UNIVERSIDAD DE SANTIAGO DE COMPOSTELA
Facultad de Farmacia
Departamento de Farmacia y Tecnología Farmacéutica

DESARROLLO DE UN NUEVO TRATAMIENTO PARA EL OJO SECO BASADO EN LA TERAPIA GÉNICA CON SISTEMAS NANOPARTICULARES

Tesis Doctoral

Giovanni Konat Zorzi
Santiago de Compostela, 2011

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DOÑA BEGOÑA SEIJO REY Y DON ALEJANDRO SÁNCHEZ BARREIRO, PROFESORES TITULARES DEL DEPARTAMENTO DE FARMACIA Y TECNOLOGÍA FARMACÉUTICA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA.

INFORMAN:

Que la presente Memoria Experimental titulada: "Desarrollo de un nuevo tratamiento para el *Ojo Seco* basado en la terapia génica con sistemas nanoparticulares", elaborada por el Licenciado en Farmacia **Giovanni Konat Zorzi**, ha sido realizada bajo su dirección en el Departamento de Farmacia y Tecnología Farmacéutica y, hallándose concluida, autorizan su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que conste, expiden y firman el presente certificado en Santiago de Compostela, el 23 de Febrero de 2011.

Fdo. María Begoña Seijo Rey Fdo. Alejandro Sánchez Barrero

A minha família

“Ninguém ignora tudo, ninguém sabe tudo.

Todos nós sabemos alguma coisa.

Todos nós ignoramos alguma coisa.

Por isso, aprendemos sempre."

Paulo Freire

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Giovanni Konat Zorzi

23 de febrero, 2010

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RESUMEN

RESUMEN

El principal objetivo de la presente memoria ha sido el diseño de nuevos sistemas nanoparticulares capaces de proporcionar una aproximación terapéutica totalmente novedosa al tratamiento del síndrome del ojo seco. En una primera etapa se ha evaluado el potencial de proteínas cationizadas como biomateriales constitutivos de nanopartículas desarrolladas mediante gelificación ionotrópica. Después de caracterizar los diferentes sistemas desarrollados, se seleccionaron nanopartículas híbridas elaboradas empleando combinaciones de gelatina cationizada con espermina y el biopolímero aniónico natural sulfato de condroitino, en base a su versatilidad, perfil de toxicidad y potencial de transfección en células del epitelio ocular. Seguidamente, se incorporó a dichas nanopartículas un plásmido que codifica la proteína MUC5AC, mucina secretada por las células caliciformes de la conjuntiva. Esta mucina es una de las principales responsables de la homeostasis del fluido lacrimal, presentándose en niveles anormalmente disminuidos en determinados procesos inflamatorios de la superficie ocular, como el síndrome del ojo seco. El tratamiento tópico en ratones sometidos a un modelo de síndrome de ojo seco con dichos nanosistemas ha permitido restablecer la producción normal de lágrimas y mejorar capacidad de protección de la superficie ocular en los animales enfermos. Estos resultados suponen la prueba de concepto sobre la aplicación clínica de las nanopartículas diseñadas al desarrollo de la terapia génica de diferentes patologías oculares como el síndrome de ojo seco.

ABSTRACT

The main goal of the present work has been the development of new nanoparticulated systems as a new therapy for the dry eye. At first, it has been evaluated the potential of cationized proteins as biomaterials for nanoparticles formulated by ionic gelation technique. After the characterization of the systems, it has been selected hybrid nanoparticles made of gelatin cationized with spermine and the natural polyanions dextran sulfate and chondroitin sulfate. The selection has been based on its versatility, toxicological profile and transfection efficiency in ocular epithelial cells. Finally, a plasmid that codifies the protein MUC5AC was associates to the nanoparticles. This mucin is one of the responsible for tear homeostasis, and presents its levels decreased in some inflammatory process in the eye surface as the dry eye. The treatment of mice submitted to a model of the dry eye, with the nanoparticles has been capable of reestablish the normal production of tears and improve the barrier function of cornea in the animals. So, these results are the proof-of-concept for the clinical application of the nanoparticles as a new gene therapy strategy for ocular diseases as the dry eye.

INTRODUCCIÓN

La Terapia Génica

La búsqueda de nuevos fármacos y herramientas terapéuticas para el tratamiento de enfermedades, constituyen un reto constante en el ámbito farmacéutico. Actualmente, entre las modalidades terapéuticas más prometedoras, la terapia génica ocupa un lugar, sin duda, preponderante. La posibilidad de inducir la expresión de una proteína terapéutica (insertando un gen funcional), o, por el contrario, de suprimir la expresión aberrante de una proteína (inhibiendo la expresión de un gen defectuoso) cuando ésta sea el origen de una determinada enfermedad, abre innumerables posibilidades que revolucionaran la práctica clínica que hoy conocemos, seguramente antes de superar el primer tercio del presente siglo.

No obstante, aunque las expectativas de futuro para la terapia génica se pueden calificar cuando menos de alentadoras, también es cierto que pueden verse fácilmente frenadas si no se encuentra respuestas o soluciones a las dos grandes dificultades que plantea su desarrollo. La primera, está relacionada con el conocimiento a cerca de si la expresión de una determinada proteína está involucrada en la fisiopatología de la enfermedad y en qué grado; lo que, en otras palabras, significa saber, si la alteración de su expresión puede conllevar una mejora del estado clínico. Tal información, puede ser más fácilmente obtenida en el caso de enfermedades causadas por el defecto de un solo gen, pero supone un verdadero reto la estimación de su importancia en las enfermedades multifactoriales, en las que interviene más de un gen. La segunda dificultad se refiere a la búsqueda de un sistema que sea capaz, al mismo tiempo, de proteger el material genético y transportarlo hasta su lugar de acción, de manera eficaz y sin representar riesgos para la salud (seguro). En este sentido, diferentes vehículos están siendo estudiados como herramientas que permiten la transferencia del material genético exógeno a la célula. Fundamentalmente, se pueden diferenciar dos tipos de

sistemas transportadores: vectores virales (adenovirus, lentivirus, etc) y vectores no virales o sintéticos (complejos, micelas, liposomas, nanopartículas, etc).

En lo que respecta a la manipulación genética propiamente dicha, es posible utilizar la terapia génica como estrategia terapéutica de dos maneras: insertando un gen mediante un plásmido para inducir la expresión de una proteína o, a través de la inhibición de la expresión de una proteína, utilizando moléculas que degradan el ARN mensajero (mRNA) y, en consecuencia, impiden la expresión de la proteína codificada; tales como oligonucleótidos antisentido, Ribozimas/ADNzimas, aptámeros o ARN interferente pequeño.
A continuación se describen brevemente las estrategias más interesantes utilizadas en terapia génica, junto con sus principales características.

- Plásmidos: son unidades circulares de ADN extracromosómico de doble hebra, de elevado peso molecular (entre 5000 y 10000 pares de bases), que son capaces de replicarse de manera autónoma y que, en su estructura, llevan por lo menos la secuencia de un gen que codifica la expresión de una proteína de interés. La terapia génica *con plásmido* presenta una gran ventaja frente a otras formas de tratamiento ya que puede corregir directamente la fuente de una enfermedad, sin limitarse simplemente a suprimir los síntomas.

- Oligonucleótidos antisentido (AS-ODNs): son moléculas sintéticas de hebra simple que establecen un enlace específico con determinadas secuencias intracelulares del mRNA. Están compuestos por secuencias cortas, en general de 13 a 25 nucleótidos, las cuales son complementarias de la secuencia de mRNA en una región determinada de la hebra sentido positivo. Al establecer un enlace con

una molécula de mRNA, los AS-ODNs son capaces de interrumpir la traducción de dicho mRNA y, en consecuencia, la síntesis de la proteína codificada por el gen[1]. El AS-ODN formivirsen sódico (Vitravene®, Isis Pharmaceutical) fue la primera terapia génica aprobada por la agencia regulatoria de medicamentos estadounidense (Food and Drug Administration – FDA) para el tratamiento de retinitis producida por citomegalovirus en enfermos con SIDA.

- ARN interferente pequeño (siRNA): son pequeñas secuencias de ARN de doble hebra (alrededor de 21-23 nucleótidos) que sirven como mecanismos reguladores de la expresión génica en la mayoría de las células eucariotas, disminuyendo la translación de proteínas por inducir específicamente a la degradación de su mRNA[2,3]. Tienen como característica la presencia de dos nucleótidos 3´ salientes en las extremidades que pueden ser reconocidos por el *RNA-induced silencing complex (RISC)*, lo que conlleva a una degradación del mRNA homologo. Al igual que los AS-ODNs, los siRNA han sido utilizados para disminuir la expresión de determinada proteína, proceso que ha sido denominado *knocking-down o silenciamiento*. Además, son más estables debido a su naturaleza doble hebra[4], se obtienen por síntesis química y, desde el punto de vista regulatorio, tienen la ventaja de que deben ser encuadrados en la categoría de fármaco (*drug*), entendiéndose como tales, estructuras químicas puras fácilmente analizables tras su síntesis.

[1] Fattal E, Bochot A. Ocular delivery of nucleic acids: antisense oligonucleotides, aptamers and siRNA. *Advanced Drug Delivery Reviews* 2006; **58:** 1203–1223.
[2] Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. *Nature Reviews - Drug Discovery* 2009; **8:** 129-138.
[3] Elbashir SM *et al.* Duplexes of 21±nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411:** 494-498.
[4] Aagaard L, Rossi JJ. RNAi therapeutics: Principles, prospects and challenges. *Advanced Drug Delivery Reviews* 2007; **59:** 75-86.

- Aptámeros: son moléculas cortas de ARN o ADN de hebra simple con ligandos, que son capaces de producir enlaces de alta afinidad con sus estructuras diana, las cuales pueden ser desde pequeñas moléculas hasta proteínas de membrana o ácidos nucleares[5]. Son estructuras relativamente nuevas, descubiertas en el inicio de la década de 90, que han sido obtenidas de librerías de oligonucleótidos por selección *in vitro*. Recientemente fue aprobado el empleo del primer aptámero para el tratamiento intravítreo de la degeneración macular, el pegabtanib (Macugen®, Pfizer), si bien existen muchos otros productos para el diagnóstico que utilizan aptámeros para sus fines[6].

- Ribozimas: son moléculas de ARN capaces de cortar específicamente secuencias de mRNA. Más concretamente, interaccionan con el mRNA formando una estructura de doble hebra, la cual es fácilmente hidrolizada debido a su conformación. La ribozimas, se utilizan sobre todo para supresión genética en la apoptosis y procesos proliferativos. La presencia de una secuencia de ARN en su estructura, las hace especialmente sensibles a la acción de ARNsas[7].

- ADNzimas: son análogas a las ribozimas pero su estructura de ARN es substituida por ADN, algo que les confiere una mayor estabilidad. Su gran actividad catalítica ha permitido su utilización como

[5] Cerchia L, De Franciscis V. Targeting cancer cells with nucleic acid aptamers. *Trends in Biotechnology* 2010; **In press:** 1-9
[6] Bunka DHJ, Platonova O, Stockley PG. Development of aptamer therapeutics. *Current Opinion in Pharmacology* 2010; **In press:** 1-6
[7] Stull RA, Szoka FCJ. Antigene, ribozyme and aptamer nucleic acid drugs: progress and prospects. *Pharmaceutical Research* 1995; **12:** 465-483.

herramientas en biología molecular, concretamente en diferentes protocolos de validación[8].

El ADN plasmídico como fármaco

Tal y como se acaba de indicar, los plásmidos son moléculas de ADN extracromosómico circular o lineal que se replican y transcriben de forma independiente del ADN cromosómico. Están presentes normalmente en bacterias, donde generalmente codifican los genes de resistencia a antibióticos. Su tamaño varía desde 1 a 250 Kbp y pueden contener desde unos pocos hasta un centenar de genes diferentes[9].

Es justamente la capacidad que tiene de operar independientemente del ADN cromosómico, lo que hace al plásmido muy atractivo en el campo de la terapia génica. Sin embargo, la correcta expresión de genes recombinantes en células de mamíferos requiere la presencia, en la estructura del plásmido, de secuencias específicas relacionadas con su producción y funcionamiento, tal y como se puede observar en el esquema de la Figura 1. En la estructura del plásmido, el gen de interés debe de estar posicionado correctamente frente a los elementos de control, como son el promotor de la transcripción, la secuencia de poliadenilación y la secuencia potencializadora[10] . Para conseguir una expresión más duradera, el plásmido se ha de replicar para aumentar el número de copias en cada célula o para mantener las copias en las células hijas, razón por la cual se incluyen secuencias que promueven su replicación, conocidas como origen de replicación. La inclusión de un gen de resistencia a antibióticos es una

[8] Silverman SK. Deoxyribozymes: DNA catalysts for bioorganic chemistry. *Organic & Biomolecular Chemistry* 2004; **2**.
[9] Devlin TM. Bioquímica. *Editora Reverté, Barcelona* 2006**:** 311.
[10] Fitzsimons HL, Bland RJ, During MJ. Promoters and regulatory elements that improve adeno-associated virus transgene expression in the brain. *Methods* 2002; **28:** 227-236.

estrategia habitual para facilitar la producción de plásmido, seleccionando las cepas que lo expresan. Finalmente, para que un plásmido pueda ser realmente útil debe estar altamente purificado, libre de nucleasas y contaminantes, lo que garantizará su estabilidad durante largos periodos de tiempo[11].

Se puede construir un plásmido insertando los elementos anteriormente descritos y que sean reconocidos por una amplia variedad de células, para así incrementar las posibilidades de éxito de cara a lograr su correcta expresión. Por consiguiente muchas veces se utilizan elementos de control derivados de virus con una amplia gama de huéspedes, porque ya poseen una habilidad natural para transfectar diferentes tipos celulares[12].

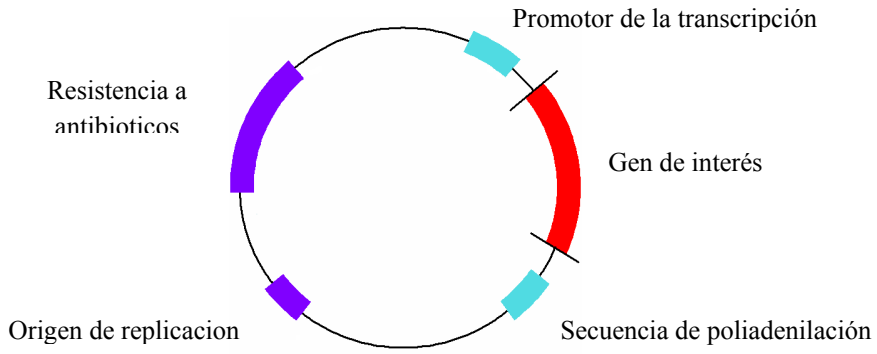

Figura 1. Representación de la estructura genérica de un plásmido

La terapia génica basada en el empleo de plásmidos presenta algunas ventajas distintas de las que se asocian a una terapia de reposición proteica. Las proteínas son muy susceptibles a la perdida de la actividad biológica como consecuencia de pequeños cambios en su estructura, tanto la cuaternaria como la terciaria. Bajo condiciones muy suaves, se puede

[11] Patil SD, Rhodes DG, Burgess DJ. DNA-based Therapeutics and DNA Delivery Systems: A Comprehensive Review. *The AAPS Journal* 2005; **7:** E61-E77.
[12] Walther W, Stein U. Cell type specific and inducible promoters for vectors in gene therapy as an approach for cell targeting. 1996; 74**:** 379-392.

producir una desnaturalización irreversible en las proteínas, mientras que, en el caso del ADN plasmídico, en estas mismas condiciones se puede llegar a restablecer su funcionalidad. Para que se produzca la perdida de actividad biológica del plásmido, generalmente se tiene que producir una modificación química en su estructura mientras que en el caso de una proteína pequeños cambios en su entorno ya son suficientes para la perdida de funcionalidad[13].

Además, una terapia génica con plásmido presenta como gran ventaja frente a otras formas de tratamiento, la posibilidad de corregir directamente la fuente de la enfermedad, sin restringirse únicamente a suprimir los síntomas. Hasta el momento, solamente un plásmido ha sido aprobado por una agencia regulatoria y el país en el que se ha producido ha sido China. Se trata de un plásmido que codifica la proteína p53 y que está indicado para el tratamiento de carcinomas en el cuello y en la cabeza (Gendicine®, SiBiono Genetech). Así mismo, actualmente se están llevando a cabo un amplio número de ensayos clínicos en el ámbito de la terapia génica empleando un plásmido como fármaco. Los diferentes estudios se recogen en la Tabla 1, que ha sido elaborada a partir de datos obtenidos de los Institutos Nacionales de Salud de los E.E.U.U[14].

Como se puede observar, la gran mayoría de los ensayos clínicos que se desarrollan en este momento están destinados al tratamiento o prevención del cáncer. Además, las instituciones públicas concentran sus esfuerzos también en la vacunación genética, en especial frente el HIV. Desafortunadamente, con excepción del plásmido de Sanofi-Aventis que codifica el factor de crecimiento de fibroblastos 1 (FGF-1) y que se encuentra en Fase Clínica III, casi todos los ensayos están todavía en etapas muy

[13] Middaugh CR, Evans RK, Montgomery DL, Casimiro DR. Analysis of Plasmid DNA from a Pharmaceutical Perspective. *Journal of Pharmaceutical Sciences* 1998; **87:** 130-146.
[14] www.clinicaltrial.gov en 07 de septiembre de 2010

preliminares de evaluación, siendo imposible predecir el futuro que van a tener tales estrategias.

Un factor que sin duda puede contribuir al fracaso de varios tratamientos utilizando plásmidos, es la falta de vehículos adecuados que, al mismo tiempo, sean capaces de proteger el ADN plasmídico y potenciar su expresión en las células.

Tabla 1. Ensayos clínicos activos que emplean ADN plasmídico como fármaco en terapia no-viral. Fuente: *U.S. National Institutes of Health*

Compañía	**Enfermedad**	**Proteína**	**Etapa**
St. Jude Children's Research Hospital	HIV-1	Env	Fase I
NIAID	HIV-1	-	Fase I
	HIV-1	-	Fase I
	HIV-1	Env (A, B y C)	Fase I
	HIV-1	Gag, Pol, Env	Fase I
	HIV-1		Fase II
	Plasmodium Falciparum	Antígeno	Fase I
	H5N1	H5	Fase I
	virus Ebola y Marburg	-	Fase I
The National Centre in HIV Epidemiology	HIV-1	Gag, Env, Pol, Tat/Rev	Fase I
Inovio Pharmaceuticals	H5N1	-	Fase I
VGX International, Inc.	H5N1	H5, NA y Ag M2e-NP	Fase I
	HIV-1	Gag, Pol, Env	FaseI
	Papiloma virus	Proteínas E6 y E7	Fase I
Genexine Co., Ltd.	Hepatitis B	Env (S, L), core protein, Polimerasa y IL-12	Fase I
Memorial Sloan-Kettering Cancer Center	Cancer Riñon	Antigeno prostático específico (PSA)	Fase I

	Cancer de mama	HER-2/Neu	Fase I
	Melanoma	GM-CSF	Fase I/II
	Melanoma (piel y intraocular)	gp100	Fase I
	Melanoma piel	gp100	Fase I
	Linfoma	CD20	Fase I
BioAlliance Pharma SA	Melanoma metastático	AMEP	Fase I
Scancell Ltd	Melanoma metastático	Modify antibody	Fase I
Vical	Melanoma metastático	IL-2	FaseI
Sidney Kimmel Comprehensive Cancer Center	Cancer de Pancreas	GM-CSF	Fase II
	Cancer de Pancreas	GM-CSF	Fase II
Ichor Medical Systems Incorporated	Melanoma (piel y intraocular)	Tirosinasa	Fase I
Penn State University	Leucemia	-	Fase I
Universidad de Ciencias Médicas de Teherán	Leucemia mieloide crónica	IL-12 y GM-CSF	Fase I
Karolinska University Hospital	Colorectal Cancer	Antigeno ACE	Fase I/II
National Institue of Cancer- Gynecologic Oncology Group	Cancer Ovario, peritoneo y trompas de Falopio	IL-12	Fase II
Jonsson Comprehensive Cancer Center	Cáncer Próstata	IL-2	Fase II
	Cáncer Próstata	IL-2	Fase II
ViroMed Co., Ltd.	Isquemia Limbal	HGH	Fase II

Sanofi-Aventis	Enfermedades vasculares periféricas	FGF-1	Fase III
BioCancell Therapeutics Ltd.	Neoplasma pancreático	Diphtheria toxin A	Fase I/II
	Cancer ovario	Diphtheria toxin A	Fase I/II
	Cáncer de vejiga superficial	Diphtheria toxin A	Fase II
National Heart, Lung, and Blood Institute	Claudicación Intermitente	VEGF-A	Fase I
Corautus Genetics	Angina Pectoris	VEGF2	Fase II
Gradalis, Inc.	Tumores sólidos	Furina y GM-CSF	Fase I
Mary Crowley Medical Research Center	Carcinoma metastático avanzado	TGFβ2 antisense y GM-CSF	Fase I
Imperial College London	Fibrosis Cística	GM169 y GL67A	Fase I/II

Las Nanopartículas poliméricas y la terapia génica

A lo largo de las dos últimas décadas, se han investigado muchos sistemas y estratégias para la vehiculización de material genético, en especial para el ADN plasmídico, tal y como se ha comentado anteriormente. Entre estos sistemas, cabe destacar el empleo de nanopartículas poliméricas para que actúen como transportadoras del mismo a los más diversos tejidos y órganos del cuerpo[15,16]. Las nanopartículas son vehículos capaces de incrementar la estabilidad de los plásmidos, de controlar su liberación, proporcionándoles además la posibilidad de escape lisosomal, así como también de incrementar su capacidad de expresión[17].

Las nanopartículas son estructuras que presentan un tamaño comprendido entre 10 y 1000 nm y que, en función del uso al que estén destinadas, pueden o no contener una molécula activa asociada. Diferentes terminologías han sido utilizadas para referirse a las nanopartículas, dependiendo de la estructura que presenten. Así, se consideran *nanocápsulas* estructuras que presentan un núcleo oleoso y una cubierta polimérica que lo rodea (sistema reservorio), mientras que las *nanoesferas* son estructuras de tipo matricial. No obstante, no es infrecuente encontrar una sustitución del termino *nanoesferas* por el nombre genérico de *nanopartículas*.

Para la preparación de estos sistemas nanoparticulados se emplean una serie de técnicas distintas, cada una con sus ventajas e inconveniente, entre las que cabe citar: precipitación/coacervación, emulsificación/evaporación de disolvente, gelificación ionotrópica,

[15] Hattori Y. Development of non-viral vector for cancer gene therapy. *Journal of the Pharmaceutical Society of Japan* 2010; **130:** 917-923.
[16] Xing J *et al.* Polycationic nanoparticles as nonviral vectors employed for gene therapy in vivo. *Mini Reviews in Medical Chemistry* 2010; **10:** 126-137.
[17] Sahoo SK, Parveen S, Panda JJ. The present and future of nanotechnology in human health care. *Nanomedicine: Nanotechnology, Biology, and Medicine* 2007; **3:** 20– 31.

emulsificación por difusión de disolvente y *cuasi* emulsión por difusión de disolvente[18]. Entre ellas, quizá sobresale la gelificación ionotrópica por su simplicidad, las condiciones suaves en las que se desarrolla y ausencia de disolventes orgánicos; que hacen de este procedimiento el más adecuado si se quieren asociar macromoléculas hidrofílicas, como es el caso del ADN plasmídico[19].

La gelificación ionotrópica consiste en el establecimiento de interacciones electrostáticas entre uno o más polímeros y un agente reticulante de carga opuesta. Tal interacción induce a una transición *sol-gel* de los polímeros, que tiene como resultado la formación de las nanopartículas[20]. Es importante resaltar que por tratarse que interacciones puramente electrostáticas, tal técnica evita el empleo de reticulantes covalentes para estabilizar el sistemas. Estos agentes reticulantes, como es el caso del glutaraldehído, son extremadamente tóxicos; conduciendo a alteraciones estruturales permanentes en organismos muy diversos. Los agentes reticulantes empleados en la gelificación ionotrópica, en general poseen bajo peso molecular y alta densidad electrónica, siendo el citrato de sodio, sulfato de sodio y tripolifosfato de sodio los más comúnmente empleados.

En los últimos años, muchos polímeros de origen sintético o natural han sido evaluados para la formación de nanopartículas por gelificación ionotrópica. Obviamente, algunos requisitos básicos para su utilización en humanos o animales, como son la biocompatibilidad y biodegradabilidad o

[18] Nagarwal RC *et al.* Polymeric nanoparticulate system: a potential approach for ocular drug delivery. *Journal of Controlled Release* 2009; **136:** 2 -13.
[19] Alonso MJ, Sanchez A. The potential of chitosan in ocular drug delivery. *Journal of Pharmacy and Pharmacology* 2003; **55:** 1451-1463.
[20] López-León T *et al.* Physicochemical characterization of chitosan nanoparticles: electrokinetic and stabilty behavior. *Journal of Colloid and Interface Science* 2005; **283:** 344-351.

que se trate de moléculas no tóxicas, deben ser respetados. Por todo ello, solo un número muy limitado de polímeros pueden ser empleados para la formación de las nanopartículas[21].

Las proteínas cationizadas como biomateriales

Entre los polímeros que han sido propuestos para la terapia génica, las proteínas cationizadas ocupan un lugar destacado. El proceso de cationización permite dotar de carga positiva a estas proteínas que de forma natural no la poseen. De este modo, es posible el establecimiento de interacciones electrostáticas con el material genético, así como también con la superficie de las células que presentan carga negativa.

Para la cationización de proteínas, se recurre principalmente a reacciones mediadas por carbodiimidas. Entre las carbodiimidas que más se utilizan, destaca la etil-3(3-diaminopropil) carbodiimida (EDC). Tal y como se puede observar en la Figura 2, se produce una reacción con formación de una amida entre la proteína y la amina de interés. Inicialmente, la carbodiimida N-substituida reacciona con los grupos carbolíxicos de la proteína para formar un intermediario altamente reactivo (O-acilisourea; paso 1). El ataque nucleofílico de la amina primaria utilizada en la cationización tiene como resultado la formación de un enlace del tipo amida, con la liberación de la carbodiimida como un derivado de isourea (paso 2).

Para la cationización generalmente se utilizan aminas de bajo peso molecular para logar el aporte de la carga positiva. Entre las más utilizadas para este propósito se pueden citar las aminas etilendiamina y colamina. En

[21] Fischer D *et al.* In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* 2003; **24:** 1121–1131.

este proceso de cationización, es importante tener en cuenta que el número de grupos amino en la molécula es un factor determinante en lo que respecta al perfil de toxicidad y a la capacidad de transfección del polímero.

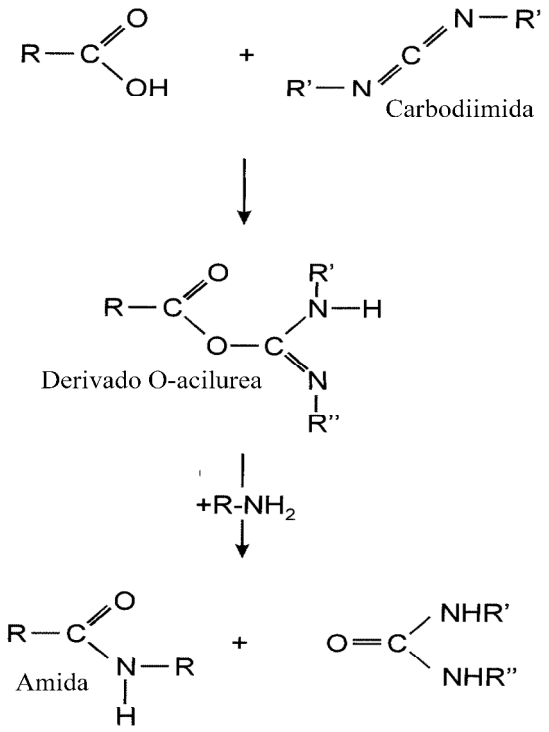

Figura 2. Reacción típica del tipo carbodiimida para la cationización de proteínas por la modificación de los grupos carboxilatos.

