nucleotide association efficiency than oleylamine-containing NE due to the higher avidity of quaternary amine group vs primary amine for the RNA molecules [29, 33]. Herein, the highest siRNA association efficiency of oleylamine might be related to its higher lipophilicity compared to cetrimide, that improved oleylamine inclusion in the NE structure and the exposure of the cationic head group on NE's surface, rather than to the type of amine presented by each surfactant (primary amine vs quaternary amine group).

Considering that oleylamine formulations combined both easiness of production as well as high cargo concentration, this prototype was selected for the addition of a protamine layer. Different protamine concentrations (1.5, 3 and 5 mg/mL in the initial polymeric solution) were tested, obtaining a formulation with a final siRNA concentration of 5 mg/mL (3.9 % loading).

Protamine NCs remained stable upon incubation in lacrimal fluid and prevented the premature release of siRNA. In addition, the release of siRNA was only observed when incubating the NCs with heparin.

With regard to the stability of the formulations during storage, protamine layer provided a better stability at 4 °C for 15 days than the corresponding siRNA-loaded NEs, maintaining not only its physicochemical properties but also a full association of siRNA. In order to try to improve their storage stability, the selected formulations containing oleylamine were freeze-dried with trehalose 10 % (w/v). The freeze-dried nanocarriers presented a neutralization of their ζ-potential, which could be due to the replacement of water by trehalose during the freeze-drying process [35, 36]. Both, the freeze-dried siRNA-loaded NEs and siRNA-loaded protamine NCs remained stable upon storage at 4 °C for 15 days.

The multilayered protamine NCs loaded with a therapeutic siRNA* (5 mg/mL siRNA* corresponding to a 3.9 % loading) were evaluated for the cytotoxicity and silencing effect in HeLa cells. The results showed that protamine NCs containing oleylamine did not compromise HeLa cells viability at a concentration of 30 µg/mL, which corresponds to the concentration of 100 nM of siRNA*, used in the silencing study. The NCs were incubated for 1 h with cells and gene expression was determined at 24, 48 and 72 h post treatment. At 72 h post-treatment the protamine NCs led to a modest but significant ($p < 0.05$) 24 % reduction of gene expression, showing no significant difference with the cells treated with siRNA*–DHF3 complex. This behavior might be indicative of a slow release of siRNA from protamine NCs. However, we cannot discard the possibility of an endosomal degradation. In order to understand the behavior of the NCs *in vitro*, further studies should be performed. For example, the analysis of gene expression for longer periods of time, as well as the use of fusogenic or pH-buffering compounds might help to elucidate the involvement of endosomal entrapment on the obtained silencing results.

The capacity of these protamine NCs to interact and penetrate through the ocular epithelium was assessed by topically administering the fluorescently labeled formulation (protamine-TAMRA) in New Zealand rabbits' eyes. After a period of 6 h post-instillation, it was possible to observe the presence of protamine NCs in the corneal epithelium probably due to the cell

penetration capacity of protamine [20], by the formation of hydrogen bonds between protamine's arginine and cell membranes' sulphate/phosphate groups [37].

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Conclusions

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The main conclusions from the work performed in this thesis are:

1. Multiple-layered protamine NCs containing different oily core compositions were produced by the solvent displacement or the self-emulsifying techniques, followed by the ionic assembling of siRNA and protamine onto the oily cores. The NCs exhibited a nanometric size (between 100 and 160) and ζ-potentials that varied from negative to positive values depending on their composition.

2. Multiple-layered protamine NCs, prepared by the solvent displacement technique and containing cetrimide, Bz CL or LAE as cationic surfactants, presented different affinity towards siRNA molecules. Those containing cetrimide were the most efficient in terms of siRNA loading, achieving final concentrations of siRNA of 2.5 mg/mL. These nanocapsules maintained their colloidal stability as well as cargo loading when incubated with simulated lacrimal fluid or in cell culture medium. Despite these promising properties, these NCs did not show a capacity to silence the expression of the target gene *in vitro*.

3. Multiple-layered protamine NCs, prepared by the self-emulsifying technique and containing oleylamine or cetrimide as cationic surfactants presented different affinity towards siRNA molecules. Those containing oleylamine were able to efficiently load a high concentration of siRNA (5 mg/mL). These nanocapsules maintained their colloidal stability as well as cargo loading, when incubated with simulated lacrimal fluid or in cell culture medium. They could be efficiently freeze-dried with trehalose, maintaining its size as well as associated siRNA upon resuspension. These NCs showed a capacity to silence the expression of the target gene, which was similar to the one observed for the commercial transfection reagent (DHF3). Moreover, *in vivo* studies performed in New Zealand rabbits indicated that siRNA-loaded protamine NCs were able to interact and penetrate through the corneal epithelium.

Overall these conclusions highlight the importance of the surfactants present in the NCs formulations in terms of *in vitro* silencing capacity and the necessity to define specific quality attributes in the early stages of the development of a siRNA nanocarrier formulation.

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Annex

Functionalization of naturally-occurring materials for RNA delivery

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Annex

Functionalization of naturally-occurring polymers for RNA delivery

This work was performed in collaboration with Laura Russo[1] and Francesco Nicotra[1]

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Abstract

Natural materials, as hyaluronic acid (HA) and collagen have been used in the development of polynucleotide delivery systems. HA, due to its mucoadhesive properties and its affinity for the CD44 receptor expressed in the ocular surface, has been widely used in the development of ocular drug delivery systems. However, its anionic nature hampers the association of nucleotides and so it has been mainly used in combination with other nanocarriers in order to improve their physicochemical properties and biocompatibility for ocular administration. On the other hand, collagen has been efficiently used for polynucleotide therapies for different applications, but scarcely for ocular polynucleotide delivery. Considering the high transfection efficiencies obtained by nanocarriers composed by materials containing quaternary ammonium groups, we functionalized both HA and collagen with quaternary ammonium linkers, without the use of organic solvents or harsh conditions. A functionalization degree of about 22 % (of HA carboxylic groups) and 97 % (of collagen lysine's primary amine groups), respectively. Transference RNA (tRNA) was used as a cargo model and different weight ratios of tRNA:polymer (up to 1:500) were tested. The physicochemical properties of the complexes were determined by dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and electron microscopy. Functionalized high molecular weight HA was able to form small round-shaped complexes of about 100-150 nm. On the other hand, functionalized collagen formed also round particles of about 200 nm with collagen characteristic fibrous structure. Unexpectedly, despite the capacity of the polymers to form well-organized structures upon interaction with tRNA, the association efficiency was very low. Therefore, this exploratory study suggests the necessity to further functionalize the polymers or the nanocomplex formulations before assessing the performance of the resulting nanocomplexes.

1. Introduction

Naturally-occurring materials such as chitosan [1-3], hyaluronic acid [1, 2, 4, 5], human serum albumin [6, 7], dextran [5, 8] and protamine [4, 5, 8], among others have been used in the development of different types of nanocarriers for polynucleotide delivery to the eye. These polysaccharides, proteins and peptides are biocompatible and biodegradable. In general, they have been reported to increase ocular residence time and/or to target specific receptors present in the eye and/or improve cellular uptake. Among them, in this work, we selected HA and collagen since they are both naturally found in ocular tissues.

HA is a polysaccharide found in different tissues of our body such as the skin, lung, intestine, umbilical cord or synovial fluid. In ocular tissues it is present in the vitreous humor, epithelium of the cornea and conjunctiva, lacrimal gland and tear fluid [9]. Its natural lubricant, water retention, mucoadhesive and viscoelastic properties have prompted its use in ophthalmic products like artificial tears (Hylo-Vision® by OmniVision; Refresh® by Allergan) and soft contact lens solutions (Maxlook All-In-One by Maxlook optics). Furthermore, HA has the capacity to target the CD44 receptors present in the corneal and conjunctival epithelia [2, 10]. Although HA, as such, has no potential for the delivery of polynucleotides due to its anionic nature, it has been used in combination with other polymers, such as chitosan [1, 2], polyethylenimine (PEI) [11] and N,N'-cystaminebisacrylamide-4-aminobutanol (p(CBA-ABOL) [12] or other carriers like solid lipid nanoparticles [13] for ocular polynucleotide delivery. A different strategy for the use of HA as a polynucleotide carrier has relied on the chemical functionalization with compounds containing amine groups, such as PEI [14-17], spermine [15, 18], polylysine, stearylamine, choline [15] and poly(2-dimethylaminoethyl methacrylate) (pDMAEMA) [19]. According to Ganesh et al. HA functionalized with polyamines, specially PEI, resulted in a higher association efficiency of siRNA, due to its high amino content that also improved the capacity of the functionalized HA to self-assemble [15].

Collagen is also found in ocular tissues like the cornea, constituting about 70 % its extracellular matrix [20], and the vitreous humor [21]. Collagen is the most abundant protein in the body, mostly found in skin, tendons and ligaments, among other tissues [22]. This protein is characterized by its amino acid sequence of Gly-X-Y (glycine-proline-hydroxyproline) and has

a unique triple-helix configuration forming fibrils [23]. Different ophthalmic collagen-based products are commercially available such as shields (Soft Shield®, by OASIS® Medical Inc), scaffolds (Ologen® Collagen Matrix, by Aeon Astron; ABCcolla® by ACRO biomedical Co.) and eye drops (Eye Repair Rx® by Hymed group). It has been used since the 70s as an ophthalmic biomaterial in the development of inserts for ocular drug delivery, shields for corneal wound healing and drug delivery to the anterior eye segment [22]. Collagen has been used for polynucleotides delivery resulting in the development of AteloGene™ (Koken, Japan) in which collagen (atelocollagen) is complexed with small interfering RNA (siRNA) [24-26], antisense oligonucleotides [27, 28] or other polynucleotides leading to an efficient transfection. Hao et al., functionalized collagen with aptamers for micro RNA targeted delivery to prostate cancer cells [29]. In the ocular field, Zorzi et al. developed collagen nanoparticles by chemically modifying collagen with ethylenediamine and spermine [30], from which collagen-spermine functionalization resulted in a more efficient pDNA and siRNA delivery to cornea and conjunctiva epithelial cell lines, due to spermine's higher amino content.

Overall, HA and collagen have been mainly functionalized with primary amine groups obtaining relevant results in polynucleotide delivery. Here our hypothesis has been that their chemical functionalization with chemical entities containing quaternary amino groups would be beneficial. Our hypothesis relies on the knowledge about the capacity of such entities, i.e. 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) [31, 32] or N-[1-(2,3-dioleyloxy)propyl-N,N,N-trimethylammonium chloride (DOTMA) [33], to achieve an efficient nucleotide delivery in ocular tissues.

2. Materials and Methods

2.1. Materials

Semed S collagen was obtained from DSM Biomedical (USA). Hyaluronic acid (1-2 MDa) was bought from Carbosynth (UK). 4-(N,N,N-trimethylammonium) butanoic acid, (2-chloroethyl)trimethylammonium chloride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), n-hydroxysuccinimide (NHS), phosphate buffer (PBS), L-arginine,

Ninhydrin reagent solution and tRNA and dialysis tubing cellulose membrane were acquired from Merck (Italy). RNAse free water was acquired from Invitrogen (UK).

2.2. Hyaluronic acid functionalization

Hyaluronic acid was functionalized with positively charge groups along its backbone, namely quaternary ammonium groups, by conjugating (2-chloroethyl)trimethylammonium chloride with HA's carboxylic group. In brief, (2-chloroethyl)trimethylammonium chloride (PBS, pH 9.0) was added to HA (10mg/mL in PBS pH 9) at different molar ratios (1:0.5 and 1:1, HA:(2-chloroethyl)trimethylammonium chloride) and left stirring overnight at room temperature (RT). Afterwards, the reaction mixtures were dialyzed for 48 h against ultrapure water using a dialysis tubing cellulose membrane. Finally, functionalized HA was freeze-dried (LIO5DGT, Pascal, Italy) and stored at - 20°C until further use.

2.3. Collagen functionalization

Collagen was functionalized with quaternary ammonium groups by conjugating the carboxylic group of 4-(N,N,N-trimethylammonium) butanoic acid with primary amino group of lysine side chains. For that, 182 mg of 4-(N,N,N-trimethylammonium) butanoic acid were dissolved in 10 ml of PBS at pH 5.5. Under stirring at RT, 192 mg of EDC and 115 mg of NHS were added to the previous solution and stirred for 30 min. The solution of the activated linker was added to a collagen solution (1 g dissolved in 20 mL of PBS at pH 5.5). The reaction was left stirring overnight at RT and then dialyzed in a dialysis tubing cellulose membrane, against ultrapure water for 4 days. Functionalized collagen was freeze-dried (LIO5DGT, Pascal, Italy) and stored at -20°C until further use.

2.4. Characterization of functionalized polymers

2.4.1. Nuclear Magnetic Resonance (NMR)

Functionalized and non-functionalized HA and collagen and their respective linkers ((2-chloroethyl)trimethylammonium chloride and 4-(N,N,N-trimethylammonium)butanoic acid) were dissolved in D_2O. A Bruker Avance I 600 spectrometer (Bruker, Italia) was used for ^1H NMR experiments.

2.4.2. Ninhydrin assay

In order to determine the functionalization degree of collagen lysine's primary amine groups, a ninhydrin assay was performed. The assay was performed according to manufacturer instructions and L-alanine was used as standard. The optical absorbance was measured at 570 nm using a Victor X3 multiplate reader (Perkin Elmer). The degree of functionalization of collagen lysine's primary amine groups was calculated from a standard calibration curve and using the following equation:

$$DF\ \% = \frac{C_{Collagen} - C_{funct.\ Collagen}}{C_{Collagen}} x\ 100$$

where $C_{Collagen}$ and $C_{funct.Collagen}$ correspond to the concentration of lysine amine groups in collagen and functionalized collagen, respectively. All the samples were analyzed in triplicate.

2.5. Preparation and characterization of t-RNA complexes

2.5.1. t-RNA complexes

In order to assess the capacity of the functionalized polymers to associate tRNA, different tRNA:polymer ratios were tested (1:100, 1:300 and 1:500, w/w). Functionalized-HA was dissolved in ultrapure RNase free water or PBS (pH 7.4 or 5.5) while functionalized-collagen was dissolved in ultrapure RNase free water or acetic acid 0.1 M, for a final concentration of 4 mg/mL. An aqueous solution of tRNA at 0.2 mg/mL was added to the polymeric solution, in

order to obtain the selected ratios, vortexed for 30 s and left incubating for at least 10 min at RT. Non-functionalized polymers were used as control, under the same conditions.

2.5.2. Evaluation of tRNA association

The effect of polymers on tRNA association efficiency was determined by gel retardation assay. Either samples and naked tRNA control were loaded in 2 % (w/v) agarose gels (in Tris-Acetate EDTA) at 500 ng of tRNA per lane. 1x SYBR® Gold nucleic acid stain was used for sample visualization. Gels were run for 1 h at 50 V, in a Sub -Cell GT cell 96/192 (Bio-Rad, Hercules, CA, USA) and imaged using a molecular Imager® Gel Doc™ XR System (UV light 302 nm; Bio-Rad, Madrid, Spain).

2.5.3. Physicochemical and morphological characterization

The capacity of the functionalized polymers HA and collagen to complex tRNA forming nanostructures was determined by Dynamic light scattering and Nanoparticle tracking analysis.

DLS analysis:

Samples were diluted in ultrapure water at 1:10 (v/v) and their particle mean size as well as polydispersity index (PDI) was measured by DLS using a Zetasizer® (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) at 25 °C with a detection angle of 173 °.

NTA analysis:

Particle size and concentration were determined by NTA analysis. Measurements were carried out using Nanosight NS300 system (Malvern Instruments, Worcestershire, UK) equipped with a 488 nm laser. Video analysis was performed by NTA image analysis software 3.3. Samples were diluted in ultrapure water at 1:100 (v/v), except for the ones of tRNA-functionalized collagen that were diluted at 1:1000 (v/v). One milliliter of sample was injected at 40 µl/min

flow rate, at 25 °C. Samples were analyzed in triplicate by recording five different videos of each.