Entre las proteínas cationizadas que han sido estudiadas como posibles biomateriales de interés en terapia génica, destaca la gelatina cationizada. En la Tabla 2, se recogen diferentes tipos de estrategias que han sido propuestas teniendo la gelatina cationizada como principal constituyente del sistema. La versatilidad de este material queda demostrada por el hecho de que pueda utilizarse en sistemas tan diferentes (hidrogeles, complejos, micropartículas y nanopartículas), pero capaces de ser explorados como vehículos para material genético.

Tabla 2. Utilización de la gelatina cationizada en la terapia génica.

Sistema	Amina	Tipo terapia	Observaciones	Ref.
Hidrogel	ED PTC SPD SPM	pDNA	Reticulación con glutaraldehído. Implantes musculares de los hidrogeles resultan en expresión más duradera. Señales de inflamación en los implantes (excepto para gelatina cationizada con ED).	[22,23,24]
Cubierta	ED	pDNA	Mejor transfección que el sulfato de protamina como agente de recubrimiento.	[25]
Complejo	ED	pDNA	Mejor transfección y perfil de toxicidad que complejos de PEI. Ultrasonido mejora transfección *in vitro* e *in vivo*. Eficazes en músculos, neuronas y riñones.	[26,27,28,29,30]

[22] Kushibiki T *et al.* Controlled release of plasmid DNA from hydrogels prepared from gelatin cationized by different amine compounds. *Journal of Controlled Release* 2006; **112:** 249-256.
[23] Kushibiki T *et al.* In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. *Journal of Controlled Release* 2003; **90:** 207–216.
[24] Fukunaka Y *et al.* Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. *Journal of Controlled Release* 2002; **80:** 333-343.
[25] Mima H *et al.* Biocompatible polymer enhances the in vitro and in vivo transfection efficiency of HVJ envelope vector. *Journal of Gene Medicine* 2005; **7:** 888–897.
[26] Aoyama T *et al.* Enhanced expression of plasmid DNA–cationized gelatin complex by ultrasound in murine muscle. *Journal of Controlled Release* 2002; **80:** 345–356.
[27] Hosseinkhani H, Aoyama T, Ogawa O, Tabata Y. Ultrasound Enhancement of In Vitro Transfection of Plasmid DNA by a Cationized Gelatin. *Journal of Drug Targeting* 2002; **10:** 197-204.
[28] Thakor D, Spigelman I, Tabata Y, Nishimura I. Subcutaneous Peripheral Injection of Cationized Gelatin/DNA Polyplexes As a Platform for Non-viral Gene Transfer to Sensory Neurons. *Molecular Therapy* 2007; **15:** 2124-2131.
[29] Kushibiki T *et al.* Enhanced anti-fibrotic activity of plasmid DNA expressing small interference RNA for TGF-beta type II receptor for a mouse model of obstructive nephropathy by cationized gelatin prepared from different amine compounds. *Journal of Controlled Release* 2006; **110:** 610-617.
[30] Kushibiki T *et al.* In vitro transfection of plasmid DNA by cationized gelatin prepared from different amine compounds. *Journal of Biomaterials Science-Polymer Edition* 2006; **17:** 645-658.

NP	CA	ON	Incremento en la estimulación del sistema inmune. Activación de grupos celulares específicos.	[31,32]
		pDNA	Menor toxicidad que complejos de PEI. Método reproducible que favorece el escalonamiento.	[33]
		siRNA	Baja estabilidad (influenciada por pH y fuerza iónica). Posibilidad de liofilización para incrementar la estabilidad.	[34,35]
MP	ED	pDNA	Empleo de disolventes orgánicos y glutaraldehído. Eficientes en la transfeción in vivo para tratamiento de diferentes modelos de cáncer y falencia cardíaca crónica.	[36,37,38,39,40]
		siRNA	Mejora clínica en ratones en modelo de alopecia	[41]

ED, etilendiamina; PTC, putrescina; SPD, espermidina; SPM, espermina; CA, colamina; PEI, polietilenimina; NP, nanopartículas, MP, micropartículas.

[31] Bourquin C *et al.* Targeting CpG Oligonucleotides to the Lymph Node by Nanoparticles Elicits Efficient Antitumoral Immunity. *Journal of Immunology* 2008; **181:** 2990-2998.
[32] Zwiorek K *et al.* Delivery by Cationic Gelatin Nanoparticles Strongly Increases the Immunostimulatory Effects of CpG Oligonucleotides. *Pharmaceutical Research* 2008; **25:** 551-562.
[33] Zwiorek K, Kloeckner J, Wagner E, Coester C. Nanoparticles as a new and simple delivery system. *Journal of Pharmacy and Pharmaceutical Science* 2004; **7:** 22-28.
[34] Zillies J, Coester C. Evaluating gelatin based nanoparticles as a carrier system for double stranded oligonucleotides. *J Pharm Pharm Sci* 2005; **7:** 17-21.
[35] Zillies JC *et al.* Formulation development of freeze-dried oligonucleotide-loaded gelatin nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics* 2008; **70:** 514-521.
[36] Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of the Progress of Disseminated Pancreatic Cancer Cells by NK4 Plasmid DNA Released from Cationized Gelatin Microspheres. 2004; **21:** 1109-1118.
[37] Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin. *Gene Therapy* 2004; **11:** 1205–1214.
[38] Kasper FK *et al.* Characterization of DNA release from composites of oligo(poly(ethylene glycol) fumarate) and cationized gelatin microspheres in vitro. *Journal of Biomedical Materials Research* 2006; **78:** 823-835.
[39] Kasper FK *et al.* In vivo release of plasmid DNA from composites of oligo(poly(ethylene glycol)fumarate) and cationized gelatin microspheres. *Journal of Controlled Release* 2005; **107:** 547-561.
[40] Thellin O, ElMoualij B, Heinen E, Zorzi W. A decade of improvements in quantification of gene expression and internal standard selection. *Biotechnology Advances* 2009; **27:** 323-333.
[41] Nakamura H *et al.* RNA interference targeting transforming growth factor-beta type II receptor suppresses ocular inflammation and fibrosis. *Molecular Vision* 2004; **10:** 703-711.

Tal y como se refleja en los comentarios incluidos en la Tabla 2, excepto en el caso de los complejos, el empleo de glutaraldehído para logar la estabilización de las estructuras basadas en gelatina es recurrente. Como se ha comentado anteriormente, esta molécula es extremadamente tóxica, siendo capaz además de inducir alteraciones estructurales permanentes en organismo, por lo que es difícil la aprobación, por las agencias regulatorias, de formulaciones en cuya elaboración se incluyese tal molécula[42,43].

Los hidrogeles de gelatina son capaces de transfectar eficazmente tejidos después de ser implantados en músculos de ratones [22,23]. Diferentes aminas de bajo peso molecular han sido empleadas para cationizar la gelatina, como la espermina, putrescina, espermidina y etiléndiamina. La toxicidad de los implantes, evaluada a través de la liberación de IL-6 es dependiente del número de grupos aminos presentes en la molécula utilizada para cationizar la gelatina[22]. Así, los hidrogeles formados a partir de gelatina cationizada con etilendiamina fueron los únicos que no indujeron inflamación, cuando se comparan con hidrogeles elaborados con gelatinas cationizadas con putrescina, espermidina o espermina.

Las únicas nanoestructuras descritas con gelatina cationizada, son partículas únicamente de gelatina en las que apenas se ha cationizado su superficie. Para ello, previamente las nanopartículas de gelatina han sido obtenidas por desolvatación con posterior reticulación empleando glutaraldehído. A continuación, se ha cationizado la superficie de esas nanopartículas utilizando la amina de bajo peso molecular colamina. La posible presencia residual de glutaraldehído y/o de la carbodiimina utilizada

[42] Zeiger E, Gollapudi B, Spencer P. Genetic toxicity and carcinogenicity studies of glutaraldehyde--a review. *Mutation Research/Reviews in Mutation Research* 2005; **589:** 136-151.
[43] Jayakrishnan A, Jameela SR. Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices. *Biomaterials* 1996; **17:** 471-484.

en la cationización, puede ser motivo de preocupación con respecto a un posible efecto tóxico de dichas nanopartículas. Asimismo, otro factor que merece ser destacado es el hecho de que la asociación en estos sistemas de los diferentes tipos de materiales genéticos, como son pDNA, ON o siRNA, necesariamente deberá producirse por interaciones electrostáticas superficiales. Por lo tanto, una vez que este material no se encuentra atrapado en el interior de la estructura de las nanopartículas, los problemas de inestabilidad de la formulación frente a ligeros cambios que se puedan producir en el medio en que se encuentran las partículas, resulta más que evidente.

En lo que se refiere a los complejos, aunque se trate de sistemas que resultan más inestables en comparación con otras estructuras más compactas o robustas como las nanopartículas o hidrogeles, se ha comprobado que los complejos formados a partir de gelatina cationizada con etilendiamina son capaces de proteger el ADN plasmídico y transfectar tejidos, con una eficacia comparable a la de agentes de transfección comercial clásicos como la polietilenimina[28,29,30].

Otra proteína que igualmente ha sido objeto de estudio como agente de transfección es la albúmina cationizada. Complejos obtenidos a partir de albumina cationizada con hexametilenodiamina, han sido utilizados para transfectar fibroblastos *in vitro*[44]. En este caso, a pesar de su baja toxicidad, se han obtenido niveles de transfección muy bajos, incluso inferiores al 1%. La inestabilidad inherente a los complejos, unida a su gran tamaño y a la falta de datos relativos a la estabilidad del plásmido, pueden ser las posibles causas del escaso éxito obtenido en este estudio.

[44] Fischer D *et al.* Cationized human serum albumin as a non-viral vector system for gene delivery? Characterization of complex formation with plasmid DNA and transfection efficiency. *International Journal of Pharmaceutics* 2001; **225:** 97–111.

La MUC5AC como diana para la terapia génica

La MUC5AC es una mucina formadora de gel, secretada por células epiteliales presentes en diferentes órganos y estructuras del organismo, como es el caso del aparato respiratorio, estomago, oídos y ojos[45,46].

Las mucinas son una clase de glucoproteínas O-glucosiladas de alto peso molecular, en las que más de 80% de su peso está compuesto por carbohidratos[47]. La MUC5AC, así como otras mucinas formadoras de gel, es codificada en el cromosoma 11p15 y el tamaño de su gen ha sido reconocido como uno de los más grandes identificados hasta el momento. Como se puede observar en la Figura 3, en su composición destaca la presencia de una serie de secuencias centrales repetidas, ricas en residuos de serina, treonina y prolina, así como dominios amino terminal ricos en cisteína[48]. La glucosilación de la molécula ocurre en sus repeticiones centrales debido a la exposición de los aminoácidos treonina y serina, mientras que la presencia de los dominios ricos en cisteína permite que se establezca la formación de puentes disulfuro entre varias moléculas de MUC5AC (alrededor de 20), lo que conduce a la formación de multímeros[49]. Estudios de microscopia revelaron que esos multímeros tienen una forma lineal, presentándose como

[45] Fujisawa T *et al.* Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. *Journal of Immunology* 2009; **183:** 6236-6243.
[46] McKenzie RW, Jumblatt JE, Jumblatt MM. Quantification of MUC2 and MUC5AC Transcripts in Human Conjunctiva. *Investigative Ophthalmology & Visual Science* 2000; **41:** 704-708.
[47] Argüeso P, Gipson IK. Epithelial Mucins of the Ocular Surface: Structure, Biosynthesis and Function. *Experimental Eye Research* 2001; **73:** 281-289.
[48] Gipson IK. The Ocular Surface: The Challenge to Enable and Protect Vision. *Investigative Ophthalmology & Visual Science* 2007; **48:** 4391-4398.
[49] Gipson IK. Distribution of mucins at the ocular surface. *Experimental Eye Research* 2004; **78:** 379–388.

un largo hilo que fácilmente exceden a los 10 µm de largo, siendo el tamaño medio de una sola molécula de la MUC5CA de 570 nm (~600 KDa)[50].

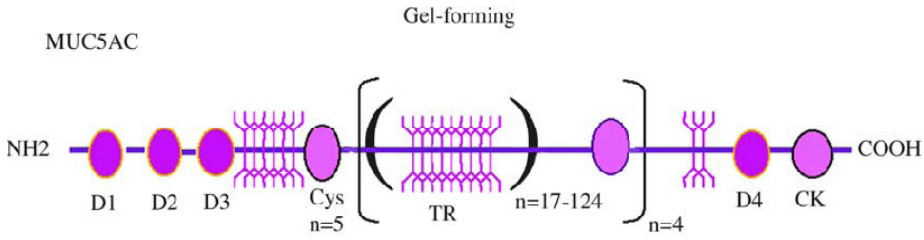

Figura 3. Estructura de la MUC5AC. Presencia de dominios ricos en cisteína (D) en la porción amino terminal que forman los puentes de disulfuro y de las secuencies centrales repetidas (TR) donde ocurre la glucosilación. La función de las cisteínas adicionales (Cys) y del nodo de cisteína (CK) en la porción carboxi-terminal es todavía desconocida.

En el ojo, la MUC5AC es secretada en la conjuntiva por un grupo de células apócrinas especializadas, conocidas como células caliciformes[51] y su producción está básicamente bajo el control del sistema central autónomo[52]. Entre las principales funciones atribuidas a la MUC5AC a nivel ocular están: (i) proporcionar las propiedades reológicas características del moco; (ii) lubricar los epitelios corneal y conjuntival durante el parpadeo; (iii) estabilizar la película lacrimal; (iv) constituir una barrera protectora frente a patógenos (v) prevenir la desecación ocular[53,53].

La disminución de la expresión de la MUC5AC en el ojo está relacionada con una serie de eventos y condiciones tales como exposición a

[50] Sheehan JK *et al.* Physical characterization of the MUC5AC mucin: a highly oligomeric glycoprotein whether isolated from cell culture or in vivo from respiratory mucous secretions. *Biochemical Journal* 2000; **347:** 37-44.
[51] Jumblatt MM, McKenzie RW, Jumblatt JE. MUC5AC Mucin Is a Component of the Human Precorneal Tear Film. *Investigative Ophthalmology & Visual Science* 1999; **40:** 43-49.
[52] Johnson ME, Murphy PJ. Changes in the tear film and ocular surface from dry eye syndrome. *Progress in Retinal and Eye Research* 2004; **23:** 449–474.
[53] Dartt DA. Regulation of mucin and fluid secretion by conjunctival epithelial cells. *Progress in Retinal and Eye Research* 2002; **21:** 555–576.

ambientes adversos[54], deficiencias nutricionales[55] , así como también procesos patológicos[56,57].

Con respecto a las enfermedades en las que la depleción de la MUC5AC ha sido observada y se supone que puede ser un factor preponderante en la patología, se pueden destacar pacientes con alergias inflamatorias como la queratoconjuntivitis atópica, donde la inestabilidad del fluido lacrimal se supone que es, al menos en parte, debida a una significativa alteración en la expresión de MUC5AC así como disminución del número de células caliciformes[58,59]. En este caso, parece que existe una sobreexpresión de otras mucinas, tales como las mucinas de membrana MUC1 y MUC4, como mecanismo de compensación para la protección del epitelio[60].

No obstante, quizá donde la importancia de la MUC5AC resulte más evidente es en la patología conocida como *ojo seco*. El ojo seco es un síndrome caracterizado por la alteración de la unidad funcional lacrimal que conlleva alteraciones relacionadas con el volumen, composición, distribución y/o depuración de la película lacrimal. Dos mecanismos principales, la hiperosmolaridad de la lágrima y su inestabilidad, han sido identificados como los más importantes en la patología. En la Figura 4, se puede observar

[54] Rummenie VT *et al.* Tear cytokine and ocular surface alterations following brief passive cigarette smoke exposure. *Cytokine* 2008; **43:** 200-208.
[55] Tei M, Spurr–Michaud SJ, Tisdale AS, Gipson IK. Vitamin A Deficiency Alters the Expression of Mucin Genes by the Rat Ocular Surface Epithelium. *Investigative Ophthalmology & Visual Science* 2000; **41:** 82-88.
[56] Berry M, Ellingham RB, Corfield AP. Human preocular mucins reflect changes in surface physiology. *British Journal of Ophthalmology* 2004; **88:** 377–383.
[57] Ramamoorthy P, Nichols JJ. Mucins in contact lens wear and dry eye conditions. *Optometry & Vision Science* 2008; **85:** 631-642.
[58] Dogru M *et al.* Alterations of the ocular surface epithelial MUC16 and goblet cell MUC5AC in patients with atopic keratoconjunctivitis. *Allergy* 2008; **63:** 1324-1334.
[59] Kunert KS *et al.* Alteration in Goblet Cell Numbers and Mucin Gene Expression in a Mouse Model of Allergic Conjunctivitis. *Investigative Ophthalmology & Visual Science* 2001; **42:** 2483-2489.
[60] Mantelli F, Argüeso P. Functions of ocular surface mucins in health and disease. *Current Opinion in Allergy and Clinical Immunology* 2008; **8:** 477–483.

las diferencias presentes en la lágrima que recubre el epitelio, en condiciones normales y patológicas[55,61].

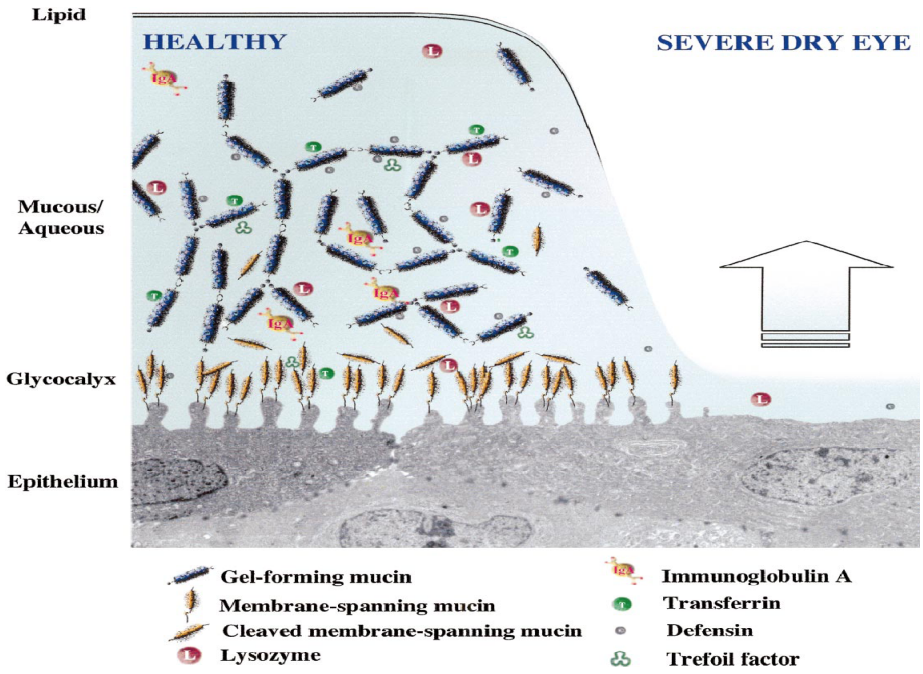

Figura 4. Hipótesis de la arquitectura del fluido lacrimal en epitelio ocular sano y en el ojo seco. En condiciones normales, la MUC5AC contribuye a la homeostasis de la lágrima con la consecuente protección de la superficie ocular. En el ojo seco, la disminución de los niveles de MUC5AC conlleva una desestabilización de la lágrima[47].

Es conocido que la homeostasis del fluido lacrimal responde a un complejo y delicado mecanismo regulatorio el que interviene tanto estimulación hormonal como fenómenos neuroregulatorios[62]. En casos de ojo seco no-inmune, como es el producido por el síndrome de insensibilidad a andrógenos que es una rara enfermedad endócrina en la que existe una producción insuficiente de andrógenos, se produce una disminución de la

[61] The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). *Ocular Surface* 2007; **5:** 65-204

[62] Baudouin, C. The pathology of dry eye. Survey of Ophthalmology, 2001, 45, S211-S220.

expresión de MUC5AC, siendo una de las principales causas de la inestabilidad de la lágrima que se traduce en una evaporación más elevada de la misma[63].

En el caso del síndrome de Sjögren, una enfermedad auto-inmune que conduce al ojo seco, se produce una reducción en la expresión de MUC5AC a nivel ocular y una disminución en el número de las células caliciformes[64]. Tal disminución se supone fundamental para la manifestación de los signos clínicos que se asocian a esta enfermedad. Además, haciendo uso de un anticuerpo especialmente diseñado (el H185) capaz de unirse a glucoproteínas como las mucinas, se ha demostrado que pacientes que presentan ojo seco del tipo no-Sjögren, poseen deficiencia en las modificaciones post-translacionales de la MUC5AC, que pueden dar como resultado la síntesis de una proteína no-efectiva.

A pesar que, como se ha indicado, la MUC5AC tiene una gran importancia en diversas situaciones, casi todas ellas de tipo patológico, hasta el momento ninguna estrategia dirigida a lograr el incremento de su expresión ha sido descrita con éxito. Es conocido que algunas citoquinas pro-inflamatorias, como el TNF-α y la IL-1β, pueden estimular la producción de MUC5AC en el ojo y en las vías aéreas cuando se administran en animales sanos[61,65], siendo ese incremento ocasionado muy probablemente por un simple mecanismo de defensa del epitelio. Sin embargo, en las condiciones patológicas que se producen en el ojo seco, la presencia de dichas citoquinas es concomitante con una disminución de la síntesis de la MUC5AC, y no su

[63] Mantelli F, Moretti C, Micera A, Bonini S. Conjunctival mucin deficiency in complete androgen insensitivity syndrome (CAIS). *Graefe´s Archives for Clinical and Experimental Ophthalmology* 2007; **245:** 899–902.
[64] Argüeso P *et al.* Decreased Levels of the Goblet Cell Mucin MUC5AC in Tears of Patients with Sjögren Syndrome. *Investigative Ophthalmology & Visual Science* 2002; **43:** 1004-1011.
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incremento, como anteriormente se ha descrito. De hecho, el tratamiento más común para el ojo seco es el tratamiento paliativo con lágrimas artificiales, con el que sólo se consigue un alivio sintomático y pasajero de los síntomas, o bien el empleo de inmunosupresores en los casos más graves, que a su vez conducen a importantes efectos colaterales debido a su absorción sistémica[66].

Teniendo eso en cuenta todo ello, el empleo de un plásmido que codifique específicamente la MUC5AC podría conducir a mejoras importantes en procesos en los que la disminución de la expresión de MUC5AC tenga una clara relación con las señales clínicas que presentan. Con la producción específica de esa proteína se pueden evitar una serie de efectos secundarios indeseados recurrentes a la administración de fármacos clásicos, como anteriormente se ha mencionado. Tal estrategia es singular en su concepción hasta el momento ya que, hasta que nosotros conocemos, no se ha planteado ninguna terapia para el tratamiento del ojo seco que de lugar específicamente al incremento de los niveles oculares de esta mucina.

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Artículo I

Nanotechnology and biomaterials: New prospects in ocular therapeutics

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ABSTRACT

Due to the unique anatomy of the eye, drug delivery to its structures has always been problematic. Intensive efforts have been made to improve ocular bioavailability, by lowering the non-specific interactions between drug and tissue, increasing drug stability or increasing the residence time in the case of topical drugs. In this context, nanotechnology has been a very helpful tool for developing suitable drug delivery systems. More specifically, different kinds of strategies in this field have been proposed to overcome the problems related to ocular administration. However, the success of a nanoparticulated system relies on the correct choice of the material which it is made of. This article offers a review of the most important biomaterials employed in nanostructured systems for ocular drug delivery, especially those designated to delivery to ocular surface.

Key words: eye, nanotechnology, nanoparticles, drug delivery, biomaterial.

Contents

1. Introduction

The eye can be divided in two different anatomic regions: anterior and posterior segments. The anterior segment consists of cornea, conjunctiva, aqueous humor, iris, lens and ciliary body, whereas the posterior segment is comprised of vitreous humor, retina, choroid, sclera and optic nerve. The structures that have more clinical relevance are the cornea, conjunctiva and the retina (Figure 1).

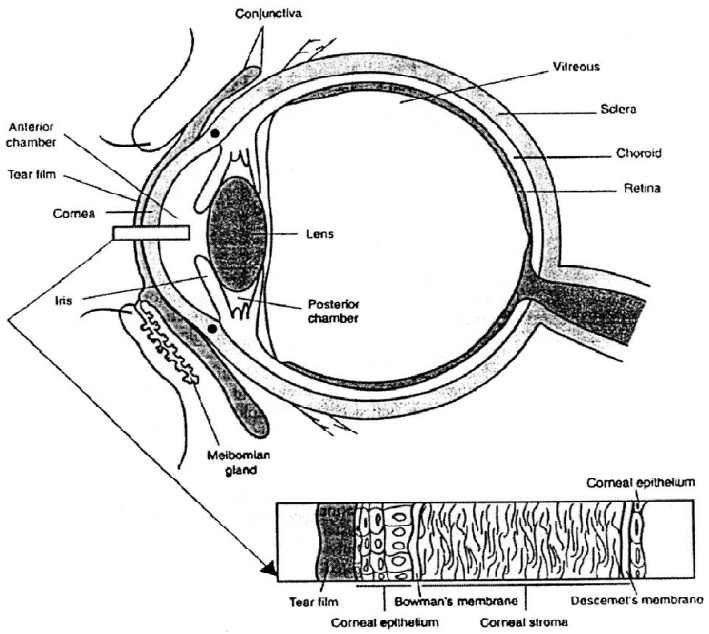

Figure 1. Eye structures.

The cornea is a clear, transparent, avascular tissue to which nutrients and oxygen are supplied by the lachrymal fluid and aqueous humour. It is composed of five layers: epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium. The epithelium is non-keratinized stratified squamous consisting of 5 to 6 layers of cells with remarkable regenerative ability due to the intense mitotic activity of basal cells and limbo stem cells (1, 2). The basal cells are columnar and have a great number of tight

junctions that act as a barrier trough the passage of microorganism and most of the drugs. Furthermore, it has important optic activity for light transmission despite of the properties of refracts and reflects light (3).

The conjunctiva is a thin transparent membrane, which covers the inner surface of the eyelids and is reflected onto the globe. At the corneal margin, it is structurally continuous with the corneal epithelium. The membrane is vascular and moistened by the tear film. The conjunctiva is composed of an epithelium, a highly vascularised substantia propria, and a submucosa or episclera. The structure resembles a palisade and not a pavement when compared to the corneal epithelium. At the surface, epithelial cells are connected by tight junctions, which render the conjunctiva relative impermeability (4). Another important element in conjunctiva is the presence of a group of specialized cells called goblet cells whose main function is the production of trefoil factor, peptides as TFF, and mucins (especially MUC5AC), among others. These molecules work as stabilizers of tear film and help in cornea regeneration (5).

The human retina is a light-sensitive tissue, part of central nervous system and it recovers the inner surface of the eye. The retina is a complex; layered structure with several layers of neurons interconnected by synapses and the only neurons that are directly sensitive to light. It consists mainly of three cell types: the photoreceptors, different nerve cells and retinal pigment epithelium (RPE). The ganglion cells, which are the output neurons, are the innermost parts of the retina, whereas the photoreceptors (rods and cones) are in the outermost layer against the retinal pigment epithelium and choroid. The rods are spread throughout the peripheral retina and the cones are concentrated in the macula, the portion of the retina responsible for central and color vision (6).

1.1 Routes of ocular administration

Even though the eye is one of the most easily accessible sites for drug administration and despite the numerous scientific efforts, efficient ocular drug delivery remains a challenge for pharmaceutical scientists. In general, a local effect is the main goal of an ocular drug administration. A major problem in ocular therapeutics is the attainment of an optimal drug concentration at the site of action. The main routes of drug administration in the eye are: intraocular, systemic, periocular and topical administration (Figure 2).