Field emission scanning electron microscopy (FESEM):

Morphological analysis of tRNA complexes with functionalized and non-functionalized HA and collagen was performed by FESEM (Zeiss Gemini Ultra Plus, Germany) using scanning transmission electron microscopy (STEM) and immersion lens (InLens) detectors for sample observation. Samples containing tRNA/polymer mixture or just the functionalized polymer were stained at 1:1 (v/v) with an aqueous solution of 2 % (w/v) phosphotungstic acid. Then, 1 µL of the sample was placed on a copper grid and allowed to dry. The grid was washed with ultrapure water and left to dry before analysis. Samples' micrographs were taken at different magnifications.

3. Results and discussion

3.1. Polymers characterization

3.1.1. Hyaluronic acid functionalization

HA was functionalized with quaternary amine groups in order to reduce its negative charge density and enable RNA association. The functionalization was performed through soft conditions, avoiding the use of solvents (Fig. 1).

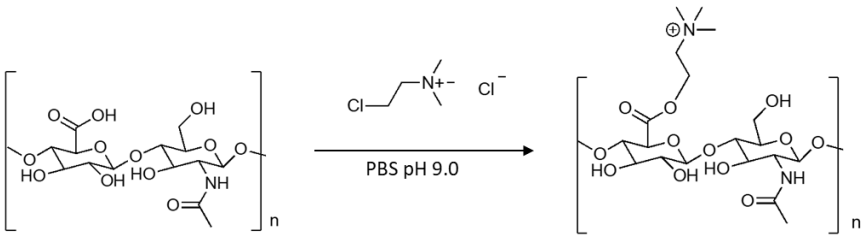

Fig. 1 - Scheme of HA functionalization with quaternary amine groups.

In ^{1}H NMR spectra of Fig. 2 we can observed that in all the samples, HA (non-functionalized), HA(1:0.5) and HA(1:1) the signal assigned to the sugar rings protons is easily recognized as a multiplet between 4.3-3.5 ppm. The two anomeric protons (α and β) between 5-4.7 ppm and the signal at 2.3 ppm represent the protons $-CH_3$ of the N-acetyl group of HA. The functionalization of both HA(1:0.5) and HA(1:1) was confirmed by the peaks at 4.4 ppm, 4.1 and 3.5 ppm corresponding to the signals of CH_2CH_2 and $-(CH_3)_3$ of the linker, respectively. The degree of functionalization (DF) was calculated using the following equalization:

$$DF\% = \frac{A_{funct.HA} - A_{HA}}{n} x\ 100$$

where $A_{funct.\ HA}$ and A_{HA} is the area of the sugar peaks in the functionalized and non-functionalized (HA) samples, respectively; and *n* is the linker protons number. HA(1:0.5) and HA(1: 1) presented a DF of 6 % and 22 %, respectively. This difference can be observed in the ^{1}H NMR spectra by the difference in the peak intensity at 3.5 ppm ($-(CH_3)_3$), which results to be more intense in HA(1:1) sample.

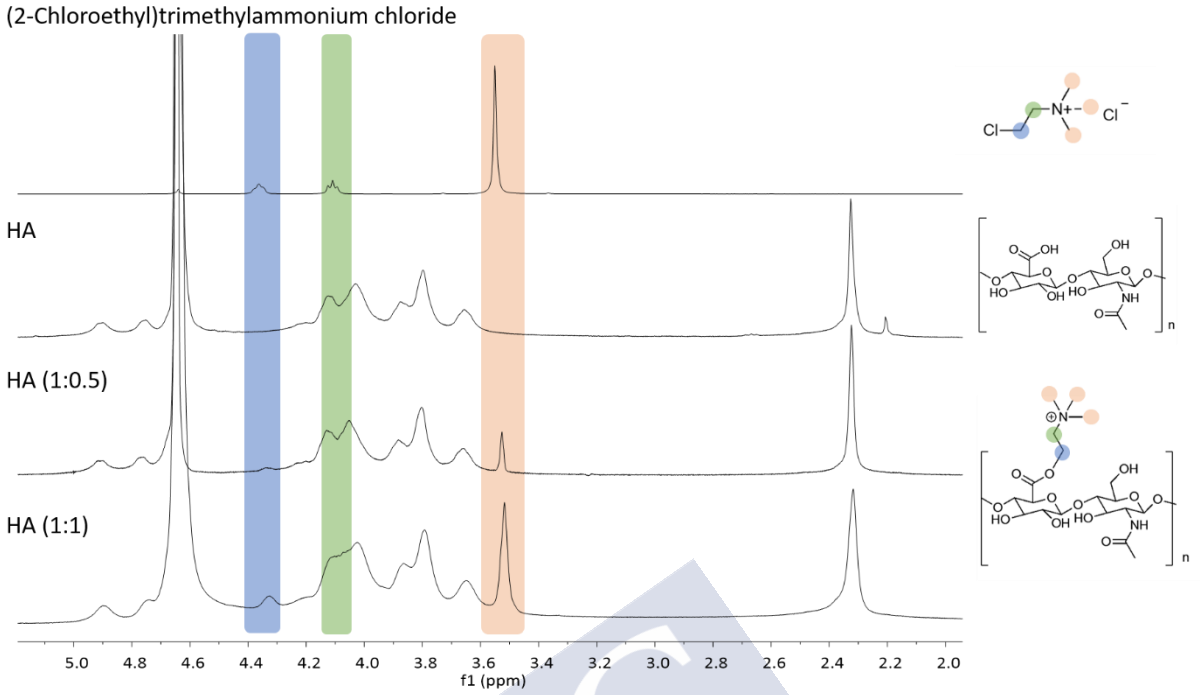

Fig. 2 - ^{1}H NMR of functionalized HA with quaternary amine groups, and the respective controls (HA and (2-chloroethyl)trimethylammonium chloride) in D2O.

3.1.2. Collagen functionalization

A similar strategy was used with collagen in order to improve its capacity to associate siRNA (Fig. 3).

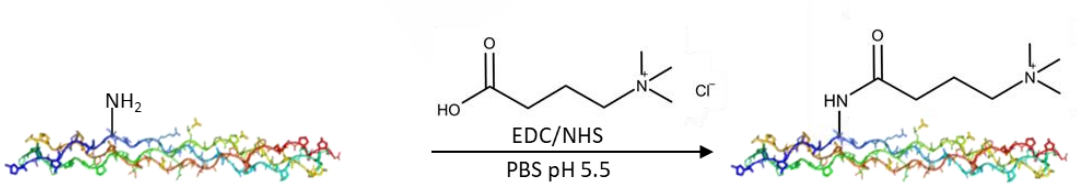

Fig. 3 - Representation of collagen functionalization with quaternary amine groups.

^{1}H NMR spectra (Fig. 4) displayed the presence of a peak at 2.6 ppm, assigned to the methylene hydrogen of lysine amines that disappears once the collagen functionalized was functionalized with 4-(N,N,N-trimethylammonium)butanoic acid. Moreover, the functionalization also led to the introduction of new signals: at 2.4 ppm, attributable to the -CH_2-N- of the linker, and between 3.1–2.8 ppm, attributable to the N-(CH_3) of the linker.

Due to the complexity of the collagen, the determination of the functionalization degree was not possible by ^{1}H NMR. Nonetheless, the disappearance of the peak corresponding to CH_2-N- indicates a high degree of functionalization of the total lysine content (5% of total collagen amino acids). In order to confirm the absence of free primary amine groups on functionalized collagen, a ninhydrin assay was performed. In this assay ninhydrin reacts with primary amine groups and the results indicated that about 97 % of lysine's primary amine groups have been functionalized.