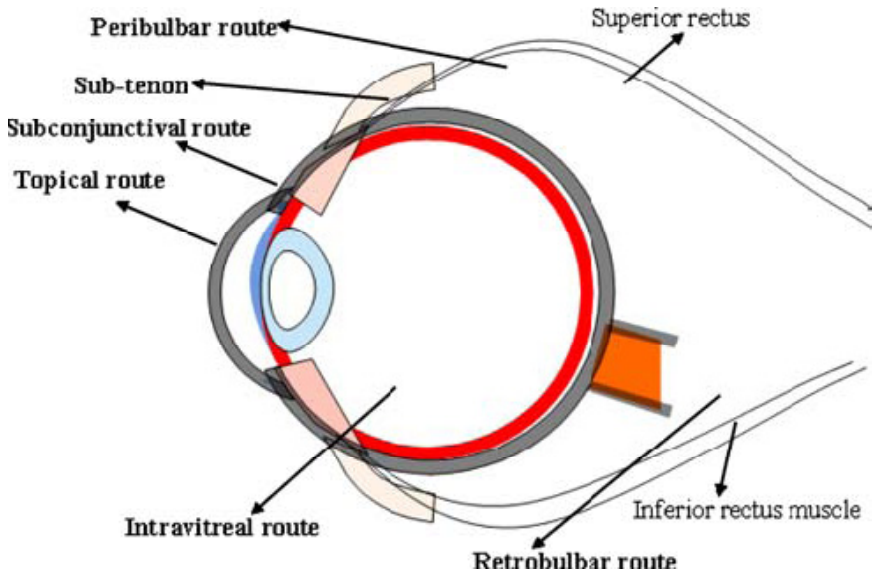

Figure 2. Ocular administration routes.

1.1.1. Intraocular administration provide

The intraocular administration can be divided between intracameral for humor aqueus and intravitreous for humor vitreus. Intravitreal injections have gained considerable attention in the past two decades. This method involves injection of drug solution directly into vitreous via *pars plana.* Unlike other routes, intravitreal injection offers higher drug concentrations in

vitreous and retina avoiding undesired side effects in others structures (7). Because of the risk of severe trauma that could end among others, in retinal detachment, blurred vision, bleeding, the nanocarriers for intraocular injection are aimed to produce a sustained release of the drug, decreased drug clearance and consequently, less administrations would be required (8).

1.1.2. Periocular administration

Periocular refers to the region surrounding the eye, which includes peribulbar, posterior juxtascleral, retrobulbar, subtenon and subconjunctival routes. The periocular route has been considered the most promising and efficient route for administering drugs to posterior eye segment. Drug solutions are placed in close proximity to the sclera which results in high retinal and vitreal concentrations because sclera which is made out of fibrous tissue offers less resistance to the passage of drugs (9). The main goals of periocular administration are to provide more effective therapy through direct targeting to the site of action and to prolong activity by providing extended high levels of drug at the targeted site (10). Subconjunctival injections can be given either under the eyeball conjunctiva (epibulbar) or underneath the conjunctiva lining the eyelid (subpalpebral). Subconjunctival injections are indicated for treatment of lesions in the cornea, sclera, anterior uvea and vitreous because of the injection underneath the conjunctiva allows drugs to bypass the epithelium, one of the main barriers that limit drug entry. Significant systemic drug exposure can occur due to rapid absorption of subconjunctivally injected drugs into the ocular venous circulation (11).

1.1.3. Systemic administration

Systemically administered drugs have poor access to the eye because of the blood-ocular barrier, which physiologically separates the eye from the rest of the body by epithelial and endothelial components, whose tight junctions limit transport from blood vessels to the eye. However, systemic

administration is an optimal therapeutic route when treating the posterior segments of the eye (retina, choroid, optic nerve and vitreous) because it is a non-invasive method. As mentioned above, in a normal eye the blood–eye barriers limit the amount of drug penetration into the eye. If the eye is inflamed the normal impermeability of these barriers is greatly reduced, increasing drug concentration in the target tissues. Systemic therapy can be used to treat conditions of the eyelids, sclera, uveal tract (both anterior and posterior), vitreous, retina, optic nerve and retrobulbar area if the drug has a large therapeutic window (11).

1.1.4. Topical administration

Topically administered drugs onto the eye surface correspond for more than 90% of ocular formulation currently commercialized. One of the main drawbacks of this route is the poor bioavailability of drugs in ocular dosage forms, which is mainly due to precorneal loss factors. Among the factors responsible for the low availability of the drug the most important are: (i) tear dynamics, (ii) non-productive absorption, (iii) transient residence time in the cul-de-sac and (iv) relative impermeability of the corneal epithelial membrane (12, 13). For this reason, only a small fraction of the drug is actually absorbed. So far, attempts to improve ocular drug bioavailability are made by extending drug residence time in the conjunctival sack using viscosizing agents to improve drug penetration across the cornea, the major pathway of drug entry into the eye (14). Prolonging pre-corneal residence time with these viscosity enhancers and gels has only a limited value because both cases are liquid formulations and therefore are eliminated by the usual routes in the ocular domain (15).

2. Nanotechnologies applied in ocular drug delivery

Nanotechnology-based drug delivery systems constitute a versatile alternative vehicle, capable of overcoming physiological barriers and guiding

the drug to specific cells or intracellular compartments either by passive or ligand-mediated targeting mechanisms (16, 17). More specifically, the potential of colloidal carriers such as liposomes and polymeric or lipid nanoparticles, among others, have been explored for ocular delivery due to their easy application as eye drops, with the advantage of being patient friendly as a consequence of their less frequent application and extended duration of the retention in the eye surface (13).

The nanotechnology begun to be explored to develop a drug delivery system that would circumvent the problems associated with the treatment of eye diseases (17). These nanosystems have a wide variety of forms (Figure 3), each one with its own advantages and limitations, and they can serve as carriers for drugs, peptides, vaccines, and genetic material which can also be delivered successfully to specific targets (18). However, a successful formulation requires the correct biomaterial (19, 20) that has special features such as biodegradability, non-toxicity, biocompatibility and mucoadhesiveness (9, 15).

This article reviews the biomaterials used today for improving drug delivery into the eye structures by using different nanotechnological strategies.

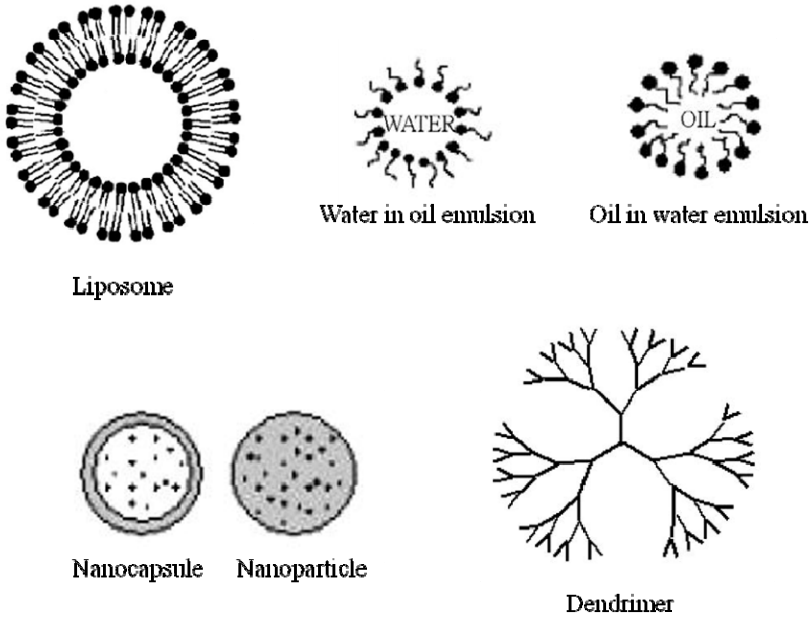

Figure 3. Different types of nanostructures employed in ocular drug delivery.

2.1. Liposomes

Liposomes are biocompatible and biodegradable vesicles composed of amphiphilic molecules, such as phospholipids and cholesterol, similar to those present in biological membranes. They have a diameter of 25 – 10,000 nm and their properties such as size, surface charge, stability and pharmacokinetics depend on its preparation method and composition. Based on their size and number of bilayers, they can be divided into small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), giant unilamellar vesicles (GUVs), and multilamellar vesicles (MLVs). The cavities within the liposomes, which are delimited by the polar heads of the phospholipids, can carry hydrophilic drugs, whereas lipophilic drugs can be solubilized within the bilayer among the hydrophobic tails (21, 22).

Vesicular systems such as liposomes are useful vehicles for sustained drug delivery to the eye. They not only help provide prolonged and controlled action to the corneal surface but also allow controlled ocular delivery by

preventing the metabolism of the encapsulated drug by the enzymes present at the tear/corneal epithelial surface (23). Liposomes can also protect the drug from enzymatic degradation and may bind to the epithelium increasing the residence time of the drug (24).

Several biomaterials, mainly of a lipidic nature, are employed in the manufacture of the liposomes and they can be divided into three groups according to their charge at physiological pH: negative, positive and neutral lipids. Table I summarizes the main materials used for the preparation of liposomes as well as its main function in the formulation.

The most common biomaterial used in the composition of liposome is lecithin/phosphatidylcholine. Lecithin is obtained from several sources, mainly from the soyabean and egg yolk, and it is comprised of a mixture of several phospholipids, mainly the phosphatidylcholine. Phosphatidylcholine (PC) is a neutral lipid at physiological pH, but liposomes formed with lecithin usually have negative zeta potential because of the presence of small amounts of acid phospholipids. Among the benefits of using lecithin/PC are the reduction of drug side effects, increased stability and modifications of the pharmacological/pharmacokinetic behavior (25-29). Lecithin/PC are employed even in the formulations of positively charged liposomes because they form the bilayer where the cationic lipid is anchored.

Cholesterol is another lipid that is usually employed in liposome formulations for ocular routes. It has the capacity of reducing the fluidity and permeability of the bilayer as a results of suppressing the *sol-gel* phase transition, thus increasing the rigidity of the liposome membrane (25). For this reason, cholesterol is widely used in liposome technology and its presence provides several benefits, such as the reduction of drug toxicity (30), increasing residence time (29) and the stability of the formulation stability (31).

Table I. Main lipids employed in liposome manufacture.

Lipid	Charge	Highlights	Ref.
Lecithin/ PC	-	Mix of several phospholipids mainly phoshatidylcholine (neutral lipid). The negative charge of liposomes based on lecithin is due the presence of small amounts of acid phospholipids in the lecithin composition.	(25-29)
PEG-DSPE	-	PEGylated phospholipid that increase liposome stability and lower its fate after *in vivo* administrations.	(25, 26, 28)
Cholesterol	0	Neutral lipid used to modify the fluidity of the bilayer. It is capable of change the transition phase of the bilayer, increasing its rigidity and as consequence lowering the leaking of drugs.	(25, 26, 28-31)
DOPE	0	Neutral lipid capable of destabilize the lysosomal membrane. Usually employed in combination with other cationic lipid in transfection assays.	(31, 37-40)
DOTAP	+	Cationic lipid recognized as transfection agent for gene therapy.	(38-40)
SA	+	Cationic lipid used to generate liposomes with positive charge. It is a fatty amine with only one lipophilic chain. Possess lower transfection efficiency than cationic phospholipids but the same ability in associate genetic material.	(27, 33, 34)
TMAG	+	Cationic lipid with great potential in transfection. Usually is employed in combination with DLPC	(35-38)
DC-cholesterol	+	Cationic lipid recognized as transfection agent that can in part combine the cholesterol properties with the better results of positive molecules.	(37, 38)

The behavior of liposomes upon ocular administration has been related to their surface charge. Many studies that have been performed in the last years confirmed the superiority of positively-charged colloids, such as liposomes, in delivering therapeutic agents to the eye. Liposomes are usually taken up by the cornea in the following order of positive > negative > neutral (32). In order to modulate of zeta potential of the formulation of liposomes, many compounds are added which results in higher interaction with charged structures. One of the compounds is the stearylamine (SA), a fatty amine employed to generate positively charged liposomes. Due to the presence of only one aliphatic chain, SA itself does not form the bilayers that are required for the vesicular structure of the liposome. Therefore, it must necessarily be employed in combination with other phospholipids (27, 33, 34). Positively charged liposomes with SA completely and uniformly covered the corneal surface, increasing the extent of acyclovir concentration in the cornea in comparison to negatively charged liposomes and the free drug (34). The use of SA in liposomes also increased the extent and the intensity of the reduction of intraocular pressure (IOP) induced by acetazolamide (27).

Other cationic lipids, such as TMAG (N-(a-trimethylammonioacetyl)-didodecyl-D-glutamate) (35-38), DC-cholesterol (3-b[N-(N0,N0-dimethylaminoethane)-carbamoyl] cholesterol) (36-38), DOTAP (1,2-dioleoyl-3-trimethyl ammoniumpropane) (39, 40) and DDBA (dimethyldioctadecylammonium bromide), enabled the design of vesicles capable of efficiently encapsulating sophisticated molecules such as nucleic acids, including plasmid DNA (pDNA) for gene therapy, oligonucleotides (ON) for immunostimulatory therapy, ribozymes and small interfering RNA (siRNA) for gene silencing applications. When associating genetic material, liposomes exhibit several differences from the vesicular systems for conventional drugs as a consequence of the electrostatic interaction with the cationic groups on lipid molecules and the phosphate present in the nucleotides of the genetic material, and they are commonly known as

lipoplex. In terms of the capacity of these cationic phospholipids to transfect ocular structures *in vivo*, the studies seem to point to the following order of effectiveness: TMAG > DOTAP = DC-cholesterol > DDBA (36-38). The use of TMAG is usually accompanied by DLPC (dilauroylphosphatidylcholine), a negatively charged phosphatidylcholine, which favored the transfection of adherent and suspended cells (38). It was observed that the use of TMGA in liposomes facilitate stable expression of beta-galactosidase for at least one week after administration onto the ocular surface of rabbit eyes and retinal ganglion cells (37).

The benefits of incorporating the neutral lipid DOPE (1,2-dioleoyl-3-phosphatidylethanolamine), to cationic liposomes have often been investigated because of its pH-sensitive ability to destabilize lysosomal membranes after cellular entry of the lipoplexes through endocytosis (31). DOPE is employed in combination with other lipids, mainly cationic ones, used as transfection agents for gene delivery (37, 39-41). But they have the disadvantage that even with better transfection efficiency than cholesterol, DOPE liposomes face the problem of aggregation when higher amounts of pDNA are loaded (31).

The coating of negatively charged liposomes with cationic polymers, such as protamine and chitosan, is a strategy to modulate the properties of liposomes, mainly the zeta potential. Coating liposomes with the polysaccharide chitosan can significantly increase the long-term stability (42) and the residence time of ciprofloxacin (43) and sodium diclofenac (42) in the eye when compared to uncoated liposomes or a solution of free drug. The coated liposomes displayed an enhanced penetration for transcorneal drug delivery. This result was a combination of the positive charge of the chitosan and its mucoadhesive properties that increase the interaction between liposomes and biological membranes (44). However, the effect of polymeric coating can vary depending on the eye structure, as it was observed for protamine sulfate coating. DOTAP/DOPE lipoplexes loaded with alkaline phosphatase and

coated with protamine sulfate showed different results when transfecting retina and cornea cells. For gene therapy in the retina, this coating has shown enhancement of the liposomal gene transfer in both *in vitro* and *in vivo* experiments, giving higher and more sustained gene transfer than the uncoated DOTAP/DOPE lipoplexes (41). Nevertheless, the response in the corneal epithelium after the transfection of the same DOTAP/DOPE lipoplex was the opposite: uncoated lipoplexes induced better expression of alkaline phosphatase in the cornea than the coated ones (40).

It is well known that coating the liposome surface with hydrophilic polymers such as PEG [poly(ethylene glycol)] chains enhances their stability and transport in serum (45). PEGylated lipids act as a barrier against the establishment of electrostatic bindings and can reduce the effect of the ionic strength of the lacrimal fluid on the stability of the vesicles and prolong the residence time of the systems. For this reason, the use of sterically stabilized liposomes for drug delivery to the eye has been exploited. The use of 1,2-distearoyl-*sn*-glycero-3 phosphatidylethanolamine-*N*-[poly(ethyleneglycol)-2000] (PEG-DSPE) in liposomes has been proposed by several authors to encapsulate several labile molecules, such as peptides (26, 28), oligonucleotides (25) and plasmids (37). This polymer showed an increase in the stability of such molecules by protecting them from the interaction with biological fluids. After intravitreal injection, PEG-DSPE liposomes loaded with ON were able to protect it from degradation and to prolong its vitreal residence time and also to reduce the distribution of oligonucleotides to non-target sites (25). The use of PEG-DSPE also has great value when an intravitreous administration of liposomes is required because the PEG chains that surround the vesicle can reduce the interactions between the liposomes and glycosaminoglycans that could lead to liposome aggregation (in the case of positively charged vesicles) and also diminish the interaction with the fibrillar network (39).

2.2. Nanoemulsions

Nanoemulsions (NEs) are a group of heterogeneous dispersions of two immiscible liquids. Their physical stability can be substantially improved by selecting suitable emulsifiers that are capable of surrounding the dispersed droplets in such way as to reduce interfacial tension or increase droplet-droplet repulsion. Depending on the concentrations of oil/water/emulsifier and the efficiency of the emulsification equipment used to obtain a reduced droplet size, the final emulsion may be in the form of oil-in-water (o/w) or water-in-oil (w/o) or, even, multiple emulsions (46).

Initially intended for parenteral applications, oil-in-water (o/w) lipid NEs now have been studied and exploited commercially as vehicles to improve the ocular bioavailability of drugs. The natural biodegradability, nanometric droplet size, sterilizability, substantial drug solubilization (mainly for lipophilic drugs) and improved ocular bioavailability make NEs promising ocular delivery systems (47, 48).

Basically, there are two types of compounds in an NE formulation that are fundamental to define the properties of the dispersed system: the oil and the surfactant. One of the most described associations for oil and surfactant has been between lecithin and soybean oil. For example, this combination was able to reduce the irritation caused by topical administration of pilocarpine onto the eye surface (49) improving its ocular penetration in the cornea, aqueous humor, conjunctiva and sclera (50) as well as increasing its miotic effect (51). The same combination was also capable of improving the effect of the anti-inflammatory drug piroxicam, and reducing the ulceration of the eye surface after an alkaline burn (52). Other oils, such as Miranol-MHT (53) and medium chain triglycerides (52, 53), have also been used in NEs to solubilize several drugs like piroxicam and HU-211, a synthetic analog of the *Cannabis sativa* alkaloid Δ^9-THC being able to protect and increase the activity of the drugs.

Poloxamer 188 is generally employed as a co-surfactant to improve the stability of the NEs (50, 52, 54). Sometimes, the incorporation of the drug into the inner phase of the NE increases its size and particle distribution, resulting in the deleterious effect of lowering the stability of the system. The use of 2% poloxamer can form NEs loaded with drug with same size and polydispersion as those seen in blank NEs (54).

Like afore mentioned for liposomes, cationic NEs have a better behavior than anionic ones in terms of stability, ocular penetration and pharmacological activity (32, 46, 48, 50). While some anionic NEs are stable only for few weeks, cationic ones last for years (50). Cationic lipids such as oleylamine (OA), DOTAP or SA are added to the formulation to bring positive charge to the surface of the NEs. SA is capable of significantly increasing the ocular concentration of pilocarpine (50) in the sclera and retina and can increase the epithelial healing process induced by piroxicam (52). Other cationic lipids as OA and DOTAP are capable of modulating the kinetic behavior of oligonucleotide release in the eye.

As well as for liposomes, the formation of cationic NEs can also be reached by polymeric coating that can invert the zeta potential of the emulsion by interacting with its surface. Once again, the polysaccharide chitosan was successfully employed, modifying not only the zeta potential of anionic NEs but also its size. Furthermore, it was capable of slightly modifying the release of the encapsulated drug from the system (55). Nowadays, it is possible to find in the market cationic NEs that are commercialized as lubricant (Cationorm®) and other new formulations that are in clinical trials: a cyclosporine A (CyA) nanoemulsion is in phase III study to treat vernal keratoconjunctivitis in pediatrics (Vesikat®) and another CyA cationic NE is under registration process to treat moderate dry eye (Cyclokat®).

A good example of the importance of the constituents in the properties of NEs was observed when the influence of different kinds of oils (isopropyl myristate, Miglyol 812, and triacetin) surfactants (Tween 80 and Cremophor

EL), and cosurfactants (propylene glycol, triacetin, Transcutol P, and Miranol C2M conc NP) both in the formation and activity of dorzolamide-loaded NEs was performed. The authors concluded that the drug release from NEs containing isopropyl myristate and Tween 80, together with Transcutol or triacetin was lower ($p<0.05$) than that from NEs containing propylene glycol (56). Clinical investigations revealed that NEs formulations were non-irritant and could be well tolerated by rabbit eyes. Figure 3 shows the effect of the materials used to evaluate the pharmacological effect over the IOP in normotensive rabbits after the administration of a single dose of dorzolamide hydrochloride NE. It was observed that NEs, in contrast to the drug solution or the market product, induced a pronounced decrease in IOP in just half-hour after administration of the eye drops (35% for NE and 20% for solution). Also the decrease of IOP was 2 times longer for some formulations. This showed that by changing the composition of a NE, it is possible to modulate the extent of the pharmacological action as well as its intensity.

A miscellaneous of several other biomaterials has an important role in the manufacture of the NEs. The use of α-tocopherol is widespread because it can avoid the oxidation of insaturations present in the oil and surfactant (50, 57). There is a preference in employing glycerol as osmotic agent because is a non-ionic molecule and it does not lead to instability of the system (49-53). Other polysaccharides usually used as thickening agents, such as methylcellulose and sodium carmellose, were employed to increase the stability of the formulation (54).

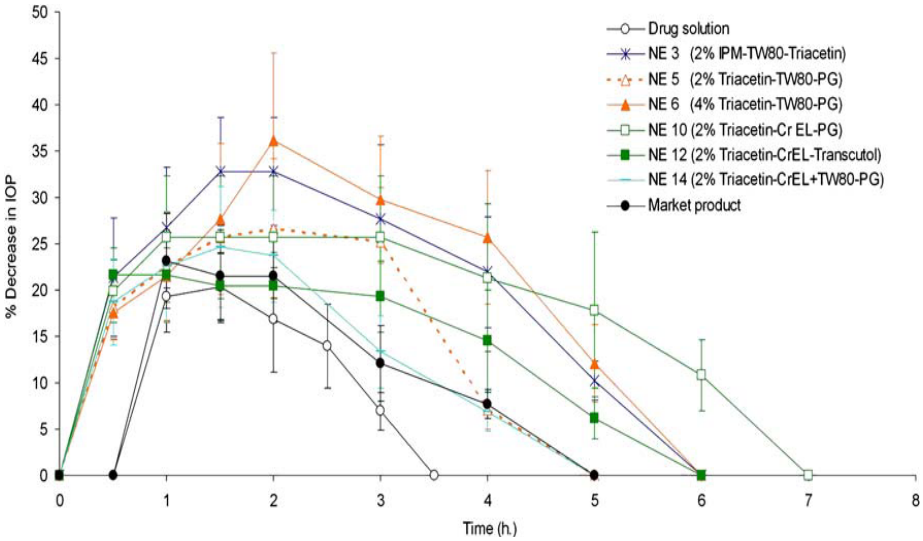

Figure 4. Influence of different biomaterials in the percentage of decrease in of the intraocular pressure (IOP) after administration of dorzolamide hydrochloride nanoemulsions, drug solution, and the market product. Adapted with permission from (56).

2.3. Nanoparticles

Nanoparticle (NP), as the name implies, are particles varying in size from 10 to 1000 nm and, depending on the end of use, may or may not include an active molecule inside their structure. This molecule can be attached to the matrix of the nanoparticle or may be dissolved, encapsulated and/or entrapped, giving rise to the different terminologies which include nanospheres or nanocapsules. The term *nanosphere* (NS) refer to a matricial structure and the term nanocapsule (NC) is applied to a system with an oil core and a polymeric shell.

For ophthalmic applications, these nanosystems have the advantage of being easy to administer in liquid form, such as eye drops. For topical application, they are well accepted by patients due to their less frequent dosing requirements as well as extended duration of retention in the pre-corneal area.

We will now review some biomaterials employed in the manufacture of this system for ocular drug delivery.

2.3.1. Synthetic polymers

The potential of different types of synthetic polymers including acrylic polymers, especially polyacrylates and poly(alkylcyanoacrylates) (PACAs) and polyesters has been evaluated in several studies and a few of them can be visualized in the Table II.

2.3.1.1. Poly(alkylcyanoacrylates)

The inherent properties of poly(alkylcyanoacrylate) (PACA), such as the biocompatibility and biodegradability, the simple preparation process of the nanoparticles and particularly, their ability to entrap a significant number of bioactives, specifically proteins and peptides, have sparked extensive interest in the use of this kind of polymers for the development of ocular drug delivery systems (58). For these reasons, a poly(butylcyanoacrylate) (PBCA), a type of PACA, was one of the first polymers that was used to develop a nanostructurated drug delivery system for the eye (59). Unfortunately, the first attempt failed to achieve good results mainly due to the low drug entrapment efficiency of pilocarpine, a higly hydrophilic drug.

PBCA presents some inconveniences in encapsulating very hydrophilic drugs, because it is a hydrophobic polymer. Nevertheless, even with a low entrapment efficiency (around 15%), PBCA NSs enhanced about 20-30% the miotic effect of pilocarpine (60). PBCA NSs are also able to modify pharmacokinetic parameters of pilocarpine after instillation in the eye. Using pilocarpine concentrations between 2-6% as well as different particle concentrations, approximately 23% an increase of the pilocarpine concentrations in the aqueous humor was observed with the NS dispersion compared to the aqueous solutions used as a reference, whereas the clearance was decreased significantly. The $t_{1/2}$ values of the aqueous humor

concentration of pilocarpine were observed to be in a similar time range as the miosis $t_{1/2}$ readings. It was found that at lower drug levels a more pronounced prolongation of miosis was achieved with NPs than with a control solution (61).

Taking into account the fact that physicochemical characteristics play a key role in the behavior of the NSs, studies were conducted to evaluate the factors that influence the formation of NSs such as temperature and the use of stabilizers (62-64). More concretely, our group has investigated the use of stabilizing agents, as dextran 70000, and found that they influence not only the physicochemical properties of the nanoparticulated system (size, zeta potential and loading of the antibiotic amikacin) but also the drug concentration in the cornea and aqueous humor (65). After 1h, almost 100% of the amikacin was still available in the eye anterior chamber while only 30-50% of the drug was available at the same time for the PBCA-NSs. However, the encapsulation process did not modify the minimum inhibitory concentration of the antibiotic assessed *in vitro* against *E. coli* and there was no difference in the corneal and aqueous humor concentration after topical administration in rabbit eyes when compared to a drug solution and the NSs (66).

As previously described for pilocarpine, the low affinity between the polymer and the drug seems to affect the encapsulation of the drug. Nevertheless, a high affinity can also be detrimental as was observed in a study using progesterone. According to the authors, the high affinity for progesterone by the PBCA matrix of the nanostructure is responsible for the fact that the concentration was generally 4-5 times lower for the NPs than the control solutions in several ocular structures (cornea, conjunctiva and humor aqueous) following topical administration of PBCA-NSs (67).

2.3.1.2. Polymethacrylates

The United States Pharmacopeia (USP-NF) describes the methacrylic acid copolymer as a fully polymerized copolymer of methacrylic acid and an acrylic or methacrylic ester. Three types of copolymers, namely Type A, Type B, and Type C, are defined in the monograph. They vary in their methacrylic acid content and viscosity in solution. Type A (*Eudragit RL*) and Type B (*Eudragit RS*), also referred to as ammonium methacrylate copolymers, consist of fully polymerized copolymers of acrylic and methacrylic acid esters with a low content of quaternary ammonium groups (68).

Polymethacrylates can modify several characteristics of drugs, including their stability, release profiles as well as their pharmacokinetic and pharmacodynamic profiles. Thus, Eudragit RS100 and RL100 were able to increase the stability of cloricromene, a synthetic coumarine with several biological activities, after its encapsulation in nanostructures made of both polymers (69). The encapsulation of the drug also led to controlled release, with ~40% of the drug released in the first 4 h instead of the burst release observed for the free drug solution. Eudragit RL100 NPs modified some pharmacokinetic parameters, such as maximum concentration (C_{max}; 1.16 µg/mL) and area-under-the-curve (AUC; 190 µg.min/mL) in respect to the control solution (0.68 µg/mL and 107 µg.min/mL respectively) (70). Also, by using Eudragit RL100 and RS100 in the composition of NSs, the anti-inflammatory effect of flurbiprofen associated to the structure was assessed *in vivo* in the rabbit eye after induction of ocular trauma and showed inhibition of the miotic response to the surgical trauma comparable to the formulation of a control eye-drop, even with a lower concentration of free drug in the conjunctival sack which was achieved using the nanoparticulated system (71). The positive polymer played a key role in modification of the pharmacokinetics of drug due to its insolubility at physiological pH and its ability to swelling. Regarding the pharmacological activity, Eudragit RS100, for example, can modify the effect of a drug. For example, when the anti-

inflammatory drug piroxicam was loaded into NSs made of Eudragit RS100, a more significant inhibition ocular inflammation was observed in rabbits than the response to the free drug and microsuspension (72).

However, positive polymers such as Eudragit RS100 and RL100 have the disadvantage of being potentially toxic, as is the case with most of the positively charged biomaterials (73). Several studies on ocular acute tolerance were performed in rabbits for both Eudragit RS100 and RL100 in the form of NSs (70, 74). The evaluation was made by applying a modified Draize test and evaluating the following events: congestion, swelling, conjunctival discharge, iris hyperemia and corneal opacity. According to the authors, even with a small irritation after the instillation of NSs suspensions, Eudragit does not seem to cause any major irritant effect on the cornea, iris, and conjunctiva up to 24 h after instillation and, for that reason it appears to be a suitable polymer for an inert nanocarrier in the ophthalmic drug delivery.

2.3.1.3. Aliphatic polyesters

Aliphatic polyesters are synthetic homopolymers or copolymers of lactic acid (PLA), glycolic acid (PGA), and ε-hydroxycaproic acid (PECL). They are biodegradable and undergo to hydrolytic and biological degradation through cleavage of the ester linkages or are metabolized via the citric acid cycle into nontoxic products. The rate of biodegradation and the characteristics of drug release of a drug delivery system made of aliphatic polyesters can be controlled by changing the physicochemical properties of the polymers, including crystallinity, hydrophobicity, monomer stereochemistry, copolymer ratio, and polymer molecular weight. These adequate properties represent some of the reasons why aliphatic polyesters were one of the first polymers to be investigated for nanoparticulated drug delivery systems for ocular administration (75).

Nanoparticles made of PECL can increase the drug concentration in the cornea, aqueous humor and iris-ciliary body by more than 3-fold after topical instillation, as it was shown for NCs and NSs loaded with indomethacin (76). The ocular bioavailability was increased by 300%, leading the authors to believe that PECL can act as a penetration enhancer. Furthermore, PECL plays an important role in stabilizing the NCs. Polymeric coating of PECL can increase the stability of the formulation of NCs when compared to the control of NEs (77).

A significant improvement of the pharmacological response and a reduction of drug side effects can also be obtained by using PECL NCs. While the 0.5% commercial betaxolol solution achieved a maximal reduction of the IOP of 20%, in alpha-chymotrypsin pretreated rabbits, betaxolol at equal concentration but encapsulated into NCs, yielded about 30% of IOP reduction and longer pharmacological response. Even a 0.1% betaxolol-loaded NCs preparation was still significantly better than the commercial 0.5% betaxolol solution. Similar results were observed with carteolol-loaded NCs when PECL was used as polymer. The encapsulation of the beta-blocker increased its therapeutic effect, as demonstrated by a prolonged reduction of the IOP of over 8 h (78). Perhaps, one of the most interesting achievements was the reduction of side effects. Significant reduction of the systemic side effects in terms of cardiac rate and arterial pressure were observed in comparison to commercial eye drops of betaxolol and cartelol-loaded NCs. Same results could be observed for isoprenaline and metipranolol-loaded PECL NCs. No differences were observed in terms of heart beat rate between animals treated with isoprenaline-NCs and the controls, whereas the commercial eye drops inhibited the heart beats about 50% (79). Even though there was no difference in the pharmacological effect between NCs and the commercial solution, the loading of metipranolol into the NCs reduced its systemic side effects (80). The bradycardia resulting from the systemic absorption of the drug was greatly reduced when the drug was encapsulated.

After 60 min, the heart beats were similar between the NCs and the control, while the reduction induced by the eye drops lasted for the whole assay (120 min). Furthermore, encapsulated metipranolol did not alter the decrease in mean arterial pressure induced by isoprotenerol at any time, while eye drops led to almost 30% of reduction. An equivalent NC system composed of a Miglyol 840 oil core surrounded by a PECL coating was able to load a high amount of CyA, of almost 50% (drug/PECL ratio). After topical administration, the drug levels in the cornea were up to 5 times higher for the encapsulated drug than under oil solution form. In addition, these levels remained significantly higher than those of the control group for up to 3 days. Also, an increase was found in AUC when compared the encapsulated CyA (320 μg.min/mL) and the oil solution (74 μg.min/mL) (81, 82). The topical administration of the formulation in keratoplasty showed no effect of rejection in the rats, and the drug was not detectable in the blood. In contrast, the oil solution delayed the onset of corneal rejection and the drug was detectable in the blood (83, 84). Higher concentrations of CyA in ocular tissues such as the cornea and conjunctiva were achieved by using positively charged PECL-NSs (85). The tissue concentration for the NPs was almost 10 times higher than that achieved with the oil solution for at least 24 h.

As well as for other nanostructurated systems, it is possible to modify surface properties of PECL nanostructures by adsorbing polymer on it. The coating of PECL nanostructures with CS or PEG influences drug penetration, retention time and also modifies its penetration pathway. PEG accelerates transport whereas CS favors retention in the outer epithelial layers (86). Higher stability can also be achieved by coating the nanostructure with poly-L-lysine. However, the polymeric coating failed to increase the ocular bioavailability of indomethacin when compared to uncoated particles. The structure of each polymer seems to play a more important role than its charge in the modification of the nanocarrier properties (87).

In addition to PECL, PLA and PLGA facilitate a sustained release of the encapsulated drug, as in the case of budesonide (88) and flurbiprofen (89). After subconjunctival administration, NPs produce sustained levels of budesonide in the retina and other ocular tissues. At concentrations below the cytotoxicity level, budesonide inhibited the secretion of VEGF as well as the expression of mRNA expression in the retinal cells ARPE-19 in a dose-dependent manner. A modification of the pharmacological effect is also described for these polymers as reported when PLA induced a more dramatic reduction of the arachidonate-induced inflammation after topical instillation of flurbiprofen-loaded NSs in the rabbit eye (90). PEG-coated and uncoated PLA-NSs showed a sustained release of acyclovir release–and were highly tolerated by the eye. Both types of PLA nanostructures were able to increase the aqueous levels of acyclovir 4-fold and improve the pharmacokinetics profile, but the efficacy of the PEG-coated NPs was significantly higher than that of the easy PLA NSs (91). Due to the nature of the polymer, PLA and PLGA-NSs accumulated in the retina and choroid of rats within 3 h and remained over the succeeding seven day period even when not administrated *in situ*, as was shown after intravenous administration of NPs loaded with betamethasone for autoimmune uveoretinitis (92).

Despite the fact that PLGA and PLA possess a limited solubility in water, they were capable of interacting with genetic material to produce gene therapy vehicles. More concretely, PLGA was used to associate VEGF-ON to NSs, which were then transfected in ARPE-19 cells and they inhibited the expression and secretion of VEGF in cells. The uptake of VEGF-ON administered in NPs was increased 4-fold with respect to free ON (93). NSs made of PLA and PLGA were also shown to be safe and effective in transfecting plasmids into the retina (94). PLGA-NSs containing red nuclear fluorescent protein plasmid were successfully transfected both in the retina of the rat and the conjunctival epithelial cells of the rabbit.

An assessment of the toxicity of PLA in nanocarriers was carried out to determine its safety. Histological studies demonstrated the anatomic integrity of the eyes injected with PLA NSs and showed no toxic effects (95). Mild inflammatory cell infiltrate was observed in the ciliary body 6 h after the injection and in the posterior part of vitreous and retina in 18 to 24 h. Inflammation was markedly decreased after 48 h. Confocal and fluorescence microscopy as well as immunohistochemistry showed the internalization of the NSs and that a gradual transretinal movement of the NSs took place followed by localization into RPE cells. The studies also showed that the NSs were still present within the RPE cells four months after a single intravitreous injection (95, 96).

2.3.2. Natural polymers

2.3.2.1. Chitosan

Chitosan (CS) is one of the most extensively studied natural polymer for the preparation of nanoparticles (97-99). Chitosan (α [1→4] 2-amino 2-deoxy β-D glucan) is a natural polymer synthesized by alkaline deacetylation of chitin, which is the second most abundant polysaccharide in nature, after cellulose. This polymer can be found in the exoskeletons of crustaceans, insects and some fungi (100). It exhibits several favorable biological properties that make it an interesting polymer for use in pharmaceutical formulations, as demonstrated by the number of scientific reports published on this topic (101). It is a mucoadhesive and biodegradable polymer that possesses penetration-enhancing properties (102-108). The applications of CS have been extensively reviewed for ocular drug delivery and more information about its use can be found in the recent works of Alonso and Sanchéz (44), Paolicelli *et al*. (98) and de la Fuente *et al*. (101).

The use of CS in ocular therapy began with its use as a coating agent of nanostructures, like nanocapsules or nanoemulsions, as previously described

(55, 87). The advantages of a CS coating are not only because of its positive electrical charge which can interact with the cell surface but also the nature of CS itself. The *in vivo* behavior of CS-coated NCs was significantly different from PLL-coated NCs when compared to the bioavailability profile of uncoated PECL-NSs, even though they both possess a similar positive surface charge. There was an increase of indomethacin bioavailability both in the cornea and aqueous humor for the CS-coated ones, but the PLL-coating did not differ from the uncoated NCs used as control (87). The effect of CS-coated nanostructures has also been observed in a study performed by Calvo *et al*. (86), in which they compared the CS coating to the PEG coating. The CS coating substantially differs from PEG coating in terms of its interaction with the ocular epithelium. Whereas PEG-coated NCs cross the whole epithelial area, the CS coating favors the retention of NCs in the superficial layers of the epithelium. Confocal laser scanning microscopy studies also showed that the CS-coated NCs are able to pass through the corneal epithelium via a paracellular pathway.

CS was also used to form nanoparticles by gelation technique This technique is performed at room temperature involves the addition, of an aqueous phase containing a low molecular weight counter-ion into an aqueous phase containing CS. NPs are formed immediately upon the mixing of the two phases through inter- and intramolecular linkages created between the phosphate groups of the counter-ion and the amino groups of CS (44). It is the most suitable technique for NPs that are intended to associate hydrophilic and unstable drug, such as genetic material (pDNA, siRNA) or proteins (109).

Our group was one of the first to investigate the potential use of CS in nanoparticulated drug delivery systems for the eye. NPs made only of CS were formulated for ocular drug delivery using a modified ionic gelation technique in which a small amount of acetronitrile was added to solubilize CyA (110). The use of CS result not only in high association efficiency

(higher than 70%) and but also was capable of interacting with the ocular surface, increasing CyA levels in the cornea and conjunctiva when compared to the controls (suspension of CyA or suspension of CyA and CS). Unfortunately, this CS formulation could not avoid systemic drug absorption, and the CyA blood levels were similar to those found after administration of the controls. Nevertheless, the levels of drug in the plasma were still much lower than toxicity levels (110).

The interactions between the CS-NPs, the ocular surface and the tear film were extensively studied. The stability of CS-NP after incubation with lysozyme, and the interactions between CS and the anionic reticulant are strong enough to maintain its physicochemical properties (size and zeta potential). This does not occur after incubation with mucin, given that the zeta potential is reduced, suggesting an interaction between CS and mucin (111). In this same study, a confocal microscopic analysis was performed with NPs made from fluoresceinamine labeled CS (CS-fl), which were administered to the cul-de-sac of conscious rabbits to quantify its *in vivo* interaction with corneal and conjunctival epithelia. CS-fl-NPs provided a greater concentration of fluoresceinamine to corneal and conjunctival tissue than CS-fl solution or free fluoresceinamine. The CS-NPs were internalized mainly by a paracellular instead of a transcellular pathway, which has a slightly different behavior when comparing with PECL or PBCA NPs. The authors concluded that not just the natural properties of CS play a role in fluoresceinamine content but also its nanoparticulated nature. An interesting finding was that the distribution in conjunctival epithelia was not homogenous (as it is in the cornea). Indeed, the conjunctival epithelium is more heterogeneous than the cornea and is made up of different cell types such as goblet cells and antigen presenting cells (111).

Another interesting study in which CS-NPs were associated with liposomes was performed by Diebold *et al.* (112). *In vitro* assays have proved the ability of these nanostructures to cross the cell membrane of the IOBA-NHC (a

nontransfected, naturally immortalized cell line derived from normal human conjunctiva) without compromising cell viability. The tolerance *in vivo* was assessed, and the clinical macroscopic signs were compatible with a non-irritated eye surface. Moreover, the pathology study showed no histological alterations in the cornea, conjunctiva nor in the eyelids (113).

The CS has also been studied for its application in gene delivery to the eye. Our group has investigated the use of CS in hybrid NPs with hyaluronic acid and we have pioneered this type of approach. These NPs were able to associate lipophilic drugs (CyA) and pDNA (114). These hybrid NPs successfully transfected *in vitro* both human corneal epithelial cells (HCE) and conjunctival cells (IOBA-NHC). Several different types of plasmid DNA, such as pEGFP, pLac-Z and pSEAP were successfully transfected in these cell lines (115, 116). An important observation was that the length of the CS chain seems to influence the transfection efficiency. The NPs made of oligomers (10-12kDa) (117) showed to be more effective in transfecting HCE cells than CS with a higher chain length, as it can be seen in Figure 5 (118). These nanoparticles also showed an acceptable *in vitro* toxicity profile (MTT assay) in IOBA-NHC and HCE cells. This reduced toxicity was attributed to the addition of hyaluronic acid to the system, an effect that will be discussed further.

Even with several reports that refer to CS as a non-toxic polymer suitable for administration in most tissues and organs (119-123) and due to the fact that its ocular topical applications seems to be well tolerated (111, 112, 118), its use in ocular therapeutics still faces the inconvenient toxic effects which occur when an intraocular administration is required. CS-NPs were shown to induce severe vitreous haze, cellular infiltration and membranous opacities, which were observed in eyes injected with CS-NPs and were often associated with inflammation. This drawback also limits its use for other ocular routes of administration, such as sub-retinal administration (124).

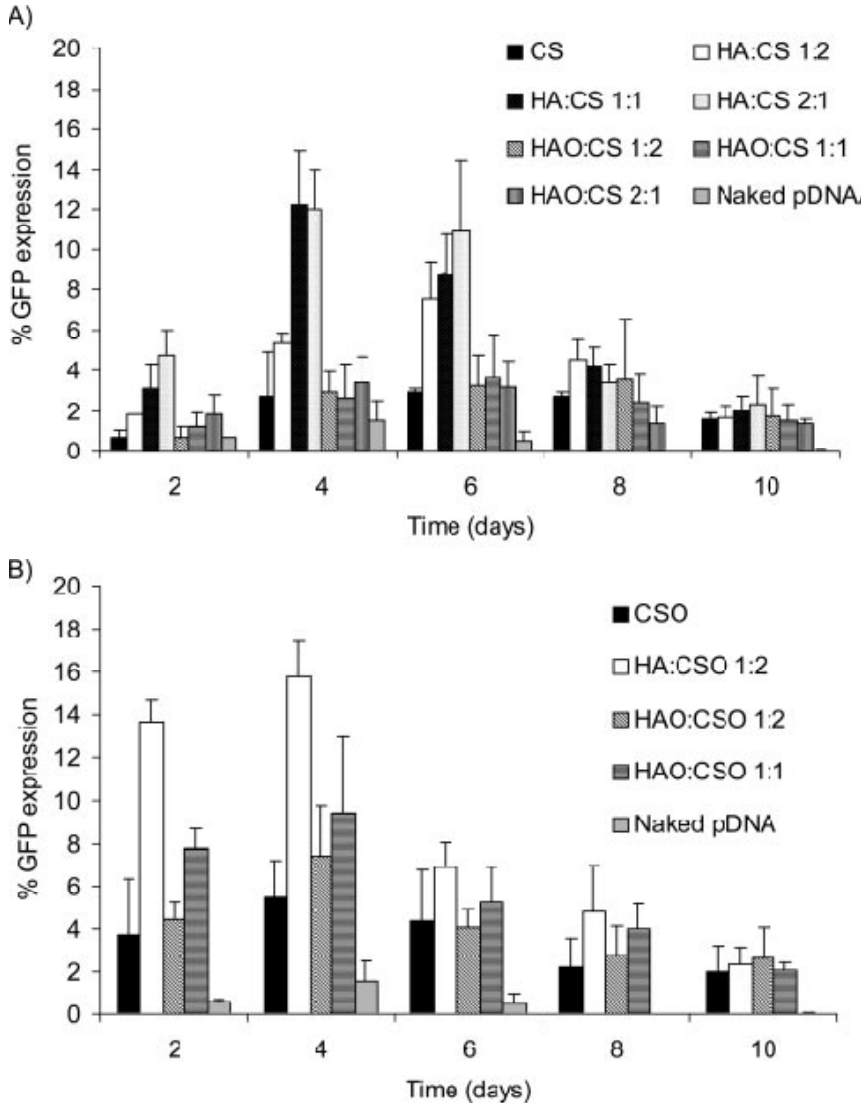

Figure 5. Evaluation of the transfection efficiency of HA-CS NPs in HCE cells. Percentage of transfected cells over 10 days after incubation of NPs composed of variable polymer ratios of hyaluronic acid (HA), chitosan (CS), hyaluronic acid oligomers (HAO) and chitosan oligomers (CSO). (**A**) NPs composed solely of CS, HA:CS 1:2, HA:CS 1:1, HA:CS 2:1, HAO:CS 1:2, HAO:CS 1:1, or HAO:CS 2:1 NPs or of naked pDNA. (**B**) NPs composed solely of CSO, HA:CSO 1:2, HAO:CSO 1:2, or HAO:CSO 1:1 NPs or of naked pDNA (mean ± SD, *n* =3). Adapted with permission from (118).

Nevertheless, the main disadvantages of the nanoparticles of CS formed by ionic gelation are unstable at physiological pH and are very sensitive to ionic strength (111, 114). NPs have a tendency to aggregate when exposed to the above mentioned conditions. Furthermore, the poor reproducibility of different commercial batches of CS is another important issue that our group faced and it must be taken into account.

2.3.2.2. Hyaluronan

Hyaluronan (HA) is a non-sulfated non-epimerized linear glycosaminoglycan existing *in vivo* as a polyanion of hyaluronic acid and composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine [→ 4GlcAβ1→3GlcNAcβ1→] (125). It is a major constituent of the extracellular matrix (ECM) of the eye and many other tissues and organs and it possesses mucoadhesive properties and low toxic behavior (126, 127). However, the most attractive property of HA for ocular drug delivery systems is its ability to interact strongly with various receptors, for example, the hyaluronic acid receptor for endocytosis (HARE) (128-132) and the CD44 receptor (133), which are expressed in the ocular epithelium (134-136).

Initially, in nanoparticulated drug delivery systems, HA, as it was described in the case of CS, was used as a coating agent (85, 137), and was later included in hybrid systems (114-116, 118) until the development of nanosystems based only in HA (138). Additionally, HA was used as coating agent of PECL-NPs, using benzalkonium chloride as an anchor to ensure attachment to the NP surface (139). An *in vivo* study in rabbits with these NPs (85), showed that corneal cyclosporine A levels were found to be higher for PECL-NPs coated with HA than for uncoated NPs.

With respect to hybrid CS/HA nanoparticles (118), the high efficiency seen in the transfection process was due to the interaction of HA with the CD44

receptor (Figure 6). When the receptor was blocked (by an antibody or an excess of HA), the transfection efficiency decreased dramatically. As occurs with CS, HA oligomers are much more effective in transfecting both conjunctival and corneal cells. The transfection effect seems to last at least 10 days, reaching its maximum level on the fourth day after transfection with around 9% and 12% of cells transfected (for HCE and IOBA-NHC respectively) (118).

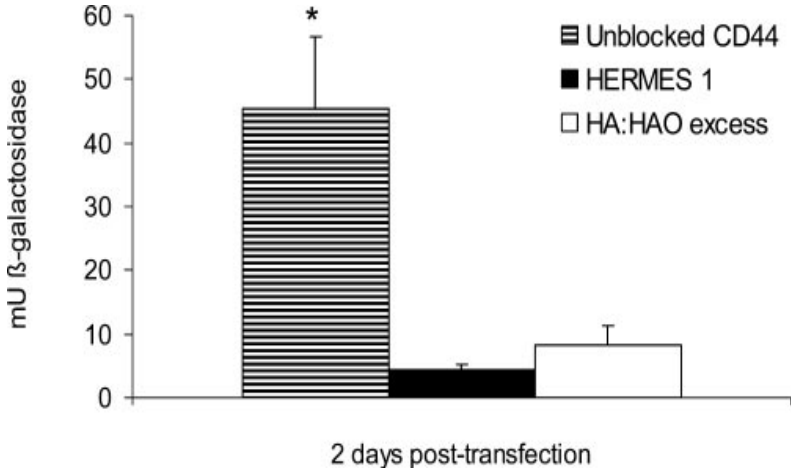

Figure 6. Transfection efficiency of pDNA-loaded NPs (HA:CSO 1:2) incubated with HCE cells. The CD44 receptor was blocked with 1 μg of the monoclonal antibody Hermes-1 or with an excess of a HA/HAO mixture (50-fold with respect to the amount of HA in the nanostructures). The expressed β-galactosidase was quantified by the ONPG reaction. (mean ±SD, n = 3). *Significant difference, P < 0.05. Adapted with permission from (118).

The activity of HA, like that of CS, seems to be influenced by its molecular weight. Hyaluronic acid olygomers (HAO) seem to be more effective than HA with longer chains. Figure 6 shows the influence of the molecular weight in the transfection of HCE and NHC cells as well as the influence of the polymer ratio in the expression of green fluorescent protein (GFP).

HA is a relatively safe compound, being widely used in cosmetic formulations and in the regenerative medicine. Usually the toxicity of systems based on HA is due to other compounds in the formulation such as polymers or surfactants, primarily those of a cationic nature (124).

Recently, a novel approach on ocular drug delivery has been proposed by the use of a modified ionic gelation technique that consists of the use of polyamines with low molecular weight as a reticulant agent, instead of the classic negatively charged crosslinkers such as tripolyphosphate or sodium sulfate. In this new approach, an inverse situation was investigated using spermine as a crosslinking agent of anionic polymers. Spermine (SPM) is a biogenic polyamine of low molecular weight which is present in almost all tissues. It is essential for the growth processes of the tissues tissue growth and has specific receptors for its internalization. It has four amine groups that are fully protonated at physiological pH. It allows the use of several naturally occurring anionic polymers such as the HA. The resulting systems were able to incorporate both drugs and genetic material (siRNA and pDNA) and werw totally biocompatible and biodegradable because most of them are composed by that are part of eye structure and eye metabolism (127, 140). Because these systems are composed of anionic polymers and possess a negative zeta potential, they can overcome the toxicity associated with cationic polymers and positive zeta potential which limits the use of nanoparticulated systems. These systems are also suited for intraocular delivery because their negative charge would not interact with intravitreous glycosaminoglycans (141), which are known to limit the delivery of certain compounds especially genetic material (39, 142).

2.3.2.3. Gelatin

Gelatin is a natural polymer that is derived from collagen and is commonly used for pharmaceutical and medical applications because of its biodegradability and biocompatibility in physiological environments. One

interesting feature of gelatin is that its isoelectric point can be modified during the fabrication process to yield either negatively charged acidic gelatin, or ~~a~~ positively charged basic gelatin at physiological pH (143, 144). Commercial gelatin is usually obtained from an animal source, so safety concerns, including possible contamination and immunogenicity can be an important issue. For this reason recombinant human gelatin can be a useful alternative because it has the same properties of the commercial gelatin but its purity is higher (145-147).

Gelatin itself does not possess enough positive charge to produce NSs by the ionic gelation technique, so they are produced mainly by the desolvation/coacervation process, a technique that uses organic solvents, mainly ethanol, to precipitate the gelatin. Because of its highly hydrophilic nature, gelatin has a tendency to swell which could compromise~~s~~ the stability of system. To avoid this, a chemical crosslinker (usually glutaraldehyde or carbodiimides) is always added to ensure the stability of the nanoparticles after their formation (148, 149). However, the use of chemical crosslinkers results in a serious safety issue because most of them exhibit non-acceptable toxicity and no regulatory agency is likely to approve a system that contains such crosslinker. Due to the nature of the gelatin, NSs based on it are able to encapsulate several types of molecules, including lipophilic (150) and hydrophilic drugs (151) as well as extremely sensitive molecules, such as genetic material (152, 153). The association of drug into gelatin NSs is capable of modulating the release of drug as occurred for pilocarpine when gelatin NSs produced by the desolvation method and using glutaraldehyde as crosslinker led to a sustained release approaching to zero-order kinetics (150).

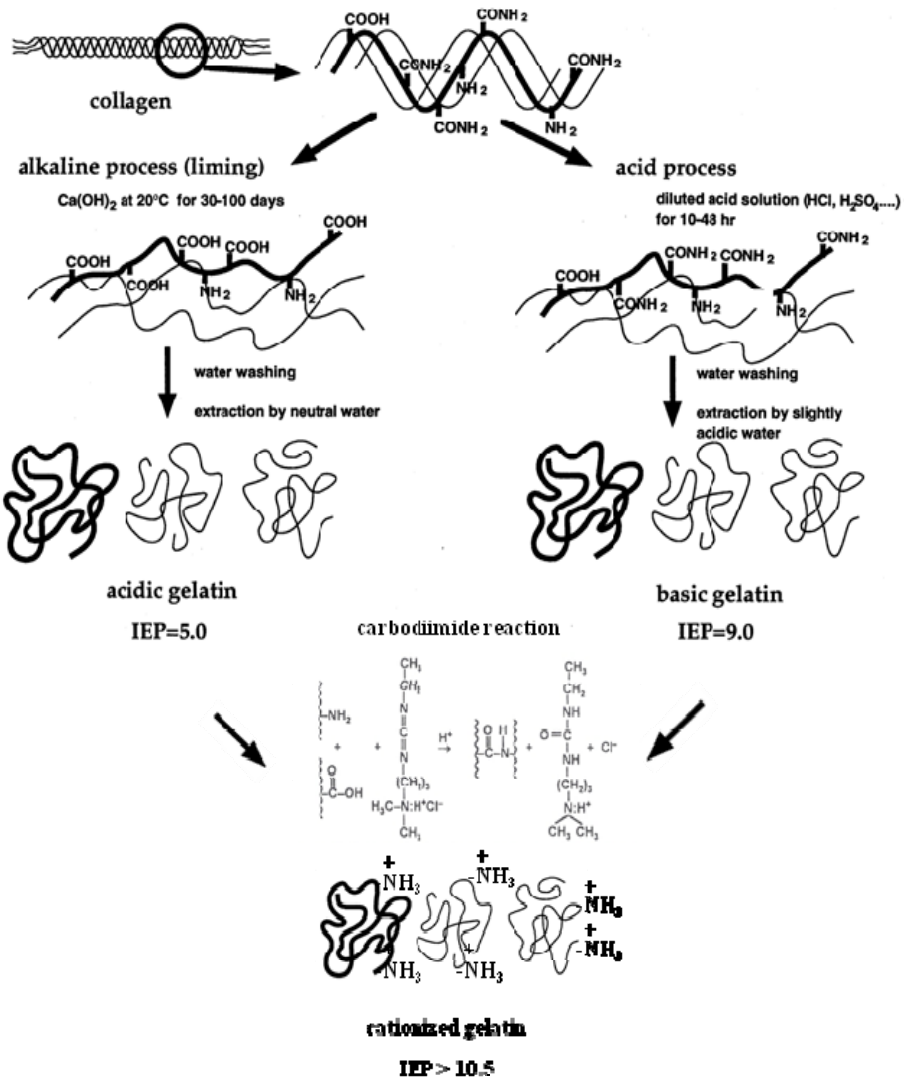

Figure 7. Obtention process of gelatin and cationized gelatin. Adapted with permission from (149).