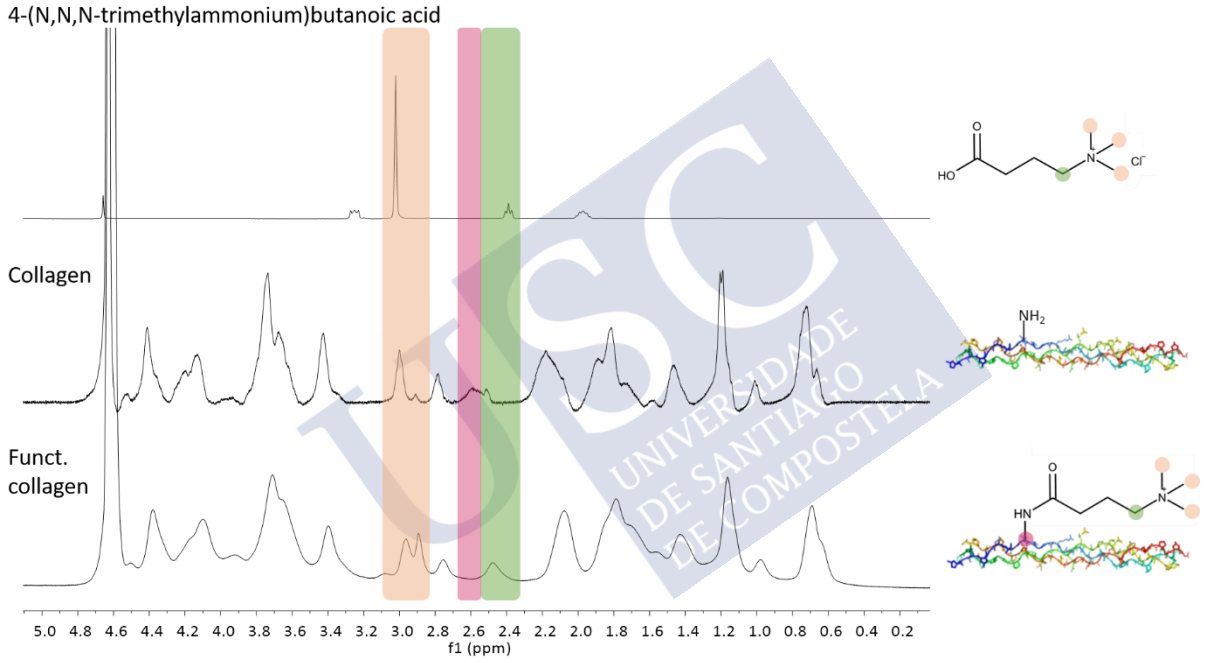

Fig. 4 - ^{1}H NMR of functionalized collagen and the respective controls (collagen and 4-(N,N,N-trimethylammonium)butanoic acid) in D_2O.

3.2. Functionalized HA-tRNA complexes

3.2.1. tRNA association efficiency

In order to determine the ability of the functionalized HA (HA 1:1) to associate tRNA, different ratios of tRNA:Funct. HA (w/w) were tested (1:100, 1:300 and 1:500). This screening was performed by adding a tRNA aqueous solution into a solution of functionalized HA formerly dissolved in ultrapure RNase free water. Samples were stained with SYBR® Gold and loaded

into 2 % agarose gels. As presented in Fig. 5A, the gel retardation assay indicates that the functionalized HA was not able to efficiently complex the RNA. Nonetheless it is possible to observe a slight mobility shift through the agarose gel (see the dashed line as reference of naked tRNA level in each gel) specially in the case of the higher tested ratio of tRNA:Funct. HA (1:500, w/w). In order to understand the influence of the presence of salts and pH on functionalized HA-tRNA association efficiency, the polymer was also dissolved in PBS at pH 7.4 and 5.5. The results showed that when the polymer was dissolved in PBS at pH 5.5 (Fig. 5B) the association of tRNA was slightly higher. The same conditions were tested using nonmodified HA as control. Similar results were obtained, but taking into consideration bands intensity, the association of tRNA seemed to be slightly higher in the case of functionalized HA dissolved in PBS pH 5.5.

3.2.2. Particle size analysis

In this work, high Mw HA was selected due its high capacity to retain water and high mucoadhesiveness which is expected to improve ocular surface hydration and enhance ocular residence time [34]. According to DLS measurements, the complexation of tRNA with functionalized HA (HA 1:1) at the ratio 1:500 (w/w) led to the formation of aggregates of about 4000 nm and 1500 nm when prepared in water or PBS, respectively (Fig. 5C). By functionalizing a low Mw HA (20 KDa) with a similar quaternary amine moiety, Ganesh et al. was able to prepare nanoparticles of about 175 nm at a RNA-HA weight ratio of 1:450 [15], however herein we used a high Mw HA that resulted in aggregation. The effect of HA Mw on particle formation was also demonstrated by Martens et al. that was able to coat pDNA complexes with low Mw HA, maintaining a nanometric size, but not with high Mw HA (~ 2 MDa) [12]. Despite the negative effect of the use of high Mw HA, its carbodiimide crosslinking onto the surface of liposomes led to an efficient HA coating [35], indicating that it is possible to use a HA with high Mw. Possibly in our case, a higher functionalization degree would be necessary to improve HA interaction with tRNA and result in an efficient complexation.

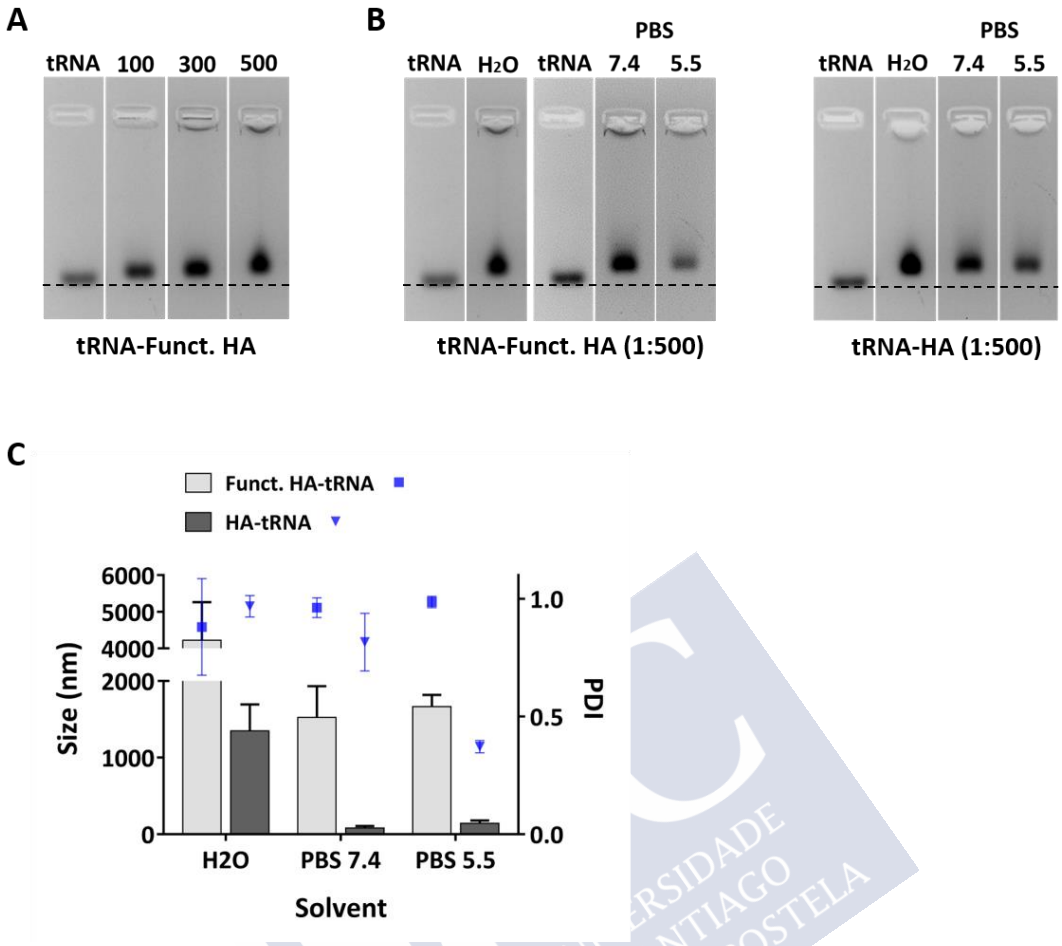

Fig. 5 - Effect of HA functionalization on tRNA association. Gel retardation assay of tRNA:functionalized HA complexes at different ratios (1:100, 1:300 and 1:500, w/w) (**A**). Effect of solvent on functionalized HA and HA (nonmodified) on their tRNA association efficiency, by gel retardation assay (**B**), and on particle size, by DLS measurements (**C**). tRNA refers to naked tRNA as control. Numerical values represent the mean ± SD (n=3).