An alternative is the use of cationized gelatin (CG), which are gelatins that are bound to low molecular amine (154), normally through carbodiimide reaction (Figure 7). It is known that CG can be transported across the epithelial mucosa (155) and can associate peptides such as insulin (156) and genetic material such as pDNA (157) and siRNA (158, 159). Usually the

delivery of genetic material using CG is done by means of complex formation (160-162) or as a hydrogel (157, 163, 164). However, our group is now testing the feasibility of employing CG to produce NSs by the ionic gelation technique that will guarantee the safety of transfection onto the ocular surface to treat inflammatory diseases (165). Recent unpublished data suggest that this new approach of using CG is quite valuable for nanoparticulated drug delivery systems, especially for gene therapy for the surface of eye.

2.3.2.4. Others polymers

The interaction with HARE and CD44 receptors is not exclusive to HA. It has been reported that chondroitin sulfate and dextran sulfate (133, 166-168) share the same ability but at different intensities, and this property can be very helpful in the internalization of NPs. Chondroitin sulfate is a widely distributed glycosaminoglycan composed of a chain of alternating N-acetylgalactosamine and glucuronic acid units, which plays an important role in the connective tissue and the metabolism of the eye (169, 170) and has been proposed as a biomimetic agent for rational drug delivery (171). Dextran sulfate is a highly sulfated glucan from anhydroglucose composed of approximately 95% alpha-D-linkages with the remaining linkages for the branching of the polymer. Currently, our group is exploiting the use of both chondroitin and dextran sulfate as new non-viral vehicles for gene therapy on the ocular surface. These systems can be hybrid systems when associated with CG for example or based only on the polyanionic polymers when using our new approach that employs SPM as crosslinking agent (138).

Glucomannan-based NPs coul have a special application in the treatment of retinal pigment epithelium (RPE) cells (172, 173). Glucomannan is an uncharged linear polysaccharide derived from the mannan family and is a β-1,4 polymer composed of D-mannose and D-glucose residues (174). It has the ability to interact with specific receptors (175), and this could it may be

interesting in the case of the mannose receptors found in RPE cells (176) which are related to the cell internalization process (177). In the nanoparticulated systems, glucomannan can be employed in two basic forms: a natural form or modified with some molecule that will provide charge. In the first case the interaction between glucomannan and the others constituents of the NP will be ruled mainly by the hydrogen bounds (172) whereas in the latter case interactions will be mainly electrostatic (173).

2.4. Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are nanostructures made from a lipid that is solid at room and body temperature (178, 179). Basically there are two preparation techniques: high pressure homogenization and the microemulsification method. Depending on the fabrication technique employed, the use of a surfactant or co-surfactant may be required (180). Despite of being an interesting delivery system, SLNs face many problems, such as low loading capacity and the potential expulsion of the drug during storage. Another disadvantage is that SLNs can incorporate efficiently only hydrophobic molecules (181), but even this molecule can be expelled from the SLNs. This can be in part overcome by the incorporation of a blend of lipids, usually a liquid one, to create a lipid matrix that is as disorganized as possible. These systems are ~~so~~ called *nanostructured lipid carriers* (NLCs). For ocular delivery, there are a few studies that describe the use of SLNs. Table III shows the composition of several formulations that were studied.

Even with lower capacity to interact with ocular epithelia, SLNs that are comprised of anionic phospholipids succeeded relatively well in increasing drug bioavailability in the eye. A suspension of tobramycin-loaded SLNs containing hydrogenated phospholipids (Epikuron 200®) administered topically to rabbits produced a significantly higher drug bioavailability in the aqueous humor after 6 h. The precorneal retention of SLNs in rabbit eyes was longer on the corneal surface and in the conjunctival sac when compared with

an aqueous fluorescent solution, even with this anionic surfactant (182). Similar results were achieved by using phosphatidylcholine (Phospholipon®) when the SLNs induced higher permeation of diclofenac using bio-engineered human cornea produced from immortalized human corneal endothelial cells, stromal fibroblasts and epithelial cells (183).

Among the lipids that have been used as the nucleus of SLNs, glyceryl behenate has been shown to be the best one for encapsulating drugs. Probably, because it induces imperfections of the lattice which allows better accommodation of the drug in comparison to lipids with only one aliphatic chain such as stearic acid. It is also the lipid that leads to superior physicochemical characteristics of SLNs (184, 185).

As well as described by liposomes and NEs, the cationic lipid DOTAP is also used in the formulation of SLN. It creates the possibility of adsorbed pDNA onto the surface of SLN mixing the transfection properties of the DOTAP and the stability of the SLN (186-188). It is interesting to notice that not only the amount of DOTAP and N/P ratio were important to successful transfection efficiency, the amount of non-ionic surfactants also plays a key role in this process (186). Others cationic lipids such as SA and octadecylamine are also used but with the aim in increasing the retention time of the SLNs (184, 189).

There are a few studies that describe the benefits of NLCs for ocular administration. The use of thiolated PEG-stearate as coating agent of NLCs can dramatically increase both the precorneal retention time and the concentration of CyA after topical administration in rabbit eyes. The CyA administered in thiomer-coated NPs is able to remain on the ocular surface for up to 6 h and when compared with the NPs without thiomer coating (190). Another way to increase the retention of NLCs is the use of the cationic lipid stearylamine, which can prolong the pre-corneal retention time of drugs. The pharmacokinetic parameters, such as AUC, can also be increased (189). For ocular therapy, the most important advantages of using

SLNs are the controlled and sustained release of the drug due to the increase of residence time (that can be extended by using positively charged SLNs). These properties are severely affected by the solid lipid and the surfactant selected.

Table III. Biomaterials used in SLN preparation

	Drug	**Surfactant**	**Lipids**	**Reference**
SLN	Tobramycin	Epikuron 200 Sodium taurocholate	Stearic acid	(182)
	pDNA	Tween 80 DOTAP	Precirol ATO5	(186-188)
	Diclofenac	Tween 80 Phospolipon	Goat fat	(183)
	CyA	Tween 80 Octadecylamine	Glyceril behendate Tripalmitin	(184)
	Gatifloxacin	Poloxamer-188 Sodium taurocholate Ethanol	Stearic acid Glyceril behendate	(185)
NLC	Ibuprofen	Crempher EL Stearylamine	Glyceril behendate Gelucire 44/14 Miglyol 812	(189)
	CyA	Tween 80 Modified-PEG	Precifac ATO 5 Miglyol 840	(190)

2.5. Dendrimers

Dendrimers consist of a central core molecule, which acts as the root from which a number of highly branched, tree-like arms originate in an ordered and symmetric way (191). Their unique molecular architecture gives to them some distinguished properties such as a defined size, relatively low polydispersion and the possibility of being easily functionalized. Even with the ability to associate drugs (192, 193), dendrimers are usually employed as non-viral gene therapy vehicles. More information about dendrimers can be found in a recent review of Raviña *et al.(194)*. In ocular treatment, there are a few reports of the use of dendrimers and they are all focus in gene therapy.

Complexes with Epstein–Barr virus based plasmid vector and polyamidoamine (PAMAM) dendrimer were assayed in suicide gene therapy for cancer (195). The direct *ex vivo* application of the complex in human and rabbit cornea resulted in transfection of 6-10% of the cells. Also, the synthesis of lipid–lysine dendrimers, that subsequently had the ability to deliver a sense oligonucleotide against age-related macular degeneration (AMD), was described by Marano *et al*. (196). The complexes mediated a reduction in VEGF concentration both *in vitro* and *in vivo*, inhibiting the choroid neovascularization for a period of up to 2 months. Still it is too soon to predict the future of dendrimers as ocular nanomedicines because the lack of studies but if these results were reproduced in other tissues were reproduced, they would be a promising vehicle for effective, no toxic, ocular gene therapy.

3. Conclusion

Despite extensive research in the field, the major problem in ocular drug delivery is the attainment of an optimal drug concentration at the site of action for a sufficient period of time. Nanotechnology offers a great opportunity for solving most of the significant barriers in ocular therapeutics and in the past years, a great number of materials have been proposed for the

development of nanotechnology-based ocular drug delivery systems. The selection of the right biomaterial depends on several factors, such as the nature of the drug, type of system, and target tissue, among others. Thus, the use of different biomaterials in the nanotechnology field can lead to the development of a wide variety of systems with different features which can be exploited to overcome the many limitations of the ocular route. Using rational design, new materials have been suggested, each one with its own important features such as mucoadhesiveness, the ability to interact with specific receptors, the ability to increase stability in the biological fluid and increase the effect of the drug. These nanotechnology-based products present some advantages over the classical systems employed today and they may fulfill current needs in the clinical practice of ocular delivery.

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Table II. Different synthetic polymers used in nanostructures for ocular treatment.

Polymer	Biomolecule	Target	Relevant aspects	Ref.
PECL	Indomethacin	Not specified	Increasing of drug concentration in different structures of the eye. Bioavailabity depends on the carrier size.	(76)
	Betaxolol	Ciliary body	PECL nanoparticles were more efficient than PIBCA and PLGA nanoparticles in reducing the intraocular pressure. Residence time is higher because the NPs undergo a process of agglomeration in the cul-de-sac.	(79)
	Carteolol	Ciliary body	Therapeutic results more pronounced with NCs than commercial solution. Reduced the cardiovascular side effects and the undesired non-corneal absorption.	(78)
	Metipranolol	Ciliary body	The NCs showed the same ability in reducing the intraocular pressure when compared with a commercial formulation, with a lower conjunctival absorption. NC led to the diminution of the cardiovascular side effects.	(77, 80)
	Cyclosporine A	Different structures	Cyclosporine A levels in cornea as well as its retention time were higher for the nanocapsules than for the oily solution.	(81-84)
		Different structures	Nanoparticles increase the amount of CyA found in cornea and conjunctiva. When covered with hyaluronic acid the NPs increased the absorption of the drug by the ocular tissues.	(85)
	Rhodamine	Corneal epithelium	PECL NCs covered with CS or PEG. Systems enhanced the penetration of the encapsulated dye. Penetration rate (transcellular pathway) depends on the coating composition: PEG accelerates the transport while CS favors the retention in the outer epithelium layers.	(86)
	-	Not specified	Nanocapsules coated with a cationic polymer (PLL) had a lower interaction with lysozyme and presented a higher stability.	(87)
PLA /PLGA	Antisense oligonucleotides	Retina	Cellular delivery was enhanced by the NPs. Protein inhibition was higher than free drug but lower than commercial transfection agent.	(93)

Polymer	Biomolecule	Target	Relevant aspects	Ref.
	Flurbiprofen	Corneal Epithelium	NPs enhanced residence time of the drug on the ocular surface as well as its sustained release. NP were also stable at long term storage at different temperatures	(89, 90)
	pDNA	Retina	NPs showed not to be toxic and its internalization depends on its concentration. Different plasmids were able to transfect RPE cells. *In vivo* expression after intravitreous administration.	(94)
	Betamethasone	Retina and choroid	NP administered intravenously accumulated in the retina and choroid, reducing the clinical signs of uveitis as well as undesired cell infiltration. Long time effect was achieved.	(92)
	6-coumarine	Conjunctive	Endocytosis of PLGA-NSs in primary cultured of conjunctival cells occurs mostly independently of clathrin and caveolin-1-mediated pathways.	(96)
	Acyclovir	Intraocular	PEG-coated and uncoated PLA-NSs showed a sustained acyclovir release and were highly tolerated by the eye. Increasing of aqueous levels of acyclovir and to better pharmacokinetics profile. The efficacy of the PEG-coated NP was higher than that of the simple PLA ones	(91)
	Fluorochrome	Vitreous	Intravitreous injection of PLA-NSs shoed a continuous liberation profile and with a preferential localization in the RPE cells.	(95)
	Budesonide	Retina	Sustained drug levels in retina and other tissues after in vivo administration. Inhibiting of VEGF secretion and its mRNA expression in ARPE-19 cells in a dose-dependent manner.	(88)
Eudragit	Blank NP	Corneal Epithelium	Eudragit RS100 and RL100 NSs appeared did not showed irritant effect on cornea, iris, and conjunctiva up to 24 h after application.	(74)
	Cloricromene	Corneal Epithelium	Eudragit RL100-NSs increased its ocular bioavailability and enhanced the biopharmaceutical profile (Cmax and AUC).	(70)
		Corneal Epithelium	Eudragit RS100 and RL100-NSs presented a controlled release profile and also increased the stability of cloricromene.	(69)

Polymer	Biomolecule	Target	Relevant aspects	Ref.
	Flurbiprofen	Intraocular	Eudragit RL100-NSs did not show to be toxic. Lower concentration of free drug was obtained for the NS in the conjunctival sac but miotic response comparable to a commercial formulation was achieved.	(71)
	Piroxicam	Uvea	Eudragit RS100-NSs exhibited a controlled released profile. More significant inhibition of inflammation *in vivo* with NC than with the drug suspension.	(72)
PBCA	Pilocarpine	Not specified	Evaluated the factors that influence the PBCA-NSs formation. Poor results after in vivo administration, mainly because of poor encapsulation.	(62-64)
			Improvement of pharmacokinetic and pharmacodynamic profile of the drug.	(60, 61)
	Amikacin	Not specified	Higher concentrations of amikacin in corneal tissue after administration. The surfactants employed are a key factor for achieve an effective formulation of PBCA-NSs.	(65, 66)
	Progesterone	Different structures	Higher concentrations of the drug were achieved when using PBCA-NSs.	(67)

PECL, poli-ε-caprolactone; PLA, poly(D-lactic acid); PLGA poly (dl-lactide-co-glycolide) CS, chitosan; PEG, polyethyleneglycol; NC, nanocapsule; NS nanosphere; PLL, poly-L-lisine; PBCA, polybutylcyanoacrilate.

List of Abbreviations

AUC= area-under-the-curve

CG = cationized gelatin

CLSM =confocal laser scanning microscopy

CS = chitosan

CyA = cyclosporine A

DC-cholesterol=3-b[N-(N0,N0-dimethylaminoethane)-carbamoyl]cholesterol

DDBA= dimethyldioctadecylammonium bromide

DOPE= 1,2-dioleoyl-3-phosphatidylethanolamine

DOTAP=1,2-dioleoyl-3-trimethyl ammoniumpropane

fl = fluoresceinamine

GM = glucomannan

HA = hyaluronan/ hyaluronic acid

HARE = hyaluronan receptor for endocytosis

HCE = human corneal cell

IOBA-NHC = normal human conjunctiva cell

NC = nanocapsule

NE = nanoemulsion

NP = nanoparticle

NS = nanosphere

OA= oleylamine

ON= oligonucleotide

PBCA = polybutylcyanoacrilate

pDNA = deoxyribonucleic acid plasmid

PECL= poli-ε-caprolactone

PEG = polyethyleneglycol

PLA= poly(D-lactic acid)

PLGA = poly (dl-lactide-co-glycolide)

PLL= poly-L-lysine

RPE= retinal pigment epithelium

SA= stearylamine

siRNA = small interference ribonucleic acid

SPM = spermine

TMAG= N-(a-trimethylammonioacetyl)-didodecyl-D-glutamate

VEGF = vascular endothelium growth factor

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ANTECEDENTES

1. La terapia génica ha despertado grandes expectativas para el tratamiento de enfermedades originadas por la expresión inadecuada de un gen[1,2]. A pesar de que estas expectativas no se han visto cumplidas en el caso de terapias sistémicas, el potencial es grande en el caso de terapias localizadas como la ocular[3]. Sin embargo, su aplicación clínica se encuentra actualmente limitada a causa de la necesidad de vehículos de material genético, inocuos y eficaces[4].

2. Entre los sistemas de vehiculización de material genético que presentan menores riesgos o toxicidad asociados se encuentran las nanopartículas. No obstante, la baja eficacia de transfección o los bajos niveles de expresión inducidos hace necesaria la optimización de los nanosistemas hasta la fecha propuestos o el diseño de nanovehículos en los que el balance eficacia/riesgo resulte más favorable.

3. La elaboración de sistemas nanoparticulares híbridos compuestos por la combinación de un polímero catiónico/aniónico ha sido propuesta con el fin de incrementar la capacidad de transfección al facilitar la interacción de los mismos con receptores específicos y, al mismo

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tiempo, reducir la toxicidad que exhiben los nanosistemas convencionales[5,6].

4. Los polímeros catiónicos empleados hasta la fecha para la elaboración de nanosistemas no se encuentran exentos de toxicidad y las limitaciones respecto a su seguridad representan un problema para las agencias regulatorias, de cara a autorizar formulaciones parenterales y oculares. Esta situación ha llevado a proponer otros polímeros catiónicos de origen natural. Las proteínas cationizadas son reconocidas en la actualidad por su potencial como agentes de transfección en diferentes tejidos, bajo la forma de complejos[7] o hidrogeles[8,9]. Sin embargo, su empleo en el desarrollo de formas farmacéuticas para la vía oftálmica todavía no ha sido explorado.

5. El sulfato de dextrano y sulfato de condroitino son polímeros aniónicos naturales, biocompatibles y biodegradables, con capacidad de interaccionar con una serie de receptores celulares[10,11], entre los

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cuales se destacan los receptores de internalización presentes en las células de la superficie ocular[12].

6. La MUC5AC es una mucina sintetizada en la superficie ocular por las células caliciformes de la conjuntiva[13]. Esta mucina es una de las principales responsables de la homeostasis del fluido lacrimal[14,15], presentándose en niveles anormalmente disminuidos en determinados procesos inflamatorios de la superficie ocular, como el síndrome del ojo seco[16,17]. En consecuencia, procesos patológicos asociados a deficiencias de mucinas como la MUC5AC serían una diana adecuada para la terapia génica. Sin embargo, hasta la fecha nadie ha propuesto este tipo de estrategia clínica.

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HIPÓTESIS

1. El diseño y desarrollo de nuevas nanopartículas basadas en proteínas cationizadas empleando la técnica de gelificación ionotrópica permitirá el desarrollo de nanosistemas capaces de asociar y proteger material genético, así como de facilitar su captación por las células de la superficie ocular e inducir su expresión significativa en la superficie ocular.

2. La incorporación de los polímeros aniónicos sulfato de condroitino o sulfato de dextrano en la estructura de las nanopartículas, permitirá el desarrollo de sistemas híbridos con mejores propiedades de los nanosistemas en lo que respecta a su toxicidad y potencial de transfección en la superficie ocular.

3. Las nanopartículas híbridas descritas en el apartado anterior tendrán capacidad para transfectar *in vivo* un plásmido que codifique la mucina MUC5AC, induciendo una expresión significativa de dicha mucina a nivel de la superficie ocular.

4. La expresión señalada en el punto anterior dará lugar a una respuesta biológica capaz de mejorar las condiciones patológicas que se presentan en el síndrome del ojo seco. Esta novedosa aproximación terapéutica se materializará al tratar animales afectados por un modelo de síndrome de ojo seco con los sistemas nanoparticulares en forma de colirio.

OBJETIVOS

Teniendo en cuenta los antecedentes expuestos y las hipótesis planteadas, el objetivo general de la presente memoria ha sido el diseño de nuevos sistemas nanoparticulares capaces de proporcionar una aproximación terapéutica totalmente novedosa al tratamiento del ojo seco. Esta aproximación terapéutica se basa en los principios de la terapia génica, empleando ADN plasmídico que codifica la mucina MUC5AC, una de las principales responsables de la homeostasis del fluido lacrimal. Para el diseño de los sistemas mencionados se ha pensado en la utilización de proteínas cationizadas conjuntamente con polímeros aniónicos de naturaleza polisacarídica. De este modo, dicho objetivo global puede dividirse en los siguientes objetivos específicos:

1. Conocer y desarrollar el potencial de sistemas nanoparticulares basados en proteínas cationizadas y evaluación de su potencial como vehículos de administración de ADN plasmídico.

Resultados recogidos en el artículo II:

"Modified protein-based nanoparticles for ocular gene therapy"

2. Optimizar y evaluar biológicamente el sistema más prometedor de proteína cationizada con la incorporación de los polianiones sulfato de condroitino y sulfato de dextrano.

Resultados recogidos en el artículo III:

"Hybrid nanoparticle design based on cationized gelatin and the polyanions dextran sulfate and chondroitin sulfate for ocular gene therapy"

3. Evaluar el potencial de los sistemas desarrollados para transfectar la superficie ocular con un plásmido de interés terapéutico que codifique la proteína MUC5AC, responsable por la homeostasis del fluido lacrimal.

Resultados recogidos en el artículo IV:

"Expression of MUC5AC in ocular epithelial cells using cationized gelatin nanoparticles"

4. Evaluación biológica del sistema nanoparticular más prometedor en animales con modelo de ojo seco inducido.

Resultados recogidos en el Artículo V:

"Treatment of dry eye using hybrid cationized gelatin and chondroitin sulfate nanoparticles loaded with MUC5AC plasmid"

ARTÍCULOS

Artículo II

Modified protein-based nanoparticles for ocular gene therapy

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Abstract

In this work we report a simple chemical modification of proteins to produce new biomaterials suitable for nanoparticles (NPs) development, and intended for ocular gene delivery. Concretely, the proteins atelocollagen, albumin and gelatin were cationized by attaching the amines spermine (SPM) or ethylenodiamine (ED) trough a carbodiimide reaction. After confirmation of the cationization process, the toxicity of the resulting cationized proteins was assayed *in vitro* in terms of cell viability in human cornea cells (HCE) and *ex vivo* hemolisys test. The toxicity profile of the cationized proteins was related to its molecular weight and the type of amine used to cationize it: spermine and high molecular weight led to a higher toxicity profile. NPs formulations based on these new biomaterials and associating a model plasmid (pEGFP) were formed by the ionic gelation technique, with size ranging from 50 nm to over 1 μm and zeta potential ranging from +9 to +19 mV. Increase in the plasmid stability against DNase I degradation was detected when associated to the NPs. Finally, the ability of NPs to transfect HCE cells was evaluated by the determination of expressed GFP fluorescence. The NPs transfection efficiency was influenced by the molecular weight of polymer as well as the amine used to cationize the protein. Based on the results of the different experiments, the most promising systems were the NPs formed with gelatin (137 kDa) cationized with SPM once exhibited acceptable toxicological profile, better protection of plasmid and higher transfection efficiency when comparing with the other formulations. In conclusion, the cationization of proteins is a useful strategy to create biomaterials able to form NPs capable to act as efficient vehicles for ocular gene delivery.

Keywords: cationized proteins, nanoparticles, gene therapy, ocular therapy.

1. Introduction

The eye possesses unique features for the development of successful gene therapy. Indeed, it is easily accessible and various routes of gene delivery can be used to target different layers or cell types in the eye; its small size and enclosed structure allow the use of low vector and/ or gene doses to achieve a therapeutic effect; and because of its status as immune privileged organ [1-3]. Therefore, gene therapy holds great promise for the treatment of eye diseases or abnormal conditions by replacing a defective gene with a normal gene[4].

In the last decade, modified proteins have got attention as new biomaterials for gene delivery. Concretely, the cationization of proteins is a new approach to favor the association to the genetic material and the internalization by the cells. Most of the studies were carried out using gelatin (CG), and only few reported other proteins as human serum albumin (HSA)[5,6]. Gelatin is a natural polymer that is derived from collagen, and is commonly used for pharmaceutical and medical applications because of its biodegradability and biocompatibility in physiological environments[7]. HSA belongs to a multigene family of proteins and is the major soluble protein of the circulating system with high blood concentration[8]. The polymers were cationized with low molecular amines such as spermine, ethylendiamine or spermidine and the new cationized polymers proved to be able to transfect several organs and tissues in a wide variety of forms as hydrogels or complexes[9-11]. Nevertheless, the use of cationized proteins in the preparation of nanoparticles was only limited. The nanoparticles (NPs) are a promising system to gene delivery due several special features as small size, ability to protect the genetic material and to be internalized by the cells. The few studies performed until now have employed techniques that require organic solvents and chemical crosslinkers[12,13]. So, important safety concerns that can limit the use of these NPs must be taken into account. In this scenario, ionic gelation technique shows as the most viable alternative to prepare NPs once it is an optimal technique for high hydrophilic drugs (as pDNA for example),

and it is very reproducible, requiring mild conditions for the NPs preparation[14].

In this context, we propose the cationization of three different proteins and their use to prepare NPs: gelatin, recombinant HSA and atelocollagen. Gelatin, because CG is successfully described as efficient for in vitro and in vivo transfection; atelocollagen because there is an available small interfering RNA (siRNA) transfection kit based on this polymer (AteloGene™, Koken, Japan); and albumin because there are already in the market a nanoparticulated medicine based on it (Abraxane®, Celgene, United States). So, the combination of the cationized polymers properties and the nanoparticulated structure could upgrade the transfection efficiency in comparison to the current available approaches for gene therapy.

2. Material and Methods

2.1 Materials

Type B Gelatin (238 KDa) was supplied by Kerala Chemicals and Proteins (Cochin, India) and type A gelatin (19 and 137 KDa) supplied by Nitta Gelatin (Ontario, Canada). Recombinant Human Serum Albumin (rHSA) was kindly gifted by Novozimes Biopharma (Nottingham, UK). Tripolyphosphate (TPP), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), spermine hydrochloride (SPM) and ethylenediamine (ED) were purchase from Sigma (Spain). The plasmid pEGFP was obtained from Elim Biopharmaceutics (US). The acid resin Amberlite IR-120 was purchased from Fluka (France) and basic resin Amberlite IRA 400(OH) was purchased from Supelco (Bellfonte, US).

2.2 Cationized polymers synthesis and characterization

The polymers were cationized as described previously[6]. Briefly, a 1% (w/v) polymer solution was prepared in 0.1 M phosphate buffer (pH 5.3), and for each mole of polymer carboxyl groups, 3 mol of EDC and 50 mol of SPM or

ED were added. The final pH was adjusted to 5.0 and the solution allowed to react for 18 h in a warm bath at 37ºC. After the reaction, the solution was dialyzed for 48 h, followed by lyophilization. For the isolectric point, a solution of 1% cationized protein (w/v) incubated at 37 ºC in a warm bath with a previously washed mix of acid and basic resins (1:2). After 30 minutes, the pH obtained from the solution, indicate the isoeletric point of the cationized protein. The characteristics of the cationized polymers are presented in the Table 1.

Table 1. Characteristics of proteins before and after their cationization

Polymer	**MW (kDa)**	**Side chain**	**$IEP_{initial}$**	**IEP_{final}**	**Final product abbreviation**
Gelatin	19	ED	5.0	9.80	CG_{19ed}
Gelatin	19	SPM	5.0	10.33	CG_{19spm}
Gelatin	137	ED	9.0	10.59	CG_{137ed}
Gelatin	137	SPM	9.0	11.03	CG_{137spm}
Gelatin	238	ED	9.0	10.67	CG_{238ed}
Gelatin	238	SPM	9.0	10.89	CG_{238spm}
Albumin	66	ED	5.0	9.35	$rHSA_{ed}$
Albumin	66	SPM	5.0	10.11	$rHSA_{spm}$
Atelocollagen	300	ED	7.0	9.87	$Atelo_{ed}$
Atelocollagen	300	SPM	7.0	9.81	$Atelo_{spm}$

2.3 Cell culture

Human corneal epithelial (HCE) cells were kindly gifted by Professor Arto Urtti (University of Helsinki, Finland). The cells were cultured in DMEN/F-12 with 15% fetal bovine serum, penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), 0.5% DMSO (Sigma, Spain), 10 ng/mL EGF (Invitrogen, Spain), and 0.1 µg/mL cholera toxin (Gentaur, Belgium). The

cells were maintained at 37ºC in a 5% CO_2 humidified atmosphere. Passage numbers 7 to 10 were used for the following experiments.

2.4 Polymer citotoxicity (XTT assay)

For the XTT assay, polymer solutions were prepared in DMEN-F12 and sterilized by filtration (0.22 μm, Millex®GV, Millipore, Ireland). HCE cells were seeded in a density of 10.000 cell/well in a 96-well microtiter plate (Nunc, Denmark). After 24 h, the culture medium was replaced by a serial dilution of polymers ranging from 50 μg/cm^2 to 500 μg/cm^2 and incubated for 3h hours. After this time the polymer solution was removed and replaced with culture media. The cell viability was assayed 24h later using XTT-based Toxicology Kit (Sigma, Spain) according to manufacturer instructions.

2.5 Hemolysis test

The blood collected in a previously weighted tube containing sodium citrate (25mM final concentration) was centrifuged at 700g for 10 minutes. The pellet was washed three times with cold PBS pH 7.4 by centrifugation at 700 g for 10 minutes and resuspended as 3% (w/v) solution in PBS. This suspension was incubated (1:1) with polymer solution dissolved in PBS (0.01 mg/mL to 5mg/mL) for 60 minutes at 37 ºC in shaking bath. After the incubation, the samples were centrifuged at 700g for 10 minutes and the supernatant was read in a multiplate reader at 520nm (Biorad, US). Red blood cells treated with PBS 7.4 were considered as 0% of lyses while those treated with a 0.2% (v/v) Triton X-100 solution of were considered as 100% of lyses.

2.6 Nanoparticle formation and characterization

The NPs were formed using the ionic gelation technique[15]. A solution of cationized polymer (2 mg/mL) was poured with magnetic stirring over a solution containing TPP (0.25 to 1.0 mg/mL) and the plasmid (0.033 mg/mL

final concentration in the NPs). The polymers and TPP solutions were previously sterilized by filtration (0.22 μm, Millex®GV, Millipore, Ireland). The mean particle size was determined by photon correlation spectroscopy (PCS). The samples were diluted to the appropriated concentration. Each analysis was carried out at 25ºC with a detection angle of 173º. The zeta potential was obtained by laser Doppler anemometry (LDA), measuring the mean electrophoretic mobility. The samples were diluted with a millimolar solution of KCl. The PCS and LDA analyses were performed with a Zetasizer 3000HS (Malvern, UK). The morphological analysis of the disperse phase was performed by transmission electron microscopy (TEM; JEM-1200 ExII, Jeol Japan). The samples were diluted in water (1:10) and 1% phosphotungstic acid solution used as a contrasting agent.

2.7 DNase I protection study

DNase I was added to the nanoparticles at a final concentration of 1 U DNase I/1 μg pDNA. The mixture was incubated at 37°C for 5 or 60 min. A 0.5 M solution of EDTA and 50 mg/mL solution of heparin were added to displace the pDNA from the nanoparticles. The samples were then analyzed by agarose gel electrophoresis and the integrity of the pDNA in each sample compared to untreated pDNA as a control.

2.8 Transfection studies in HCE cells and qualitative analysis of transfection

To evaluate the ability of the nanocarriers to transfect HCE cells, the cells were plated in a 24-well plate (Nunc, Denmark) at a density of 80,000 cells per well 24 h before the experiment. The NPs were incubated with the cells for 3 h (1 μg plasmid per well). Lipofectamine (Invitrogen, Spain) was used for a positive transfection control according to the manufacturer instructions (1 μg plasmid per well). The cell medium was changed each day after the transfection. The expression of GFP was detected at 48 h post-transfection

using an inverted microscope equipped with an attachment for fluorescent observation (Leica DMI 6000B, Leica Microsystems, GmB). Observations were made and images captured using a 40× objective.

2.9 Statistical analysis

The results are expressed as mean ±standard error of three independent experiments and were statistically analyzed by ANOVA, followed by Tukey test.

3. Results and Discussion

The potential of cationized proteins as biomaterials for nanoparticles prepared by ionic gelation technique is a new approach for safer gene therapy vehicles. This strategy can combine several important characteristics such as: the high transfection properties of cationized polymers; the nanoparticulated structure able to protect the pDNA; the mild conditions of ionic gelation technique for NPs preparation.

After the synthesis of new chemical entities it is important to certify that these molecules are suitable for pharmaceutical applications. Although cationized gelatin and cationized albumin have been described for gene therapy, there is a lack of information about the toxicity of these polymers[10,16]. Once it is the first time that these polymers are proposed for ocular route, a cell viability assay with the cationized polymers was performed in HCE cells. As it can be observed in the Figure 1, the toxicity depends mainly on the type of amine attached to the polymer backbone. None of the polymers cationized with ED induced to changes in the cell viability while those cationized with SPM, affected cell viability depending on the type of protein and its MW.

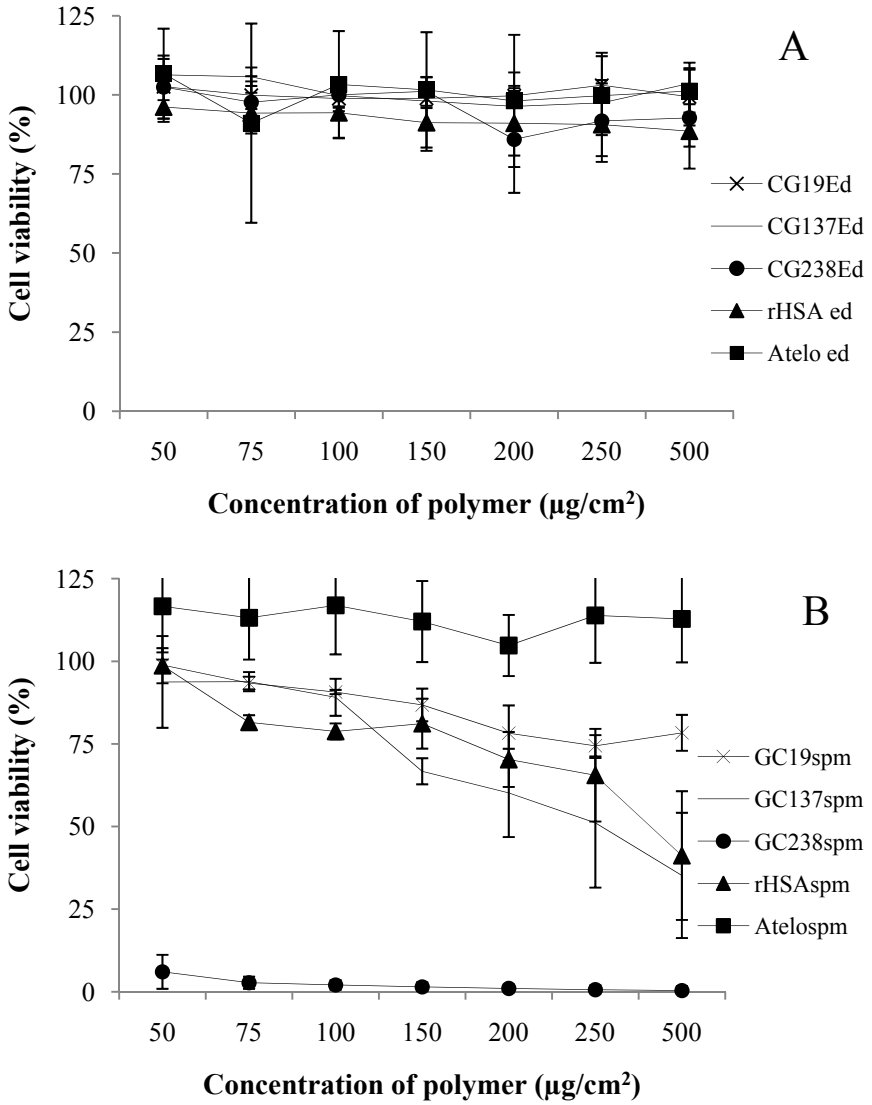

Figure 1. Percentage of viability of HCE cells after incubation of the different proteins CG_{19} cationized with ethylenediamine (A) or spermine (B).

This can be explained by the number of amine groups presented in each molecule, 2 for ED and 4 for SPM, being the isoelectric point (IEP) of the polymers cationized with SPM is higher than those cationized with ED (Table 1). Indeed, the amount of free amine groups is an important factor

when analyzing toxicity of any material once the positively charged groups usually are responsible for the toxic behavior. Other important factor in the polymers toxicity is their molecular weight (MW). The polymer toxicity was clearly influenced by the MW: CG_{238spm} > CG_{137spm} > CG_{19spm} (Figure 1B). Molecules with high MW usually have higher toxicity then their analogous with smaller MW, as previously reported for some cationic polymers as observed for polyethylenimine[17], DEAE-dextran[18] and quarternized cellulose[19]. The reason why $Atelo_{spm}$ exhibited low toxicity (even with high MW) can be attributed to its lower IEP (Table 1).

Table 2. Percentage of hemoglobin released upon incubation of the different cationized proteins at different concentrations with red blood cell suspension.

	Concentration (mg/mL)				
	0.1	**0.5**	**1.0**	**2.5**	**5.0**
CG_{19ed}	1.5 ±0.5	1.6 ±0.5	1.0 ±0.3	0.7 ±0.5	0.1 ±1.0
CG_{19spm}	1.4 ±0.2	1.2 ±0.5	1.4 ±0.3	1.1 ±0.4	0.6 ±0.3
CG_{137ed}	1.1 ±0.2	0.9 ±0.6	0.8 ±0.6	0.5 ±0.4	0.3 ±0.6
CG_{137spm}	1.6 ±1.0	1.7 ±1.0	1.9 ±1.2	1.6± 2.4	1.1 ±1.2
CG_{238ed}	2.7 ±1.1	2.5 ±1.3	2.6 ±1.6	3.1 ±2.3	2.9 ±2.8
CG_{238spm}	2.7 ±0.9	2.1 ±0.3	1.9 ±0.2	1.4 ±1.2	0.5 ±0.9
$rHSA_{ed}$	0.1 ±0.6	-0.4 ±0.5	-0.5 ±0.7	-0.4 ±0.2	-0.4 ±0.3
$rHSA_{spm}$	0.8 ±1.8	1.7 ±3.2	1.2 ±2.1	2.8 ±5.0	2.8 ±3.3
$Atelo_{ed}$	0.1 ±0.6	-0.4 ±0.5	-0.5 ±0.7	-0.4 ±0.2	-0.4 ±0.3
$Atelo_{spm}$	0.4 ±0.8	0.9 ±0.5	0.7 ±1.2	0.30 ±1.2	0.6 ±0.9

Despite of the cell viability assay, the membrane-damaging property of cationized proteins was evaluated by determining the percentage of hemoglobin released upon incubation of the polymer solution with freshly extracted red blood cells, as it directly correlates with the degree of hemolysis caused. The results presented in Table 2 clearly shows a high

compatibility of the polymers with red blood cells, as low levels of hemoglobin release were detected in all cases (<5%). It is important to observe that the results of in vitro toxicity assays for eye cells and structures must be analyzed carefully. There is almost instantaneous dilution, tear clearance and continuous action of the eye lids in vivo cannot be reproduced. Therefore, the concentrations used in vitro usually cannot be recapitulated in vivo, and consequently the toxic effects observed in vitro can be overestimated[20].

The physicochemical parameters of the NPs could be modulated in a very limited extend by changing the ratios of the crosslinker (TPP) and the polymer. As it can be observed in the Table 3, the size of the NPs remained practically unchanged in respect of ratio changes for all types of CG and $Atelo_{spm}$. Meanwhile, cationized rHSA and $Atelo_{ed}$ NPs were formed in a narrow extension. The sizes of the CG-NPs ranged from 60-190 nm and were inferior to those described for CG complexes (200-700 nm)[21] and CG-NPs prepared by coacervation (200-300 nm)[12,13,16]. The formation of NPs from cationized HSA was only possible for small amounts of crosslinker once precipitation phenomena took place and its size (around 60-80nm) was smaller than those related to cationized HSA complexes[5]. The difficulty of $Atelo_{ed}$ in forming NPs is probably due the lack of polymer flexibility and less strength of ethylendiamine in interacting with TPP and the pDNA. The positive zeta potential of the NPs ranged from +9 to +19 mV (Table 4). These values are in accordance to those previously reported for gelatin complexes (around +10 mV)[21] and gelatin NPs prepared by coacervation (+1 to +25 mV)[16]. Keeping the zeta potential far from a neutral value is important for increasing the stability of the systems once electrostatic repulsion will take place. Also, positive zeta potential seems to be very important for increasing the interactions among the NPs and the cell membrane, leading to a higher efficacy of the system, particularly for ocular drug delivery[22].

Table 3. Influence of the ratio between the cationized polymers and the crosslinker in the mean size (nm) of the nanoparticles (2.5% pDNA loading).

	Ratio (Polymer:TPP)		
	4:1	**8:1**	**20:1**
GC_{19ed}	ppt	190 ±82	94 ±25
GC_{19spm}	ppt	76 ±2	160 ±14
GC_{137ed}	99 ±17	113 ±10	116 ±33
GC_{137spm}	81 ±7	187 ±23	134 ±29
GC_{238ed}	84 ±1	64 ±8	79 ±15
GC_{238spm}	73 ±12	190 ±8	131 ±35
$rHSA_{ed}$	ppt	ppt	77 ±12*
$rHSA_{spm}$	ppt	ppt	58 ±2
$Atelo_{ed}$	-	-	1629 ±128
$Atelo_{spm}$	237 ±22	277 ±80	236 ±26

* ratio 40:1; "-" no nanoparticle formation, "ppt" precipitation

Table 4. Influence of the ratio between the cationized polymers and the crosslinker in the zeta potential (mV) of the nanoparticles (2.5% pDNA loading).

	Ratio Polymer:TPP		
	4:1	**8:1**	**20:1**
GC_{19ed}	ppt	+12 ±1	+14 ±1
GC_{19spm}	ppt	+14 ±2	+13 ±6
GC_{137ed}	+10 ±2	+12 ±1	+13 ±1
GC_{137spm}	+12 ±1	+16 ±2	+15 ±5
GC_{238ed}	+12 ±1	+ 19 ±3	+18 ±4
GC_{238spm}	+13 ±2	+19 ±2	+16 ±7
$rHSA_{ed}$	ppt	ppt	+18 ±5*
$rHSA_{spm}$	ppt	ppt	+15 ±4
$Atelo_{ed}$	-	-	+11 ±1
$Atelo_{spm}$	+9 ±1	+9 ±1	+12 ±4

* ratio 40:1; "-" no nanoparticle formation; "ppt" precipitation

The morphology of the NPs was analyzed by TEM (Figure 2). The cationized rHSA and CG systems presented homogenous populations of spherical particles with a nanometer size similar to the one obtained by light scattering technique. The different MW of gelatin did not influenced in the shape and structure of the NPs, as well as the fact of being cationized with ED or SPM. However, the $Atelo_{ed}$ and $Atelo_{spm}$ NPs presented peculiar structures (Figure 2I and 2J) more like aggregates or complexes. As afore mentioned, it could be due the more rigid structure of atelocollagen that difficult the formation of NPs.

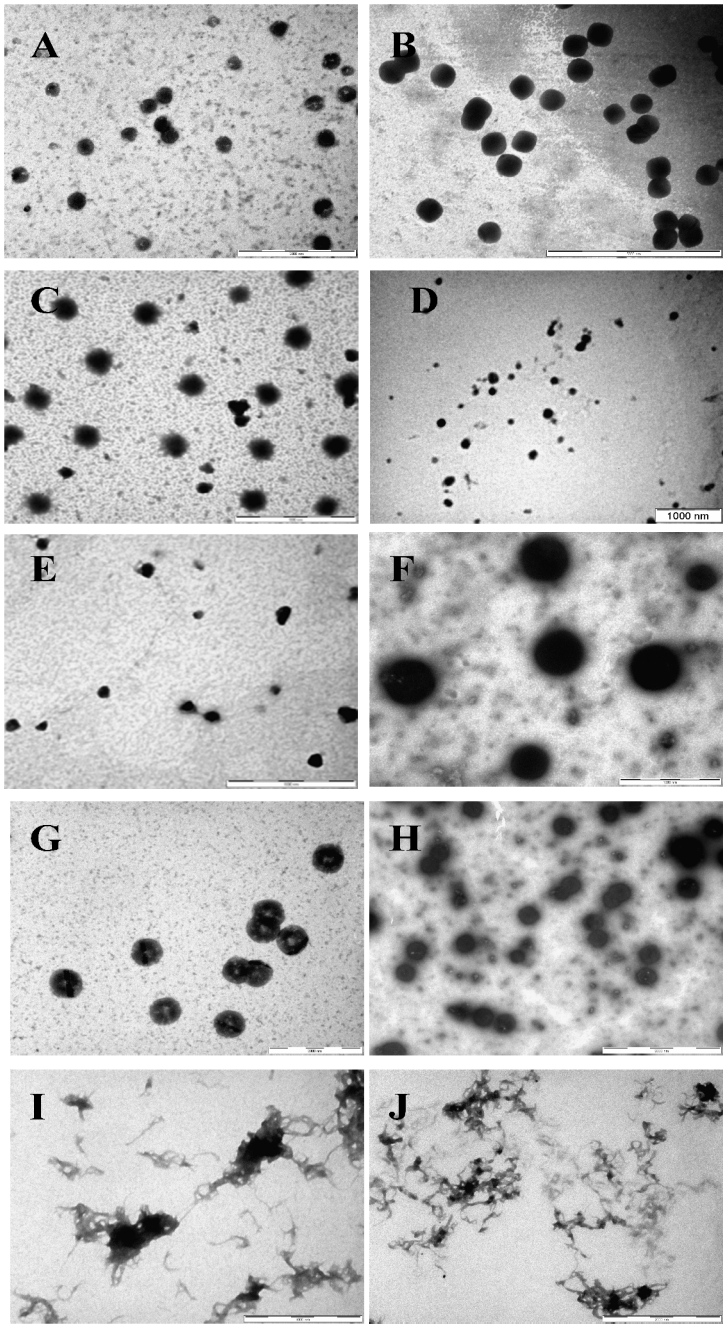

Figure 2. Transmission electron microscopy images at different magnifications of the nanoparticles formed with the cationized proteins GC_{19ed} NPs (A), GC_{19spm} NPs (B), GC_{137ed} NPs (C), GC_{137spm} NPs (D), GC_{238ed} NPs (E), GC_{238spm} NPs (F), $rHSA_{ed}$ NPs (G), $rHSA_{spm}$ NPs (H), $Atelo_{ed}$ NPs (I) and $Atelo_{spm}$ NPs (J).

The plasmid DNA is a molecule extremely labile, susceptible to the degradation by the nucleases spread over the different tissues. In the eye surface, the presence of nucleases in tears is one important barrier that the carrier must overcome[23,24]. The DNase I protection assay is generally recognized as valuable to estimate the protection of pDNA. For that, the NPs were incubated for 1 h in the presence of DNase I and the integrity of pDNA was accessed by means of electrophoresis migration. As it can be seem in Figure 3, except for the cationized atelocollagen NPs, all other systems protect the plasmid DNA from DNase I degradation. The difficulty of cationized atelocollagen in forming NPs could be the reason for this lack of protection. It is known that in complexes of pDNA-atelocollagen, the plasmid DNA surrounds the atelocollagen backbone[25]. Without the formation of the NPs there is an exposure of the DNA structure to DNase degradation. In comparison between ED and SPM cationized polymers, those cationized with SPM were more efficient in protect the plasmid DNA from the enzymatic degradation. This was expected once polyamines, such as SPM, are able to inhibit the activity of endonucleases, including DNase I. This protective ability of polyamines is attributable to the formation of a steric barrier against DNA-damaging agents and their ability to condense the DNA[26-28].

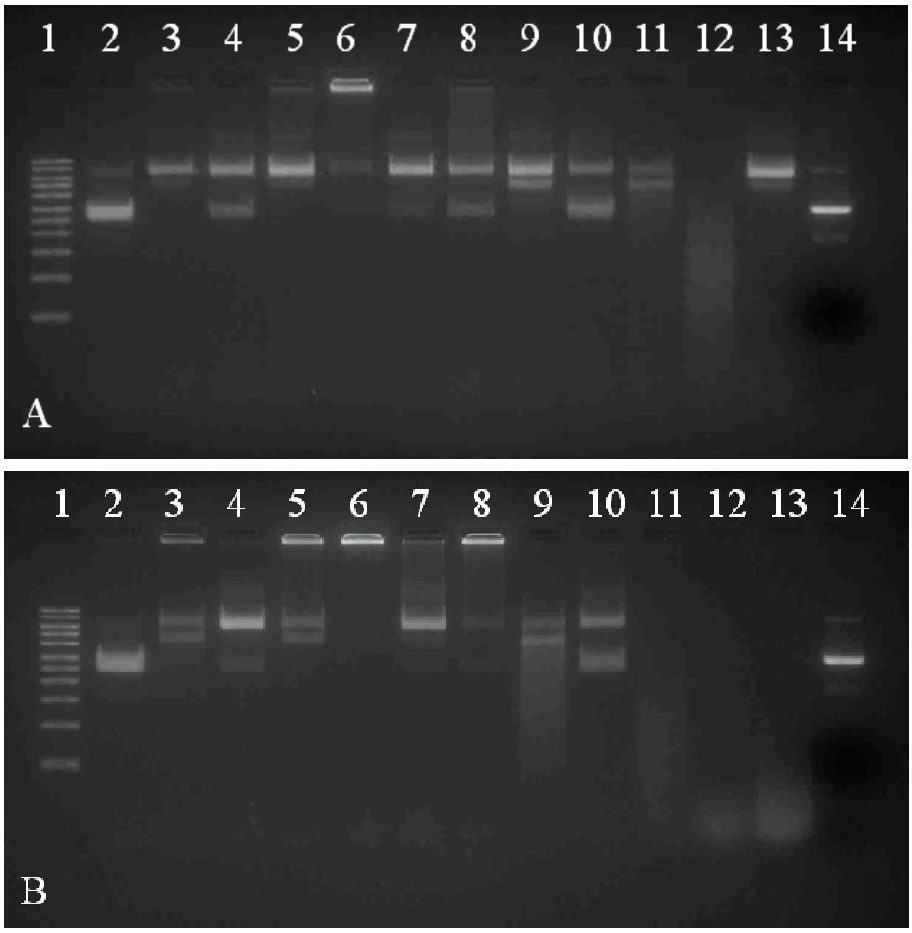

Figure 3. Nuclease stability of pDNA associated to different cationized proteins nanoparticles at 15 (A) and 60 minutes (B). Lane 1: DNA ladder; lane 2 naked pDNA + heparin; lane 3: GC_{19ed} NPs (20:1); lane 4: GC_{19spm} NPs (8:1); lane 5: GC_{137ed} NPs (20:1); lane 6: GC_{137spm} NPs (8:1); lane 7: GC_{238ed} NPs (20:1); lane 8: GC_{238spm} NPs (8:1); lane 9: $rHSA_{ed}$ NPs (40:1); lane 10: $rHSA_{spm}$ NPs (20:1); lane 11: $Atelo_{ed}$ NPs (20:1); lane 12: $Atelo_{spm}$ NPs (20:1); lane 13: pDNA + DNAse I; lane 14: naked pDNA.

Finally, we evaluated the ability of the NPs to transfect HCE cells using the model plasmid that encodes enhanced green fluorescent protein (EGFP). As it can be seem in the Figure 4, the transfection efficiency strongly varied among the systems. However, none of them was capable to induce the same

expression level as the Lipofectamine control. The order of efficiency to transfect corneal cells was: $GC_{19ed} < GC_{19spm} = Atelo_{ed} = rHSA_{ed} < Atelo_{spm} = rHSA_{spm} < GC_{137ed} < GC_{238ed} < GC_{137spm} = GC_{238spm}$ NPs. All NPs based on polymers cationized with SPM had better performance in transfect HCE cells when comparing to those based on polymers cationized with ED. For hydrogels implants in rat femoral muscle, it is reported no difference between the tranfection efficiency of gelatin cationized with SPM and ED[10]. The MW influenced the transfection in a lower extend. As it can be observed for the CG, higher MWs led to higher transfection efficiency. For other polymers like cationized cellulose[19] and poly-L-lysine (PLL)[29], higher MW chains seems to increase the transfection in vitro too. This has been attributed to more stable interactions between the pDNA and the high MW polymers. Based on these results the gelatin with intermediate molecular weight cationized with SPM resulted to be the best polymer among all assayed.

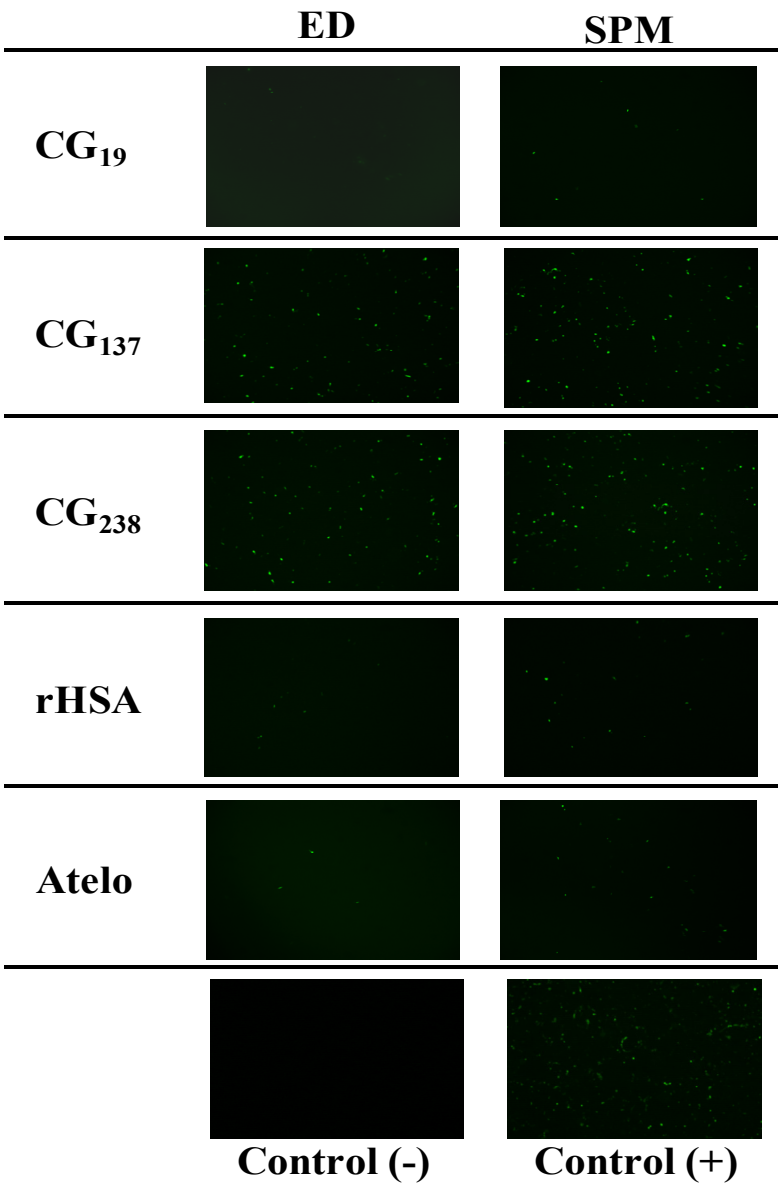

Figure 4. Expression of enhanced green fluorescent protein after transfection of HCE cells with NPs of different cationized proteins. The nanoparticles were incubated over the cells for 3h (1µg pEGFP/well). The cells were examined under a microscope of fluorescence at 48 h post-transfection and the fluorescence compared to untreated cells (Control -) and Lipofectamine®-treated cells (Control +). GC_{19ed} NPs (20:1); GC_{19spm} NPs (8:1); GC_{137ed} NPs (20:1); GC_{137spm} NPs (8:1); GC_{238ed} NPs (20:1); GC_{238spm} NPs (8:1); $rHSA_{ed}$ NPs (40:1); $rHSA_{spm}$ NPs (20:1); $Atelo_{ed}$ NPs (20:1); $Atelo_{spm}$ NPs (20:1).

4. Conclusion

The feasibility of using cationic proteins as biomaterial for the preparation of NPs by ionic gelation technique has been studied. Concretely, the proteins gelatin, albumin and atelocollagen have been selected for this study and spermine and ethylendiamine as amines to be attached to the protein backbone. The toxicity and effectiveness depends on the type of protein as well as the amine linked to its chains. Among the cationized polymers, nanoparticles based on gelatin cationized with spermine proved to be the best nanoparticulated system for protecting pDNA and transfecting cells of human corneal epithelium. In conclusion, we propose cationized proteins as promising biomaterials to form nanoparticles intended for gene therapy, especially in the eye surface.