For NTA analysis, the functionalized-HA formulations had to be further diluted in water at 1:50 or 1:100 (v/v) but almost no particles were observed. Due to their viscosity the undiluted samples could not be used, thus in order to confirm the results obtained by DLS we performed electron microscopy analysis of the samples prepared in water or PBS at pH 5.5.

The functionalized-HA dissolved in water (with no tRNA) was also analyzed and as it is shown in Fig. 6A, it forms round-shaped structures having about 650 nm with rough surface that seem to be agglomerates of the smaller structures of 20-30 nm also present in the sample. The complexation of tRNA with the functionalized HA in water led to the formation of abundant, uniform and round-shaped nanostructures with about 100-150 nm. Despite the DLS results,

herein we could observe that we were able to form nanoparticles of about the same size as the one obtained by Ganesh et al., that functionalized low Mw HA (200 KDa) with a similar quaternary amine [15]. Nonetheless, is worth to note that herein we used a high Mw HA with ~1.5 MDa. Furthermore, when PBS (pH 5.5) was used, less and bigger particles of 300 nm were formed and at high magnifications it is possible to observe a mucky background.

On the other hand, HA (nonmodified) and tRNA mixture formed big and irregular structures of 500 nm that looked like clusters of the smaller particles of about 70 nm. Nonetheless, when PBS was used as solvent, there was no formation of nano-like structures, it is only possible to observe something like ramified agglomerates and a blurred background, contradicting the DLS results that indicated that there was the formation of nanostructures of 80 to 150 nm, respectively. The results are in agreement with Paidikondala et al. study reporting that HA was not able to form nanostructures with tRNA when using PBS while some kind of small nanostructures and clusters were formed when using water.

Unlike DLS measurements, FESEM analysis was performed without diluting the samples. Therefore, we also used this technique to determine the effect of sample dilution on particle size, but in this case only the formulation of functionalized HA-tRNA prepared in water was used. As shown in Fig. 7A while the undiluted samples present uniformly dispersed round particles, their dilution in water at 1:10 (v/v) lead to particle aggregation or disruption since structures like the ones present in Fig. 7B were observed, or even no sample at all in other parts of the grid (not shown). The results seem to indicate that the dilution of the sample has a great impact on particles' structure. Therefore, it seems that the results obtained for functionalized HA-tRNA (in water) by DLS were mainly driven by sample dilution. In order to improve particle stability as well as tRNA association natural crosslinkers like tripolyphosphate (TPP) or other polymers like chitosan could be explored [30, 36].

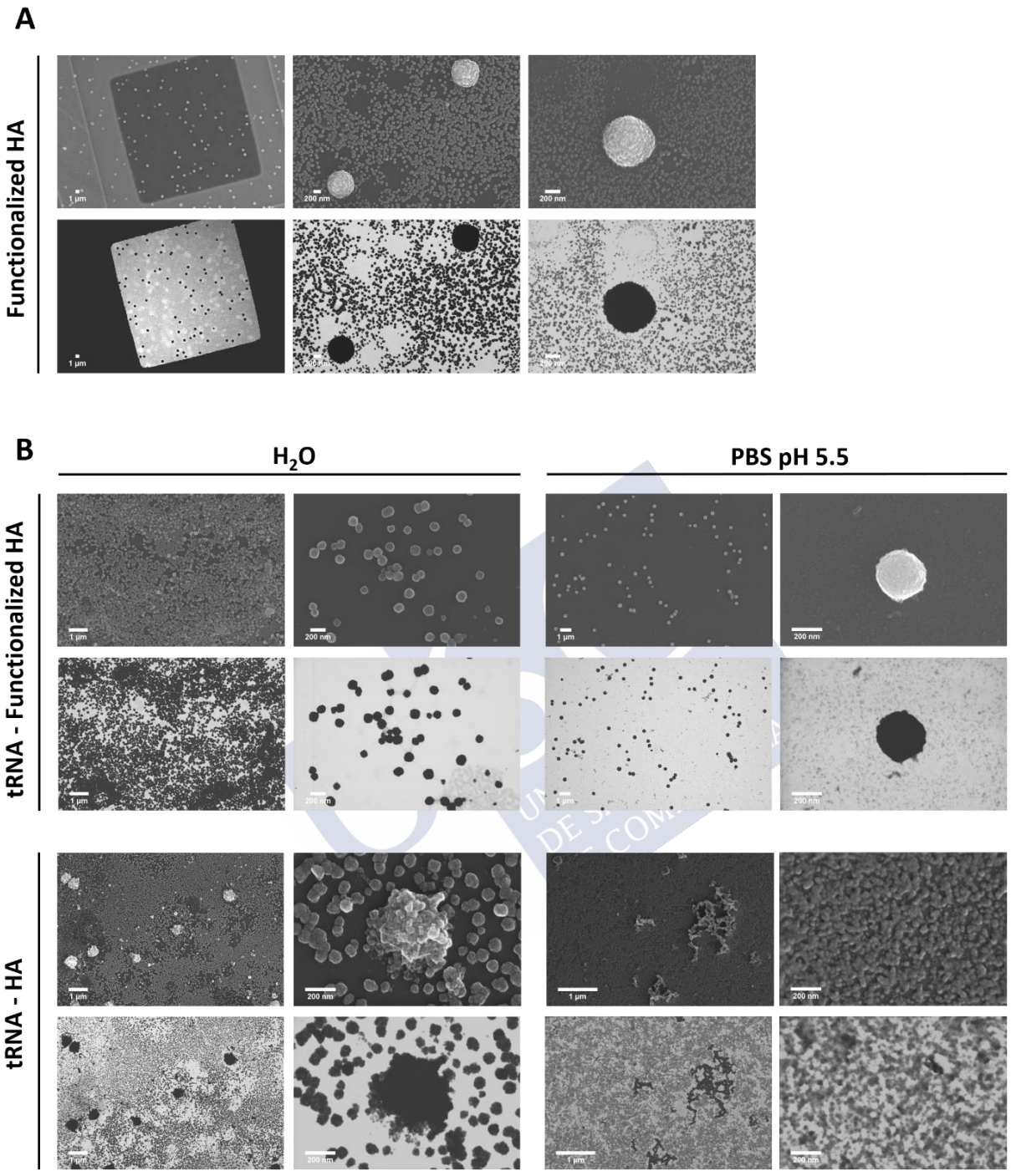

Fig. 6 - FESEM micrographs of functionalized HA and non-functionalized HA (HA). Functionalized HA dissolved in H_2O at 4 mg/mL (**A**). Functionalized HA and HA dissolved in H_2O (**B**, left hand) or PBS at pH 5.5 (**B**, right hand) and complexed with tRNA (tRNA:HA w/w, 1:500). For each group of samples there is a low and high magnification (left and right side) whose scale bars correspond to 1 µm and 200 nm, respectively. Micrographs were also taken using STEM and InLens detectors, presented at upper and lower side of each sample at both magnifications.