5. Acknowledgements

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Artículo III

Hybrid nanoparticle design based on cationized gelatin and the polyanions dextran sulfate and chondroitin sulfate for ocular gene therapy

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Submitted for evaluation

Abstract

We describe the development of hybrid nanoparticles composed of cationized gelatin and the polyanions chondroitin sulfate (CS) and dextran sulfate (DS) for gene therapy in the ocular surface. The physicochemical properties of the nanoparticles that impact their bioperformance, such as average size and zeta potential, can be conveniently modulated by changing the ratio of polymers and the crosslinker These systems associate plasmid DNA and are able to protect it from DNase I degradation. We corroborated that the introduction of CS or DS in the formulation decreases the in vitro toxicity of the nanoparticles to human corneal cells without compromising the transfection efficiency. These nanoparticles are potential candidates for the development of safer and more effective nanomedicines for ocular therapy.

Keywords: drug delivery systems, nanotechnology, ocular gene therapy, hybrid nanoparticles, ionic gelation

Introduction

Gene therapy may become a powerful therapeutic modality in the treatment of several ocular diseases by introducing, into the ocular cells, genes that encode down-regulated proteins.[1] This approach has potential as both a therapy and a method for studying disease mechanisms, holding great promise for the treatment of diseases and as a proof-of-principle of its efficacy in animal models and humans.[2]

The ocular surface is an attractive target for gene therapy because of its immune-privileged nature and accessibility.[3] Local gene delivery has the potential to achieve low and continuous concentrations of biologically active molecules, thereby improving treatment efficacy and safety. Due to the importance of gene therapy and its inherent advantages, some studies have investigated new treatments for major eye disorders.[4]

The use of nanoparticles as carriers in the delivery of therapeutic genetic materials to target tissues has become popular in recent years for the treatment of a wide range of ocular diseases. However, the success of a nanoparticulate system is closely related to the selection of the material on which it is based.[5,6] Among the polymers currently under investigation for non-viral gene delivery, cationized gelatin (CG) has demonstrated great potential in transfecting several types of cells in the form of hydrogels or complexes.[7-10] We were the first to attempt the use of CG to form nanoparticles using the ionic gelation technique; only recently, the use of these CG nanoparticles for transfecting the ocular epithelium was proposed.[11] Gelatin cationized with the low molecular polyamine spermine was the most promising among the studied cationized proteins in terms of safety, formation of nanoparticles with suitable physicochemical properties, protection of plasmid DNA, and transfection efficiency in human corneal cells.

A new generation of polymeric nanoparticles, the so-called hybrid nanoparticles, is based on the combination of different polymers in a manner

that takes advantage of the distinguished properties of each polymer.[12] This approach has been successfully described for chitosan/hyaluronic acid nanoparticles, which are capable of transfecting ocular epithelial tissues after topical instillation both *in* vitro and in vivo, in greater extent than non-hybrid nanoparticles.[6,13]

The polymers chondroitin sulfate and dextran sulfate have been suggested as materials for the design of drug delivery systems and are potential candidates for hybrid systems.[14-17] Chondroitin sulfate (CS) is a glycosaminoglycan, a major component of the extracellular matrix, and important in maintaining the structural integrity of tissues and organs, including the eye. Dextran sulfate (DS) is a natural sulfated polysaccharide used in the biomedical field, as it is both biodegradable and biocompatible. One of the most attractive properties of these polymers is likely the ability to interact with the hyaluronic acid receptor for endocytosis (HARE) and CD44, both of which are found in the eye and related to the internalization of macromolecules.[18-21] Furthermore, the addition of anionic polymer scan decrease the toxic behavior exhibited by positively charged molecules.[22]

The objective of the present study was to prepare hybrid nanoparticles made of CG and CS or DS using the ionic gelation technique and induce gene expression in the ocular epithelium. The presence of these polyanions is expected to favor transfection in ocular cells and the toxicological profile of the nanoparticles.

Experimental Part

Materials

Type A gelatin (137 KDa) was purchased from Nitta Gelatin (Canada). Tripolyphosphate (TPP), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), spermine hydrochloride (SPM), DS, and heparin were purchased from Sigma (Spain). The CS was supplied by Calbiochem (United

States). The pEGFP plasmid was obtained from Elim Biopharmaceutics (United States), and DNase I was purchased from Qiagen (Spain).

Synthesis of cationized gelatin

The gelatin was cationized as described previously.[8,9] Briefly, a 1% (w/v) gelatin solution was prepared in 0.1 M phosphate buffer (pH 5.3), and for each mole of gelatin carboxyl groups, 3 mol of EDC and 50 mol of spermine were added. The final pH was adjusted to 5.0 and the solution allowed to react for 18 h in a warm bath at 37ºC. After the reaction, the solution was dialyzed for 48 h, followed by lyophilization. The resulting polymer was called CG_{137spm}.

Nanoparticles formation and characterization

The nanoparticles were formed using the ionic gelation technique.[23] A solution of CG_{137spm} (1 mg/mL) was poured with magnetic stirring over a solution containing TPP (0.1 to 1.0 mg/mL), the plasmid (0.05 mg/mL final concentration in the nanoparticles), and CS or DS solution (0.1 to 1.0 mg/mL). The polymer and TPP solutions were previously sterilized by filtration (0.22 μm, Millex®GV, Millipore, Ireland).

The mean particle size and the size distribution of the nanoparticles were determined by photon correlation spectroscopy (PCS). Samples were diluted with filtered water and the analysis was carried out at 25ºC. The zeta potential values of the nanoparticles were obtained by Laser Doppler Anemometry (LDA), measuring the mean electrophoretic mobility. Samples of the nanoparticle suspensions were diluted with 1 mM KCl. The PCS and LDA analysis were performed with a Zetasizer® 3000HS (Malvern Instruments, UK).

The morphological analysis of the disperse phase was performed by transmission electron microscopy (TEM; JEM-1200 ExII, Jeol Japan). The samples were diluted in water (1:10) and 1% phosphotungstic acid solution

was used as a contrasting agent. The association efficiency was evaluated using the PicoGreen® reagent (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes, Spain) following the manufacturer instructions. The amount of free pDNA in the nanoparticle supernatants was quantified after centrifugation in a Microfuge 22-R (Beckmann Coulter, GmB) for 30 minutes at 11,000 g.

DNase I protection study

DNase I was added to the nanoparticles associating pDNA at a final concentration of 1 U DNase I/1 µg pDNA. The mixture was incubated at 37°C for 5 or 60 min. A 0.5 M solution of EDTA and 50 mg/mL solution of heparin were added to displace the pDNA from the nanoparticles. The samples were then analyzed by agarose gel electrophoresis and the integrity of the pDNA in each sample compared to untreated pDNA as a control.

Cell culture

Human corneal epithelial (HCE) cells were kindly gifted by Professor Arto Urtti (University of Helsinki, Finland). The cells were cultured in DMEN/F-12 with 15% fetal bovine serum, penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), 0.5% DMSO (Sigma, Spain), 10 ng/mL EGF (Invitrogen, Spain), and 0.1 µg/mL cholera toxin (Gentaur, Belgium). The cells were maintained at 37ºC in a 5% CO_2 humidified atmosphere. Passage numbers 38 to 44 were used for the following experiments.

Nanoparticles cytotoxicity: XTT assay

HCE cells were seeded in a 96-well microtiter plate (Nunc, Denmark) at a density of 10,000 cells/well. The culture medium was replaced after 24 h by a serial dilution of nanoparticles ranging from 1 to 150 µg nanoparticles/cm^2 CG and the plates incubated for 3 h. Finally, the nanoparticles were removed and their viability assayed using an XTT-based *in vitro* toxicology assay

(Sigma, Spain) according to the manufacturer instructions. The IC_{50} represents the inhibitory concentration of nanoparticles that produced 50% cell viability.

Cellular uptake of nanoparticles

Nanoparticles were prepared with 7.5% Cy3-labeled pDNA (Mirus, US) according to the previously described procedure. HCE cells were seeded on multi-chamber covers (Nunc, Denmark) at a density of 4×10^4 cells per well. Twenty-four hours before the experiment, 40 μL of nanoparticles were incubated with the cells (1 μg pDNA/well). After 1 h incubation, the cells were rinsed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X100. The cell nuclei were stained with DAPI, and F-actin was stained with BODIPY-phalloidin according to the manufacturer's instructions. The fluorescence was analyzed with a confocal laser scanning microscope (Leica TCS SP2, Leica Microsystems, GmB). Free labeled plasmid solution (0.05 μg/μL) was used as a control and incubated as described.

Transfection analysis

To evaluate the ability of the nanocarriers to transfect HCE cells, the cells were plated in a 24-well plate (Nunc, Denmark) at a density of 80,000 cells per well 24 h before the experiment. The nanoparticles were incubated with the cells for 3 h (1 μg plasmid per well). Lipofectamine (Invitrogen, Spain) was used for a positive transfection control according to the manufacturer's instructions (1 μg plasmid per well). The cell medium was changed each day after the transfection. The expression of GFP was detected at 24, 48, and 72 h post-transfection using an inverted microscope equipped with an attachment for fluorescent observation (Leica DMI 6000B, Leica Microsystems, GmB). Observations were made and images captured using a 40× objective.

Statistical analysis

The results were expressed as mean ± standard error of three independent experiments and statistically analyzed by ANOVA, followed by the Tukey test.

Results and Discussion

Nanoparticles formation and characterization

The use of CG associating DNA is of interest for gene therapy. Most studies have focused on employing the CG as a hydrogel or complex, but some have used it to prepare nanoparticles by desolvation/coacervation. Even though nanoparticles have been formed, this technique has two major drawbacks: the use of organic solvents and the use of toxic chemicals as crosslinkers. Thus, the clinical application of the resulting nanoparticles is hampered by safety concerns. Recently, we suggested CG as a biomaterial for forming nanoparticles using the ionic gelation technique in order to more safely deliver genes to the eye surface. We found that gelatine cationized with spermine of intermediate molecular weight (CG_{137spm}) is the most promising biomaterial among several other cationized proteins.[11]

In order to improve the properties of CG_{137spm} nanoparticles, we combined a natural polyanion, CS or DS, to generate new hybrid nanoparticles. The influence of polyanions in the morphology of the nanoparticles was analyzed by TEM. The systems have homogenous populations of spherical particles with a nanometer size similar to the one obtained by light scattering technique (**Figure 1**). Apparently, the presence of the anionic polymers CS and DS in the nanoparticles (Figure 1B and 1C, respectively) did not induce any alteration in the shape of the nanostructure compared to nanoparticles based only on CG_{137spm} (Figure 1A).

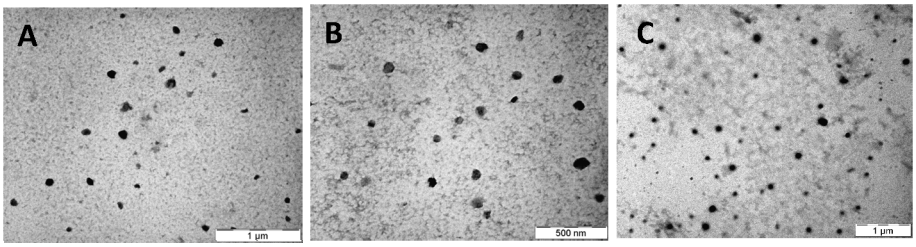

Figure 1. TEM images of CG_{137spm} nanoparticles (A), CG_{137spm}/CS nanoparticles (B), and CG_{137spm}/DS nanoparticles (C).

The effects of the addition of the anionic polymers on the mean size and zeta potential of the particles are presented in Table 1 to 5. The physicochemical parameters of the nanoparticles can be conveniently modulated in order to obtain systems with different properties simply by changing the proportions of the reticulant agent and/or anionic polymers in relation to CG. Both positive and negative zeta potential can be obtained, ranging from -38 to +48 mV when using CS and from -55 to +39 mV for DS. Keeping the zeta potential far from a neutral value is important for increasing the stability of the systems once electrostatic repulsion occurs.[24] Indeed, zeta potentials close to neutral result in a tendency to aggregate for both CS- and DS-based systems (**Table 1 and 2**). A positive zeta potential seems to be very important for increasing the interactions among the nanoparticles and the ocular surface, leading to a higher efficacy of the system, particularly for ocular drug delivery.[25] However, the possibility of producing nanoparticles with negative zeta potential may be of interest for other administration routes in which a negative zeta potential is vital for safe and successful carriers once minimal interaction among the nanoparticles and the biological environment (i.e. intravitreous glycosaminoglycans or blood platelet) is achieved.[15] Differences in the pattern of nanoparticles formation were observed for both anionic polymers; DS systems can be obtained just in a narrow range of polymer ratios and, concretely, at ratios lower that 1.5:1 no nanoparticle formation occurred, whereas CS nanoparticles can be obtained at ratios as

low as 0.75:1. The explanation for the different formation conditions can be found in the structure of both polymers; DS is more sulfated than CS. In protein-polyanion interactions, the interaction between basic amino groups and sulfate groups is one of the most important.[26,27] Binding affinity basically depends on the ability of the glycosaminoglycan sequence to provide optimal charge (orientation of sulfate groups) and surface (van der Waals contact) complementarity with the protein, but after the cationization process, the electrostatic interactions become more important than the hydrophobic interactions.[26] This difference is also reflected in the size of the nanoparticles; the incorporation of DS (**Table 4**) leads to particles with a slightly smaller size than using CS (**Table 3**), with the particles reaching a maximum value of 200 nm for DS and 300 nm for CS.

Table 1. Influence of the polyanion chondroitin sulfate (CS) and the reticulant agent tripolyphosphate (TPP) on the size (nm) of the nanoparticles loaded with 5% pDNA.

		Ratio CG_{137spm}/TPP[a]			
		30/1	**12/1**	**6/1**	**4/1**
Ratio CG_{137spm}/CS	15/1	167 ± 7	218 ± 34	253 ± 12	301 ± 28
	6/1	186 ± 20	258 ± 23	290 ± 20	+
	3/1	253 ± 47	278 ± 32	270± 76	+
	2/1	202 ± 11	197 ± 7	+	+
	1.5/1	173 ± 20	+	+	+
	1/1	+	+	+	236 ± 13
	0.75/1	168 ± 2	170 ± 17	155 ± 14	153 ± 14

[a] "+" precipitation

Table 2. Influence of the polyanion chondroitin sulfate (CS) and the reticulant agent tripolyphosphate (TPP) on the zeta potential (mV) of GC_{137spm} nanoparticles loaded with 5% pDNA.

		Ratio CG_{137spm}/TPP[a]			
		30/1	**12/1**	**6/1**	**4/1**
Ratio CG_{137spm}/CS	15/1	48 ± 2	42 ± 4	35 ± 7	25 ± 3
	6/1	40 ± 2	40 ± 5	37 ± 11	+
	3/1	42 ± 3	38 ± 2	35 ± 14	+
	2/1	36 ± 3	29 ± 4	+	+
	1.5/1	35 ± 3	+	+	+
	1/1	+	+	+	-10 ± 16
	0.75/1	-36 ± 1	-34 ± 7	-37 ± 2	-38 ± 2

[a] "+" precipitation

Table 3. Influence of the polyanion dextran sulfate (DS) and the reticulant agent tripolyphosphate (TPP) on the size (nm) of GC_{137spm} nanoparticles loaded with 7.5% pDNA.

		Ratio CG_{137spm}/TPP[a]			
		30/1	**12/1**	**6/1**	**4/1**
Ratio CG_{137spm}/DS	15/1	113 ± 17	121 ± 20	140 ± 27	+
	6/1	127 ±15	118 ± 23	+	+
	3/1	75 ± 7	+	+	+
	2/1	73 ± 5	76 ± 4	87 ± 13	102 ± 15
	1.5/1	21 ± 24	216 ± 23	216 ± 24	213 ± 58
	1/1	-	-	-	-
	0.75/1	-	-	-	-

[a] "+" precipitation; "-" no formation

Table 4. Influence of the polyanion dextran sulfate (DS) and the reticulant agent tripolyphosphate (TPP) on the zeta potential (mV) of GC_{137spm} nanoparticles loaded with 5% pDNA.

		Ratio CG_{137spm}/TPP[a]			
		30/1	**12/1**	**6/1**	**4/1**
Ratio CG_{137spm}/DS	15/1	36 ± 6	39 ± 8	32 ± 6	+
	6/1	47 ± 18	39 ± 8	+	+
	3/1	35 ± 6	+	+	+
	2/1	-32 ± 6	-32 ± 4	-32 ± 2	-42 ± 3
	1.5/1	-44 ± 7	-44 ± 14	-55 ± 6	-47 ± 5
	1/1	-	-	-	-
	0.75/1	-	-	-	-

[a] "+" precipitation; "-" no formation

In order to proceed with the biological evaluation of the nanoparticles in cell culture, three different systems were chosen: (i) CG_{137spm} nanoparticles, (ii) CG_{137spm}/CS nanoparticles, and (iii) CG_{137spm}/DS nanoparticles (**Table 5**). The selection was based on their size (<200 nm) and positive zeta potential, important features that facilitate interactions with the cell membrane[25] and increase the internalization process[28] in order to achieve higher transfection levels. The association efficiency of pDNA to the nanoparticles was determined using the PicoGreen® reagent. The results showed a high association efficiency, over 85%, for the systems, which can be attributed to the interaction between the spermine linked to the gelatin and DNA.[29]

Table 5. Characteristics of 5% pEGFP-loaded nanoparticles.

Formulation[b]	Mass ratio	Size (nm)	ζ Potential (mV)	AE[a] (%)
CG_{137spm}:TPP	12:1	179±18	37±2	93±2
CG_{137spm}:CS:TPP	12:0.1:0.5	165±16	48±2	90±3
CG_{137spm}:DS:TPP	15:1:0.6	143±21	36 ± 6	88±4

[a] AE: association efficiency.
[b] $CG_{137spm:}$ cationized gelatin 137 KDa with spermine; CS: chondroitin sulfate; DS: dextran sulfate; TPP: tripolyphosphate.

DNase I protection study

DNA is a molecule extremely susceptible to degradation by nucleases, with a half life of a few minutes in serum.[30] The presence and activity of nucleases in the tear film have been described by many authors and any vehicle candidate for ocular gene therapy must be able to protect the genetic material from degradation until it is internalized by the cells.[31-34] The DNase I protection assay is a general procedure to evaluate the protection of pDNA. In order to verify whether the nanoparticles were able to efficiently protect pDNA from nuclease degradation, they were incubated for 1 h in the presence of DNase I. Naked DNA was completely digested in 5 min, and CG_{137spm}, CG_{137spm} /CS, and CG_{137spm}/DS nanoparticles significantly protected the associated pDNA from degradation for at least 60 min (**Figure 2**). Therefore, the developed nanosystems significantly increases pDNA stability against DNase I degradation. The presence of CS and DS in the formulations did not interfere in the protection from DNase I. The protective effect of the nanoparticles could be attributed to the presence of the spermine attached to the gelatin backbone. Interactions between spermine and pDNA has been described for a long time.[29,35] Polyamines, such as spermine, are able to

decrease the degradation of polynucleotides when they are capable of inhibiting the activity of endonucleases, including DNase I.[36,37] This protective ability of polyamines is attributable to the formation of a steric barrier against DNA-damaging agents and their ability to condense the DNA.[38]

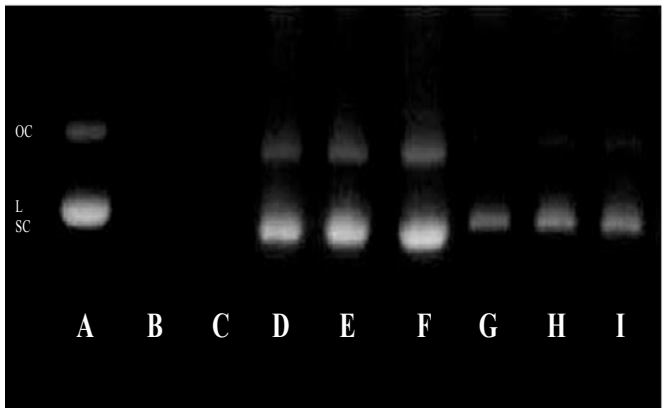

Figure 2. Stability of the pDNA associated to the nanoparticles after incubation with DNase I as determined by agarose gel electrophoresis: (A) Naked pDNA, (B) Naked pDNA incubated with DNase I for 5 min and (C) 60 min. (D) CG_{137spm} nanoparticles incubated with DNase I for 5 min and (G) 60 min. (E) CG_{137spm}/CS nanoparticles incubated with DNase I for 5 min and (H) 60 min. (F) CG_{137spm}/DS nanoparticles incubated with DNase I for 5 min and (I) 60 min.

Nanoparticles cytotoxicity

The development of a non-toxic, biodegradable, and biocompatible vehicle for gene delivery still remains a challenge despite numerous efforts in the pharmaceutical field. Many carriers that have shown efficiency in transfecting cells and tissues still face important safety issues for practical application in medicine.[39,40] The influence of CS and DS in the cytotoxicity profile of the CG nanoparticles was evaluated in the HCE cell line. We incubated the nanoparticles with these human corneal cells at varying concentrations, ranging from 1 to 150 μg/cm^2, for 3 hours, and their

metabolic activity was measured by the XTT assay. The percentage of cell viability as a function of the nanoparticle concentration is presented in **Figure 3**. The toxicity of the nanoparticles remained similar for all formulations at lower concentrations of 1 and 5 μg/cm^2. For higher concentrations, the introduction of CS or DS in the nanoparticles significantly decreased the toxicity. Concretely, for these particles, toxic behavior was achieved for 100 μg/cm^2, whereas the formulations comprised of only GC_{137spm} achieved toxic behavior at 25 μg/cm^2. The differences decreased for higher concentrations of nanoparticles, when the polyanions cannot overcome the toxicity of the cationic polymer. The IC_{50} is perhaps the best way to see improvements in the toxicological profile; the IC_{50} of CG_{137spm}/CS (107 μg/cm^2) and CG_{137spm}/DS nanoparticles (79 μg/cm^2) was 3-fold and 2-fold higher, respectively, than that of CG_{137spm} nanoparticles (35 μg/cm^2). These results are in accordance with the hypothesis that cytotoxicity decreases as a result of the presence of the natural polyanions. Such an approach was previously reported for nanoparticles and complexes using hyaluronic acid and DS using greater amounts of cationic polymer.[6,41] Unlike the in vitro situation, in vivo models exhibit almost instantaneous dilution and continuous eye lid action. Therefore, the concentrations used in vitro cannot be correlated in vivo, and the toxic effects observed in vitro may be less important in vivo.[42]

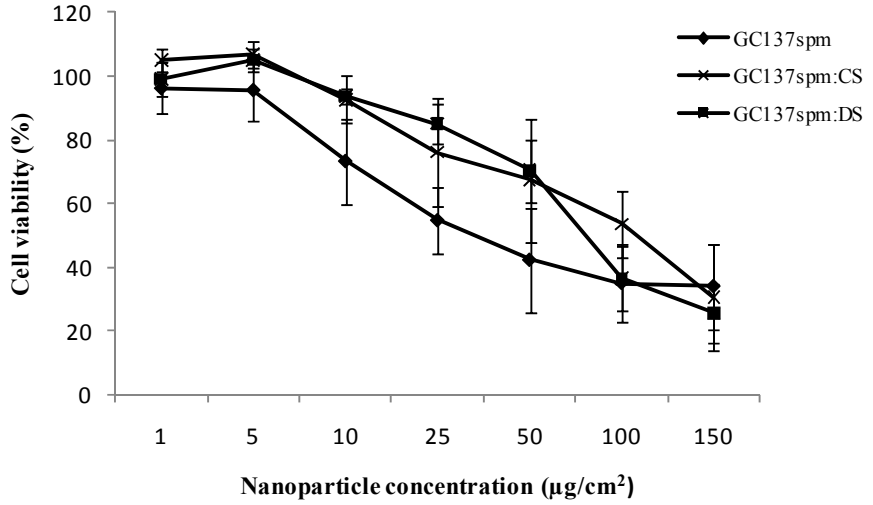

Figure 3. HCE cell viability determined by XTT assay after incubation for 3 h with CG_{137spm}, CG_{137spm}/CS, or CG_{137spm}/DS nanoparticles at increasing concentrations. Data are average ±SEM; n=3.

Cell uptake

In order to observe the influence of polyanions on the internalization of the nanoparticles, Cy3-labeled pDNA (Cy3-pDNA) was associated to nanoparticles and its uptake evaluated in HCE cells. The cell nuclei were stained with DAPI (blue) and the cytoskeleton stained with phalloidin (red). The confocal images of HCE cells (**Figure 4**) showed an intense green signal corresponding to the effective internalization of the pDNA by CG_{137spm} (Figure 4B), CG_{137spm}/CS (Figure 4C), and CG_{137spm}/DS (Figure 4D). A signal was also detected after cell incubation with free Cy3-pDNA (Figure 4A). The background fluorescence was the same for all treatments. The images of the x-y, x-z, and y-z cross sections illustrate the localization of the pDNA inside the cells. The absence of free or naked Cy3-pDNA internalization clearly indicates that the nanoparticles are fundamental for the efficient intracellular delivery of pDNA, reinforcing the potential of these nanoparticles as new gene therapy vehicles.

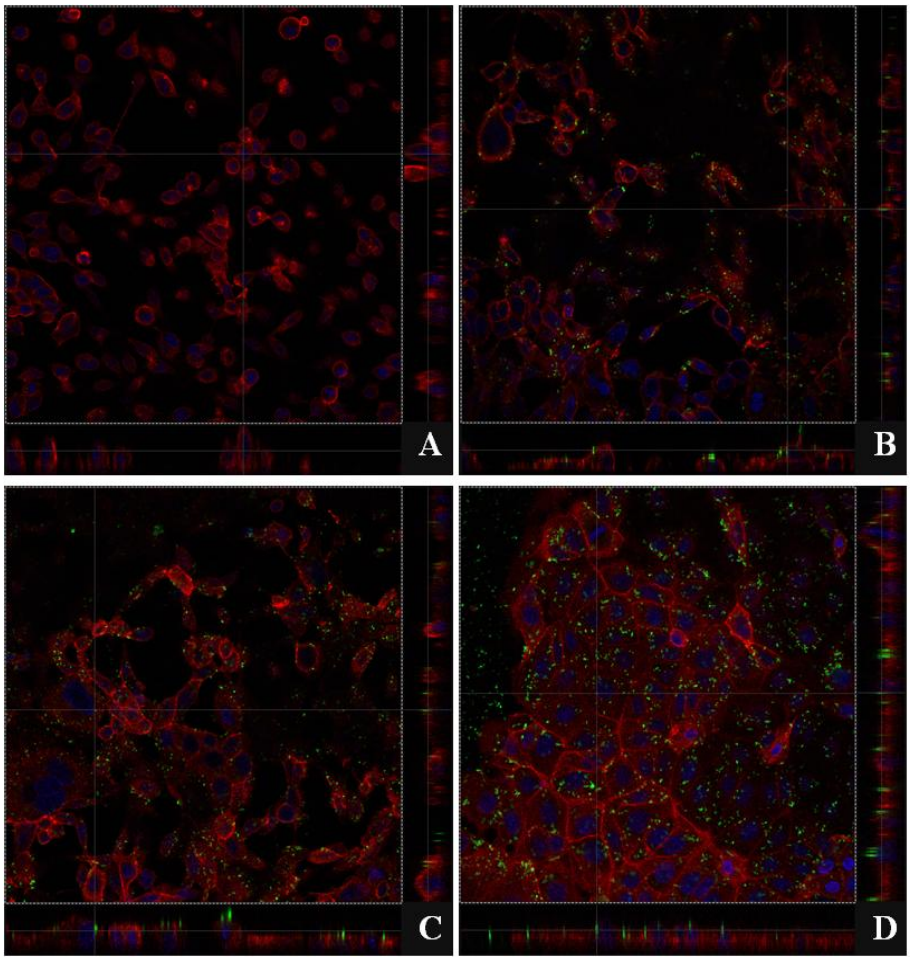

Figure 4. Confocal fluorescence microscope images of HCE cells after incubation with Cy3-labeled pDNA (green): (A) naked pDNA, (B) CG_{137spm}, nanoparticles associating pDNA (C) CG_{137spm}/CS nanoparticles associating pDNA, and (D) CG_{137spm}/DS nanoparticles associating pDNA. The cell nuclei were stained with DAPI (blue), and the F-actin filaments of the cytoskeleton were stained with phalloidin (red). Magnification 40x.