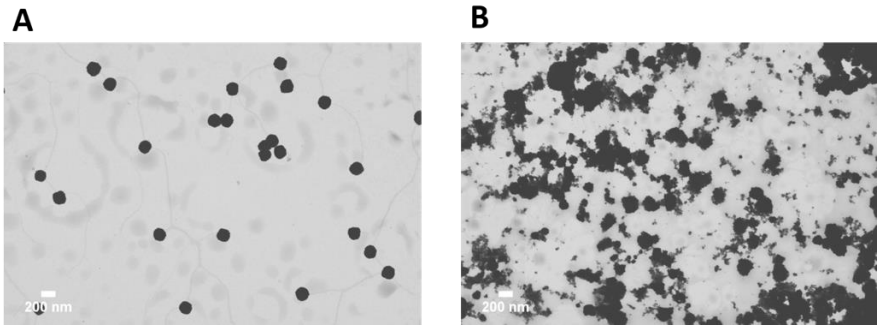

Fig. 7 - FESEM micrographs of tRNA-functionalized HA complexes (1:500, w/w) before (**A**) and after (**B**) dilution at 1:10 (v/v) in H_2O. Micrographs were taken using InLens detector. Scale bars correspond to 200 nm.

3.3. Functionalized Collagen-tRNA complexes

3.3.1. tRNA association efficiency

Collagen contains in its structure amino acids with amine groups. In this work we functionalized some of those amine groups in order to have quaternary ammonium groups, since materials like DOTAP and DOTMA containing this type of amines are highly efficient associating and delivering polynucleotides. Moreover, to the best of our knowledge ocular polynucleotide delivery using collagen has only been performed by functionalizing it with ethylenediamine and spermine (containing primary amine groups) [30].

Collagen presenting a 97 % degree of lysine's primary amines functionalization with quaternary ammonium groups, was complexed with tRNA at different weight ratios (1:100, 1:300 and 1:500, tRNA:functionalized collagen). As it is shown in Fig. 8A gel, the increase of tRNA:funct. collagen ratio to 1:500 lead to an increase of tRNA association, since it is possible to observe that there is a mobility shift on the gel and, at this ratio, no defined RNA band was formed.

Considering that collagen solubility can be improved by using acidic solutions, we also decided to assess the effect of using a common collagen solubilizer (acetic acid) on particles' properties. As it is possible to observe through gel retardation assay similar results were obtained independently of dissolving using water or acetic acid (0.1 M). Nonmodified collagen was also used under the same conditions as control. In the case of tRNA association there was free RNA (Fig. 8B, lower side). The presence of well-defined RNA band and the absence of RNA

dragging as in the case of the functionalized collagen, might indicate that functionalized collagen presented a higher association efficiency though not stable enough to hold the RNA during gel run.

3.4. Particle size analysis

Particle size and PDI were determined by DLS. As we can see in Fig. 8A (upper side) in the absence of tRNA, collagen seems to be able to form nanostructures of about 208 ± 9 nm. On the other hand, when tRNA was associated to functionalized collagen, there was a slight decrease of particle size of about 30 nm, reaching 182 ± 11 nm in the case of ratio 500, while the PDI remained at around 0.3, indicating some polydispersity. The dissolution of collagen in acetic acid led to an increase of 100 nm on particle size and PDI decrease from 0.4 to 0.2. Nonetheless, the use of acetic acid did not seem to influence collagen capacity to associate tRNA nor its particle size, maintaining a similar size of and PDI. Taking this into account and the fact that water would be preferable for future *in vitro* or *in vivo* applications, we selected this solvent for further studies.

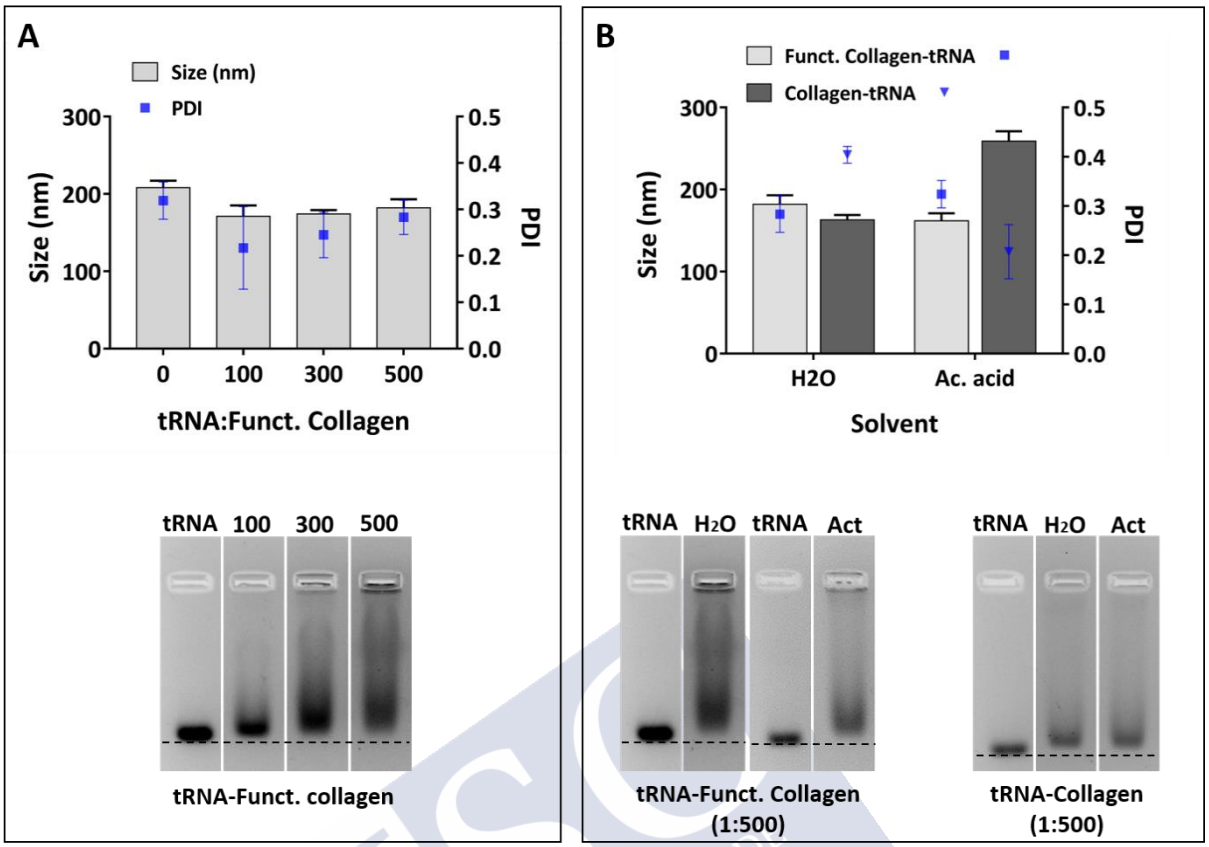

Fig. 8 - Effect of Collagen functionalization on tRNA association efficiency. Particle size and PDI (**A**, upper side) and gel retardation assay of tRNA:funct. Collagen complexes at different ratios (1:100, 1:300 and 501:0, w/w) (A, lower side). Effect of solvent on Functionalized collagen and collagen (nonmodified) on their particle size by DLS measurements (**B**, upper side) and tRNA association efficiency (weight ratio 1:500, tRNA:funct. collagen) by gel retardation assay (B, lower side). Numerical values represent the mean ± SD (n=3, except in the case of Funct. collagen n=2).

Despite the similarities presented by functionalized collagen and nonmodified collagen on tRNA complexation, we went further in the characterization of these systems and performed NTA analysis. The commonly used DLS technique measures particles light scattering and Brownian motion (diffusion coefficient) finally determining particles' size by Stokes Einstein Equation. Unlike DLS, NTA analyses a formulation on a particle-by-particle basis, which reduces the interferences usually caused by the higher light scattering of big particles or aggregates and therefore leads to a more accurate result. Moreover, by analysing particle-by-particle, NTA also determines particle concentration and its integrated microscope and camera allows a real time visualization and video recording of particles under movement. Plus, this technique requires sample concentration to be adjusted to the recommended concentration range of 10^7-10^9 particles/mL [37-40].

According to NTA measurements, functionalized collagen-tRNA formed particles of about 220 ± 28 nm while collagen-tRNA presented 286 ± 14 nm, which are about 40 and 120 nm bigger than the sizes obtained by DLS, respectively. By functionalizing collagen with ethylenimine Zorzi et al. obtained particles of about 600 and 1700 nm when complexing collagen with siRNA or pDNA, respectively, despite using TPP as crosslinker [30]. The difficulty of forming nanoparticles with collagen was attributed rigid structure of collagen.

By DLS and NTA we could observe that both collagen and functionalized collagen seem to form nanostructures with tRNA, but more importantly, as we can observe in Fig. 9A, despite the high SD, functionalized collagen-tRNA led to the formation of a much higher concentration of particles than the nonmodified collagen, which might indicate its higher affinity to tRNA. Fig. 9B represents the general view of the captured videos for both samples, giving an idea of the higher particle concentration presented by tRNA-functionalized collagen samples.

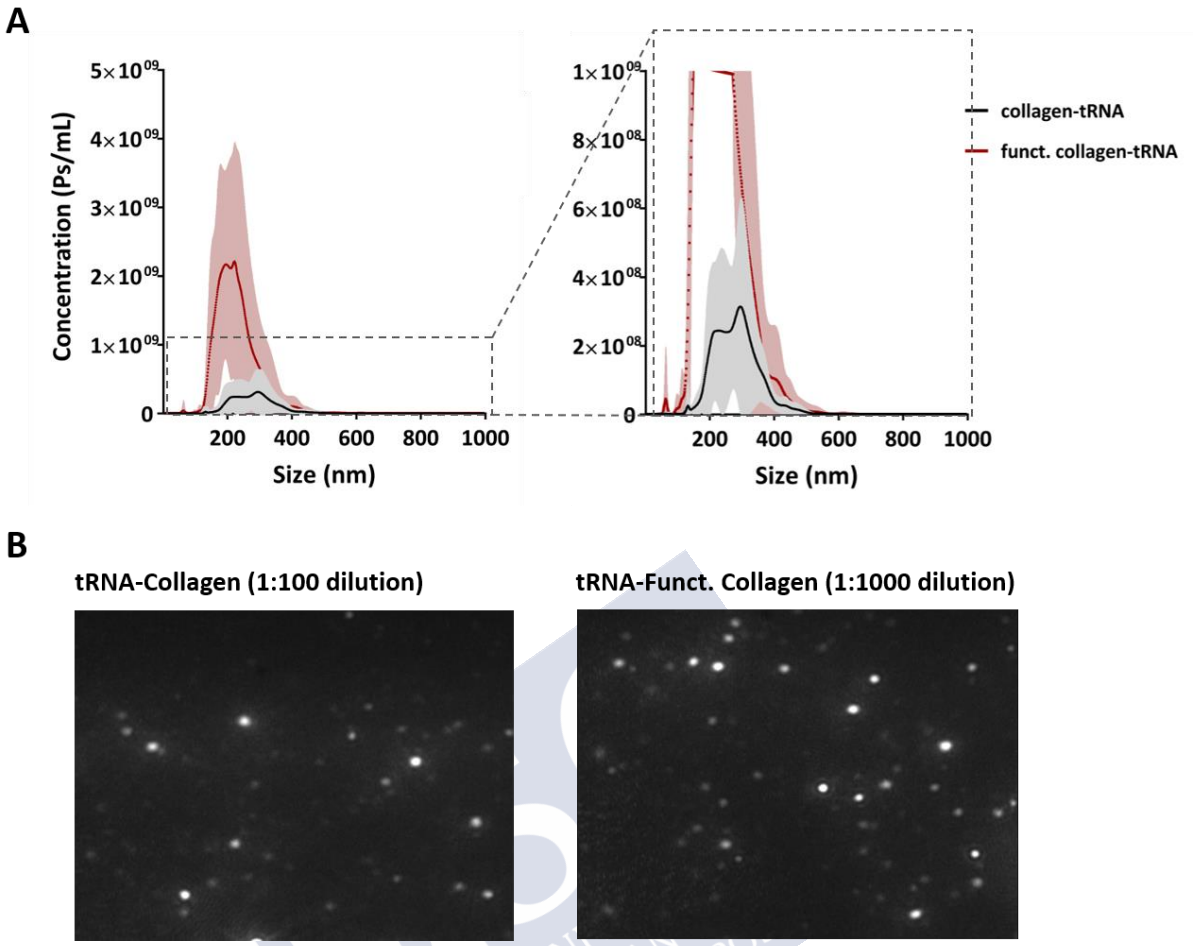

Fig. 9 - NTA analysis of tRNA-functionalized collagen (**A**) and tRNA-collagen (**B**) complexes in water (weight ratio 1:500). Particle size distribution and concentration (Ps/mL). NTA video frame images of Functionalized and nonmodified collagen tRNA complexes at different dilutions (1:1000 and 1:100 v/v, respectively). Numerical values represent the mean ± SD (n=3, five measurements per sample).

3.5. Morphological assessment

As can be observed in Fig. 10A, functionalized collagen (without tRNA) presented fibrous structures typical of collagen but also formed round particles of about 100 to 400 nm. When tRNA was associated to functionalized collagen, as expected there were fibrous structures but also round particles with sizes between 150 and 250 nm. Nonmodified collagen aside from the native fibrous structures also presented round particles, but in this case, they presented a bigger size of about 250 to 350 nm, which is in agreement with NTA results. Functionalized collagen-tRNA complexes were also diluted in water (1:100, v/v) in order to have the same

conditions as in NTA analysis (Fig. 10B), and presented a particle size of about 200 nm, which is in agreement with the previous results.

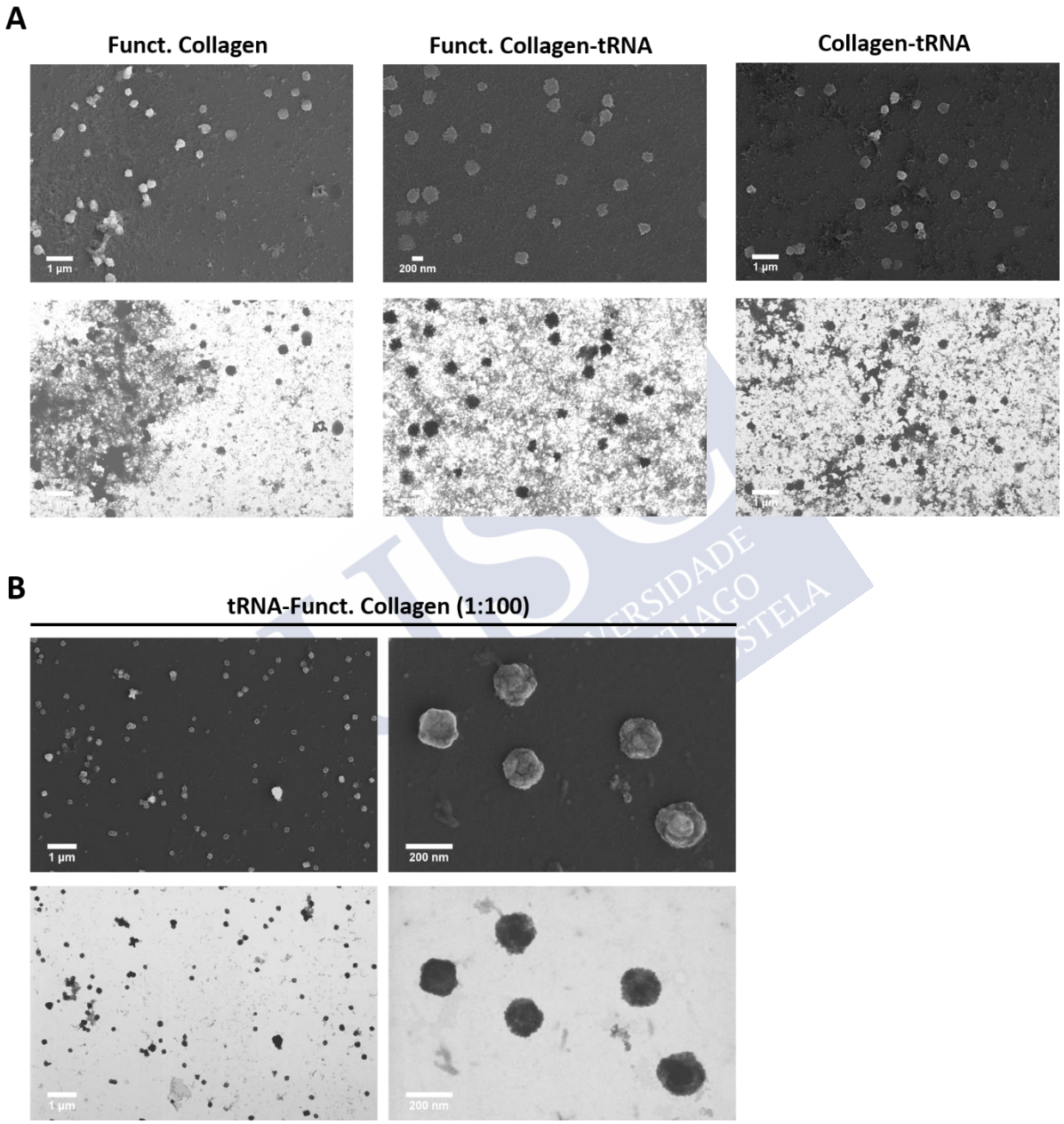

Fig. 10 - FESEM micrographs of functionalized collagen and nonmodified (collagen). Functionalized collagen dissolved in H_2O at 4 mg/mL and tRNA complexes of functionalized collagen and collagen (**A**). tRNA-functionalized collagen complexes (1:500 w/w) diluted in water at 1:100 (v/v) (**B**). Each micrograph was taken using STEM and InLens detectors, presented at upper and lower panel of each group. Scale bars correspond to 1 µm or 200 nm.

4. Conclusions

HA and collagen were chemically functionalized with quaternary ammonium groups, in order to improve their ability to associate RNA. The reactions were performed under mild conditions, without the use of organic solvents. As determined by ^{1}H NMR a functionalization degree of about 22 % of HA carboxylic groups and 97 % of collagen lysine's primary amine groups, was achieved. Unfortunately, despite the capacity of these materials to form complexes with tRNA, the association efficiency was very low. In the future, different strategies could be used to improve the capacity of these polymers to associate tRNA and form stable nanostructures, such as the increase the degree of functionalization, combine them with other polymers and use natural crosslinkers as TPP.

Acknowledgments

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List of abbreviations

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List of abbreviations

3D, 3 dimensions

5-TAMRA-SE, 5-Carboxy tetramethyl rhodamine succinimidyl ester

AAVs, adeno-associated virus

Adv, adenovirus

AIDS, acquired immune deficiency syndrome

AMD, age-related macular degeneration

APACG, Primary angle closure glaucoma

ARVO, Association for Research in Vision and Ophthalmology

AS-ODNs, antisense oligonucleotides

BAK, benzalkonium chloride

Bz Cl, benzethonium chloride

CCM, cell culture medium

cDNA, complementary DNA

CMV, citomegalovirus

CNV, choroidal neovascularization

CQAs, critical quality atributes

CTGF, connective tissue growth factor

CTGF, connective tissue growth factor

DC-cholesterol, 3-β[N-(N',N' imethylaminoethane)- carbamoyl] cholesterol

DES, dry eye syndrome

DHF3, DharmaFECT™ 3

DLS, dynamic light scattering

DMD, Duchene muscular dystrophy

DOPE, 1,2-dioleoyl-3- hosphatidylethanolamine

DOTAP, 1,2-dioleoyl-3- trimethylammonium propane

DOTMA, N-[1-(2,3-dioleyloxy)propyl-N,N,N-trimethylammonium chloride

DPBS, Dulbecco's Phosphate-Buffered Saline

DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

dsRNA, double-stranded RNA

EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

EMA, European medicines agency

EMEM, Eagle's Minimum Essential Medium

FBS, fetal bovine serum

FD, freeze-dried

FDA, Food and drug administration

FESEM, field emission scanning electron microscopy

FITC, Fluorescein isothiocyanate

GAGs, glycosaminoglycans

GCL, ganglion cell layer

GCL, ganglion cell layer

gDNA, genomic DNA

GMP, Good Manufacturing Practices

GRAS, generally recognized as safe

HA, hyaluronic acid

hATTR, hereditary transthyretin amyloidosis

HIF-1α, hypoxia-inducible factor-1α

HMG1, high-mobility group 1

HSA, human serum albumin

HSK, herpes simplex keratitis

HSV, herpes simplex virus

HVJ-AVE, hemagglutinating virus of Japan - artificial viral envelope

iCRVO, Ischemic central retinal vein occlusion

INL, inner nuclear layer

IOP, intraocular pressure

IPCV, idiopathic polypoidal choroidal vasculopathy

IRS-1, insulin receptor substrate-1

IVT, intravitreal

KCl, potassium chloride

LAE, ethyl lauroyl arginate HCl

LCA, Leber's congenital amaurosis

LDA, laser-doppler anemometry

LPL, lipoprotein lipase

MCT, medium-chain triglycerides

mRNA, messenger RNA

Mw, molecular weight

NAION, Non-arteritic anterior ischemic optic neuropathy

NCs, nanocapsules

NEs, nanoemulsions

NHS, n-hydroxysuccinimide

NIR, near infrered

NMR, Nuclear Magnetic Resonance

NPH, Neutral protamine hagedorn

NPs, nanoparticles

NTA, nanoparticle tracking análisis

OIR, oxygen-induced retinopathy

PAMAM, Poly(amidoamine)

PDGF-B, platelet-derived growth factor subunit B

PDI, polydispersity index

pDMAEMA, poly(2-dimethylaminoethyl methacrylate)

pDNA, plasmid DNA

PEG, polyethylene glicol

PEI, polyethyleneimine

PEO–PPO–PEO, poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide)

PK, pharmacokinetic

PLGA, poly(lactic-co-glycolic acid)

PLL, poly-L-lysine

POD, peptide for ocular delivery

Prot-T, protamine labeled with TAMRA

PS, protamine sulfate

RGD, arginine-glycine-aspartic acid

RISC, RNA-induced silencing complex

RKO, rhodopsin knockout

RPE, retinal pigment epithelium

RS1, retinoschisis gene

RT, room temperatura

RTP801, hypoxia-inducible factor 1-responsive gene

S/MAR, scaffold matrix attachment region

SD, standard deviation

shRNA, small hairpin RNA

siRNA*, therapeutic siRNA

siRNA, small interfering RNA

SLF, simulated lacrimal fluid

SLN, solid lipid nanoparticles

SMA, Spinal muscular atrophy

SOD1, superoxide dismutase gene 1

STEM, scanning transmission electron microscopy

TAT, transactivator of transcription

TEM, transmission electron microscopy

TGF-β2, transforming growth factor β2

TMAG, N-(alpha-trimethylammonioacetyl)-didodecyl-D-glutamate

TNF-α, tumor necrosis factor α

TPGS, D-α-tocopheryl polyethyleneglycol 1000 succinate

TPP, tripolyphosphate

tRNA, transference RNA

TRPV1, transient receptor potential vanilloid 1

UV, ultraviolet

VEGF, vascular endothelial growth factor

VEGF-A, vascular endothelial growth factor A

XLRS, X-linked juvenile retinoschisis

β2 ADR, β2-adrenergic receptor

β-Gal, β-Galactosidase

µg, micrograms

µl, microliters

µm, micrometers

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Ethical considerations

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Cell culture

The cell line (HeLa CCL-2™) used in this thesis (Chapter 1 and Chapter 2) was acquired from the American Tissue Culture Collection (ATCC®). Cells were cultured according to the manufacturers recommendations and only used for the research purposes described in this thesis.

Animal studies

The animal experiments (Chapter 2) were performed in Universidad de Oviedo, authorized by the Consejeria de desarrollo rural y recursos naturales of the Gobierno del principado de Asturias. The experimental protocol was approved by the ethical committee of the Animal Welfare Committee of Universidad de Oviedo and followed the national regulations for transport, housing and care of laboratory animals (RD 53/2013).