Transfection analysis

Finally, we evaluated the ability of the nanoparticles to transfect HCE cells using a model plasmid that encodes enhanced green fluorescent protein (EGFP). This plasmid was chosen because of its easy evaluation by a simple

visual analysis under fluorescence microscope. The cells were observed 24, 48, and 72 h after transfection. The maximum expression of EGFP for all of the systems was reached 48 h after transfection and maintained at least to 72h post-transfection (Figure 5), which is in accordance with the period related by other authors.[43,44] None of the formulations could achieve the same expression level as the Lipofectamine control. However, Lipofectamine is a tool for in vitro positive transfection and is not applied in clinical practice due to stability and safety problems. The presence of the polyanions decreased the system toxicity without compromising transfection efficiency. Indeed, all three systems had a similar response in HCE cells. Most of the systems designed for gene therapy fail because of face safety problems, but the hybrid nanoparticles were able to improve the in vitro toxicity profile of the nanoparticles in human corneal cells without compromising their transfection efficiency.

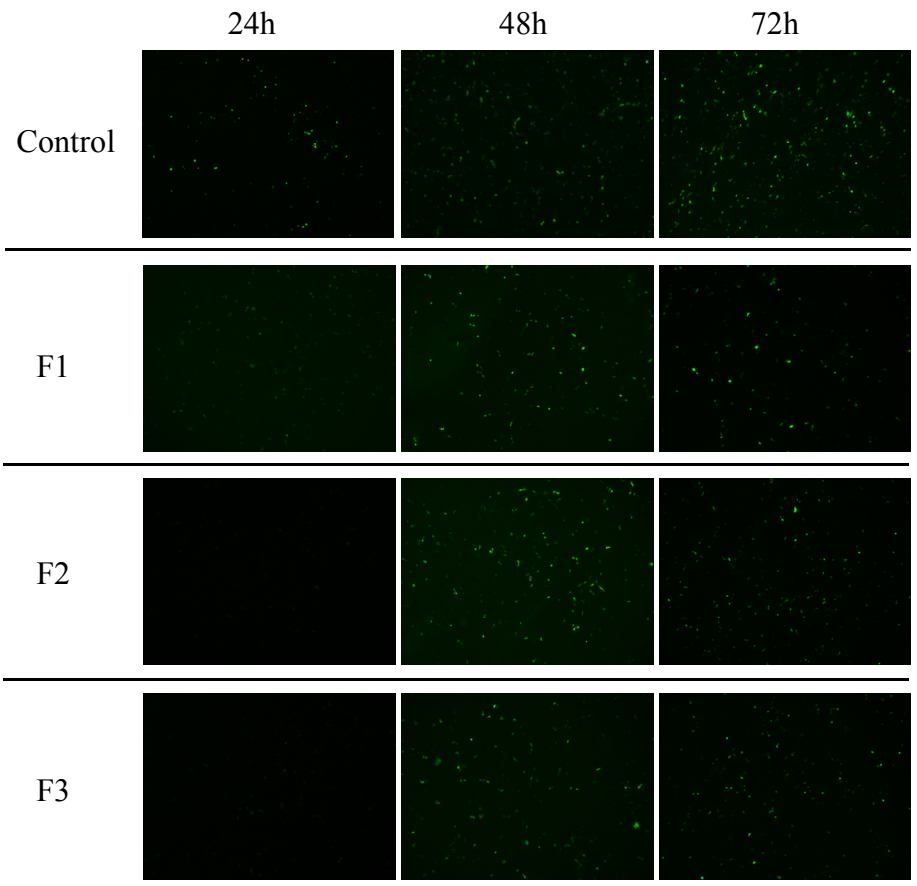

Figure 5. Expression of green fluorescent protein after transfection of HCE cells with pDNA associated to different formulations: CG_{137spm} nanoparticles (F1), CG_{137spm}/CS nanoparticles (F2), CG_{137spm}/DS nanoparticles (F3), or pDNA-Lipofectamine as a control. The nanoparticles were incubated with the cells for 3 h (1 μg pEGFP/well). The cells were examined under a fluorescence microscope at 24, 48, and 72 h post-transfection.

Conclusion

The association of the anionic polymers DS and CS with CG to form hybrid nanoparticles was achieved using a mild ionic gelation technique. The possibility of combining different proportions of positive and negative polymers gives these systems important versatility, conveniently modulating

the physicochemical parameters of the nanoparticles and improving the toxicological profile of the system in human corneal cells without compromising transfection efficiency. In conclusion, the hybrid nanoparticles are promising systems for gene therapy in the ocular surface.

Acknowledgements

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Artículo IV

Expression of MUC5AC in ocular epithelial cells using cationized gelatin nanoparticles

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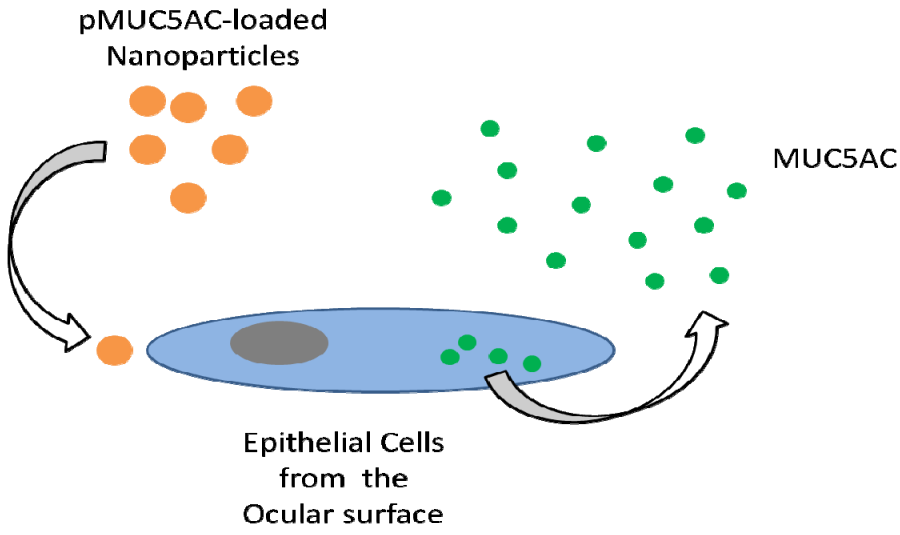
Submited for evaluation

Abstract

Decreased expression of the mucin MUC5AC in the eye is related to several pathological conditions, including dry eye syndrome. A specific strategy for increasing the ocular levels of MUC5AC is not yet available. Using a plasmid specially designed to encode human MUC5AC, we evaluated the ability of hybrid cationized gelatin nanoparticles (NPs) containing polyanions (chondroitin sulfate or dextran sulfate) to transfect ocular epithelial cells. NPs were developed using the ionic gelation technique and characterized by a small size (<200 nm), positive zeta potential (+20/+30mV), and high plasmid association efficiency (>95%). MUC5AC mRNA and protein were detected in conjunctival cells after in vitro transfection of the NPs. The in vivo administration of the NPs resulted in significantly higher MUC5AC expression in the conjunctiva compared to untreated control and naked plasmid. These results provide a proof-of-concept that these NPs are effective vehicles for gene therapy and candidates for restoring the MUC5AC concentration in lacrimal fluid.

Keywords: nanoparticle, gene therapy, dry eye, ocular surface, MUC5AC.



Introduction

The causes of dry eye are multifactorial and can be related to deficiencies in any of the components of the ocular surface and tear film. The tear film responds to very delicate regulatory mechanisms, notably those involving neuroregulatory phenomena and hormonal stimulation.[1] The inhibition of lacrimal gland secretion, decreased tear production, and hyperosmolar tears with the expression of several pro-inflammatory cytokines and chemokines are signs associated with dry eye syndrome. Among all of the factors related to tear film stability under normal conditions, a type of mucin called MUC5AC receives special attention because its decrease has been associated with several conditions, such as dry eye syndrome.[2, 3]

Mucins are a class of heavily O-glycosylated glycoproteins in which the mass may reach 80% carbohydrate. As a result of their heavy glycosylation, mucins have been difficult to characterize, and it is only with the relatively recent application of molecular cloning techniques that mucin genes have been identified.[4] MUC5AC is a gel-forming mucin secreted by conjunctiva goblet cells. MUC5AC plays a key role in the homeostasis of lacrimal fluid, and alterations in either the distribution of the protein or its glycosylation has been described in dry eye syndrome.[5] Also, a reduction in the number of goblet cells that synthesize MUC5AC may correlate with decreased MUC5AC expression and constitute one possible mechanism responsible for tear instability.[6]

Despite the lack of options for increasing the level of MUC5AC when necessary, the *in vivo* delivery of exogenous gene that codifies the MUC5AC has potential for treating ocular diseases. Gene therapy has many advantages over conventional drugs because, once inside the cells, the genes are able to express their products for periods of time that greatly exceed the duration of action for currently available drugs.[7] The use of nanoparticles (NPs) as carriers of the therapeutic genetic materials for delivery to target tissues has become popular in recent years for the treatment of a wide range of ocular

diseases. However, these systems are only effective with the right selection of biomaterials.[8]

We recently described the use of NPs produced by ionic gelation technique and based on gelatin cationized with spermine to efficiently transfect cornea cells. These NPs were able to protect the plasmid DNA from degradation. The properties of these NPs can be significantly improved by incorporating the polyanions chondroitin sulfate (CS) and dextran sulfate (DS). These systems have been shown to successfully induce plasmid internalization and are promising new carriers for gene delivery.[9]

The present study aimed to achieve successful transfection in the ocular surface using a new plasmid encoding a modified human MUC5AC protein associated to hybrid NPs based on cationized gelatin and CS or DS.

Materials and methods

Chemicals and Reagents. Type A gelatin (137 KDa) was purchased from Nitta Gelatin (Ontario, Canada). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), tripolyphosphate (TPP), and spermine hydrochloride (SPM) were purchased from Sigma (Madrid, Spain). The pEGFP-c1 plasmid was obtained from Elim Biopharmaceutics (Hayward, CA). Plastic culture ware was obtained from Nunc (Roskilde, Denmark). DMEM/F12 culture medium and other cell culture reagents were from Invitrogen-Gibco (Inchinnan, UK). The plasmid pMUC5AC, codified for a modified MUC5AC protein and for the green fluorescent protein (GFP) as a marker, was designed by our group and supplied by Biomedal (Seville, Spain) [10].

Synthesis of cationized gelatin. The gelatin was cationized as described previously[11]. Briefly, a 1% (w/v) polymer solution in 0.1 M phosphate buffer (pH 5.3) was prepared, and for each mole of gelatin carboxyl groups, 3 mol of EDC and 50 mol of SPM were added. The final pH was adjusted to 5.0 and the solution reacted for 18 h in a warm bath at 37ºC.

After the reaction, the final solution was dialyzed for 48 h, followed by lyophilization.

Preparation and characterization of nanoparticles. The NPs were formed using the ionic gelation technique as described previously.[12] Cationized gelatin (CG) was dissolved in water at a concentration of 1 mg/mL, CS at a concentration of 0.125 mg/mL, DS at 0.1 mg/mL, and TPP at varying concentrations (0.125 or 0.25 mg/mL). All solutions were sterilized by filtration (0.22 μm, Millex®GV, Millipore, Billerica, MA). The plasmid and one of the anionic polymer solutions were incorporated with the TPP solution. NPs were obtained by adding the resulting mix to the CG solution with magnetic stirring at room temperature. The mean particle size was determined by photon correlation spectroscopy (PCS). The samples were diluted to the appropriate concentration. Each analysis was carried out at 25ºC with a detection angle of 173º. The zeta potential was obtained by laser Doppler anemometry (LDA), measuring the mean electrophoretic mobility. The samples were diluted with a millimolar solution of KCl. The PCS and LDA analyses were performed using a Zetasizer 3000HS (Malvern, Malvern, UK). The association efficiency was evaluated using the PicoGreen® reagent (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes, Madrid, Spain) according to the manufacturer's directions. The amount of free pDNA was quantified using the nanoparticle supernatant obtained after centrifugation in a Microfuge 22-R (Beckmann Coulter, Krefeld, Germany) for 30 minutes at 12,000 rpm. For the *in vivo* studies, the nanoparticles were optionally concentrated by centrifugation (Beckman CR412, Beckman Coulter) at 10,000 rcf for 30 minutes at 4ºC with 0.1% glycerol (v/v). The NPs were then resuspended in 5% glucose at a final concentration of 0.5 μg/μL of plasmid.

Cell culture. Two different cell lines were used. The IOBA-NHC cell line [13] is a nontransfected, spontaneously immortalized conjunctival epithelial cell line used in passages 71 to 87. Cells were grown in DMEM/F-

12 supplemented with 10% fetal bovine serum (FBS), 5000 U/mL penicillin, 5 mg/mL streptomycin, 2.5 μg/mL fungizone, 2 ng/mL human epidermal growth factor (EGF), 1 μg/mL bovine insulin, 0.1 μg/mL cholera toxin, and 0.5 μg/mL hydrocortisone. The HCE cell line [14] is a SV40-immortalized human corneal epithelial cell line kindly gifted by Professor Arto Urti (University of Helsinki, Finland). Cells from passages 42 to 52 were cultured in DMEM/F-12 supplemented with 15% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 ng/mL EGF, 0.5% DMSO, 5 μg/mL insulin, and 0.1 μg/mL cholera toxin. Both cell lines were cultured at 37ºC in a 5% CO2-95% air atmosphere. Media were changed every other day, and daily observations were made by phase contrast microscopy.

Transfection studies. In order to evaluate the ability of the pMUC5AC-loaded NPs to transfect HCE and IOBA-NHC cell lines, cells were seeded onto 24-well plates (8 x 10^5 cells/well) and grown for 24 h reaching a final confluence of 75%. Then HCE and IOBA-NHC cells were incubated with the NPs for 3 h (5 μg of pDNA/well). Controls included cells alone and cells transfected with the naked pDNA (1μg pDNA/well) using JetPEI™-RGD (Polyplus Transfections, Illkirch, France) as transfection reagent. The expression of GFP was evaluated 72 h post-transfection using an inverted fluorescence microscope (Leica DMI 6000B, Wetzlar, Germany). Observations were made and images captured using a 40× objective.

Cell Viability. The Viability of NP-exposed cells was measured using the XTT toxicity test (Sigma)[15].Cells were seeded onto 96-well plates (2 x 10^5 cells/well) and grown until 75% confluence. HCE and IOBA-NHC cells were incubated with the NPs for 3 h (5 μg of pDNA/well). Culture medium was replaced with fresh phenol red-free RPMI, 72 h after NP incubation. Then XTT solution was added and cells were incubated at 37ºC for 15 h. Plates were read in a SpectraMAX®M5 multidetection microplate reader (Molecular Devices, Sunnyvale, CA,) at 450nm (reference wavelength: 620nm). Controls included cells alone and cells exposed to 0.5%

benzalkonium chloride (BKC), which induces a significant decrease of ocular cell viability in concentrations greater that 0.05%[16]. Cell viability was calculated as a percentage with regard to control cells. Each test was repeated three times in quadruplicate.

Real Time RT-PCR of MUC5AC. MUC5AC mRNA expression was quantified using real time RT-PCR. Total RNA from NP-exposed cells and control cells were isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer´s protocol. The RNA concentration was measured using the Quant-it RNA Assay Kit (Invitrogen, Barcelona, Spain) and it was stored at -80ºC before used. cDNA was generated from 1µg of total RNA with the SuperScript® Vilo™cDNA kit (Invitrogen), according to the manufacturer´s protocol.

Real Time RT-PCR was performed on the 7500 Real Time System (Applied Biosystem, Foster City, CA) using SYBR Green (Applied Biosystem) to quantify levels of MUC5AC mRNA in the different treatment groups. For this purpose MUC5AC primers (forward 5´-CCCACAGAACCCAGTACAA-3´ and reverse 5´-AATGTGTAGCCCTCGTCT-3´) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (forward 5´-GAAGGTGAAGGTCGGAGTCAAC-3´ and reverse 5´-CTGGAAGATGGTGATGGGATTTC-3´) were used. Relative quantification of the signals was done by normalizing the signal of MUC5AC mRNA with the GAPDH signal. The expression was referred to that in control cells.

ELISA of MUC5AC. MUC5AC protein was quantified by Enzyme-Linked ImmunoSorbent Assay (ELISA).[17] Cell lysates (50 µL) were incubated with bicarbonate-carbonate buffer (50 µL) (0.05 M, pH 9.6) at 37ºC overnight in *MaxiSorp*® flat-bottom *96 well* plates (Nunc) until dry. Plates were washed three times with PBS and blocked with 2% bovine serum albumin (BSA fraction V; Sigma, Madrid, Spain) for 1 h at room temperature. Plates were again washed three times with PBS and then

incubated with 50 µL of mouse anti-MUC5AC antibody (Chemicon, Billerica, MA), which was previously diluted (1:500) in PBS with 0.05% Tween 20. After 1 hour, the wells were washed three times with PBS and 100 µL of horseradish peroxidase-conjugated secondary donkey anti-mouse IgG antibody (Jackon Inmuno Res., Madrid, Spain) previously diluted (1:10,000) in PBS with 0.05% Tween 20 and 0.1% BSA, added to each well. After 1 h, the plate was washed three times with PBS. Color reaction was developed with 3,3´,5,5´-tetramethylbenzidine peroxide (TMB) solution (Invitrogen) and stopped with 1M H_2SO_4. Plates were read in a SpectraMAX®M5 multidetection microplate reader (Molecular Devices, Sunnyvale, CA) at 450nm. MUC5AC analog B (AnaSpec, Fremont, CA) was used as a standard. The obtained values were normalized to the total amount of protein quantified by the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's directions. The results were expressed as a percent of the expression of MUC5AC in the untreated control.

In vivo assay. In vivo studies adhered to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional research committee. To evaluate the ability of pMUC5AC-loaded NPs to produce an efficient transfection in vivo, normal conscious New Zealand rabbits were placed in a restraint box and 30 µL concentrated NPs dispersion (0.5 µg/µL of pDNA) administrated topically into the cul-de-sac. The dispersion was applied every 30 minutes until reaching 75 µg of plasmid. To facilitate the administration, the NPs were previously 10-fold concentrated. Naked pMUC5AC was used as control, following the same protocol and all results were compared to control animals treated with 5% glucose solution**.** Also, the in vivo tolerance to pMUC5AC-loaded NPs ocular exposure was studied. Clinical signs (ocular discomfort, presence of corneal and/or conjunctival alterations, discharge and eyelid swelling) were macroscopically evaluated 4 days post-transfection. Animals were euthanized 4 days post-

transfection and corneal and conjunctival tissues collected. Tissues were chopped by a scissor and were homogenized. Expression of MUC5AC in cornea and conjunctiva was evaluated by ELISA.

Statistical analysis. The results were expressed as mean ± standard error of the mean (SEM) of three independent experiments and statistically analyzed by ANOVA, followed by the Tukey test.

Results

Preparation and characterization of the nanoparticles. Hybrid NPs were recently developed as a non-viral delivery system for ocular genes. We evaluated the ability of such hybrid systems in the transfection of a plasmid encoding MUC5AC. All of the systems were based on cationized gelatin and reticulated with TPP: (i) CG NPs, (ii) CG/CS NPs, and (iii) CG/DS NPs. All systems were less than 150 nm in size and positive zeta potential between +20 and +30 mV (Table I). The amount of pDNA associated with the nanoparticles determined using the PicoGreen® reagent showed a high association efficiency of over 90% for the systems.

Table 1. Characteristics of 7.5% pMUC5AC-loaded nanoparticles.

Formulation	Mass ratio	Size (nm)	ζ (mV)	AE (%)
CG/TPP	12/1	131 ±3	+22 ±2	99.2±0.5
CG/CS/TPP	12/1/0.5	135±14	+33 ±3	97.6±1.3
CG/DS/TPP	15/1/0.6	106 ±4	+31 ±2	95.7±3.2

CG, cationized gelatin with spermine; AE, association efficiency; CS, chondroitin sulfate; DS, dextran sulfate; TPP, tripolyphosphate. Data are mean ± S.D; n=3.

In vitro transfection studies. To evaluate the ability of pMUC5AC-loaded NPs to produce an efficient transfection of HCE and IOBA-NHC cells, first GFP expression was evaluated by fluorescence microscopy and then MUC5AC mRNA and protein expressions were measured by Real Time RT-PCR and ELISA, respectively. In addition, cell viability was measured in both cell lines 72 h after NP incubation. There were no significant differences in cell viability between control HCE and IOBA-NHC cells and those exposed to the three formulations (Figure 1). Both HCE and IOBA-NHC cells incubated with NPs had a well-preserved morphology that was similar to controls, as determined by phase contrast microscopy.

GFP expression evaluated 72 h post-transfection showed a little fluorescence in cells transfected with the positive transfection control Jet-PEI™-RGD, but no fluorescence was detected in the HCE and IOBA-NHC cells after transfection with the NPs (data not shown).

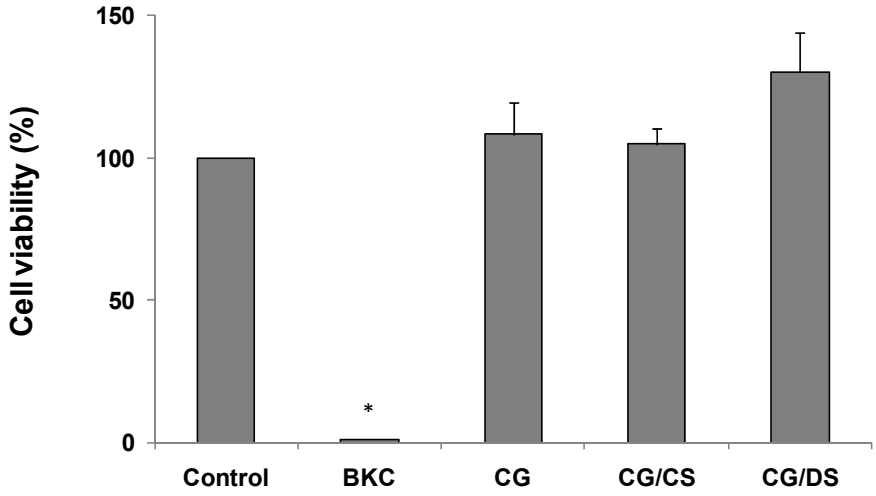


Figure 1. Cell viability 72 h after NPs treatment in HCE and IOBA-NHC cells. Results are expressed as % of control. Benzalkonium chloride (BKC) was use as toxicity control. There were no significant differences between NP-treated and non-treated corneal and conjunctival cells. n=3 independent experiments in triplicate, data are mean±SEM. *$p<0.05$ vs. control

MUC5AC mRNA expression. Both HCE and IOBA-NHC cell lines showed detectable MUC5AC mRNA expression in cells exposed to pMUC5AC-JetPEI™-RGD-complexes and to the 3 formulations, as determined by real time RT-PCR. This expression was significantly higher in pMUC5AC-JetPEI™-RGD exposed cells than in cells treated with the formulations. The 3 formulations, CG, CG/CS, and CG/DS, produced an efficient transfection of MUC5AC in both cell lines, influenced by the presence of the anionic polymer in the formulation (Figure 2). MUC5AC mRNA expression in HCE cells following incubation with CG NPs (1091±137) was 2-fold higher than the expression induced by CG/CS (543±141) or CG/DS (407±89) in this cell line. In IOBA-NHC cells, the presence of CS in the formulation significantly increased the expression of MUC5AC (198±36), which was 2- and 3–fold greater than the expression induced by CG (106±11) and CG/DS (69±10) NPs, respectively.

MUC5AC protein expression. MUC5AC protein expression was achieved in both cell lines when using the commercial transfection agent JetPEI™-RGD, as determined by ELISA. This expression was always significantly higher than that of untreated cells and cells exposed to the formulations. In IOBA-NHC cells, the level of expression was higher ($p<0.05$) for CG NPs (764±189) compared to the NPs possessing polyanions: CG/CS (337±71) and CG/DS (317±81) (Figure 3). Although mRNA production was induced in HCE cells, none of the formulations were capable of inducing protein expression.

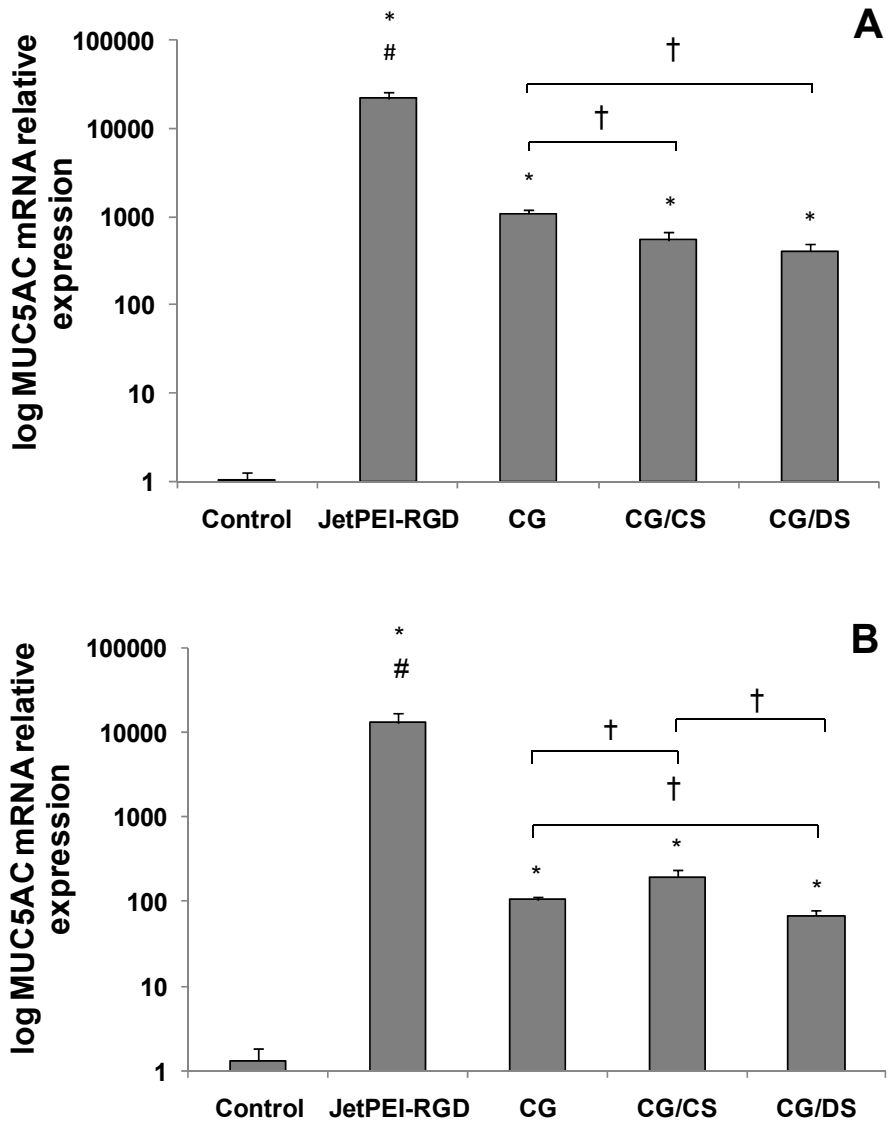


Figure 2. Relative expression of MUC5AC mRNA was quantified by real time RT-PCR in HCE cells (A) and in IOBA-NHC cells (B) 72h post transfection. Untreated cells (Control) and pMUC5AC-JetPEI™-RGD (JetPEI-RGD) were used as a control. CG NPs (CG), CG/CS NPs (CG/CS), and CG/DS NPs (CG/DS) were used to transfect the cells. n=3 independent experiments in triplicate, data are mean±SEM. * $p<0.05$ vs. control, # $p<0.05$ vs all formulations, † $p<0.05$ between formulations.

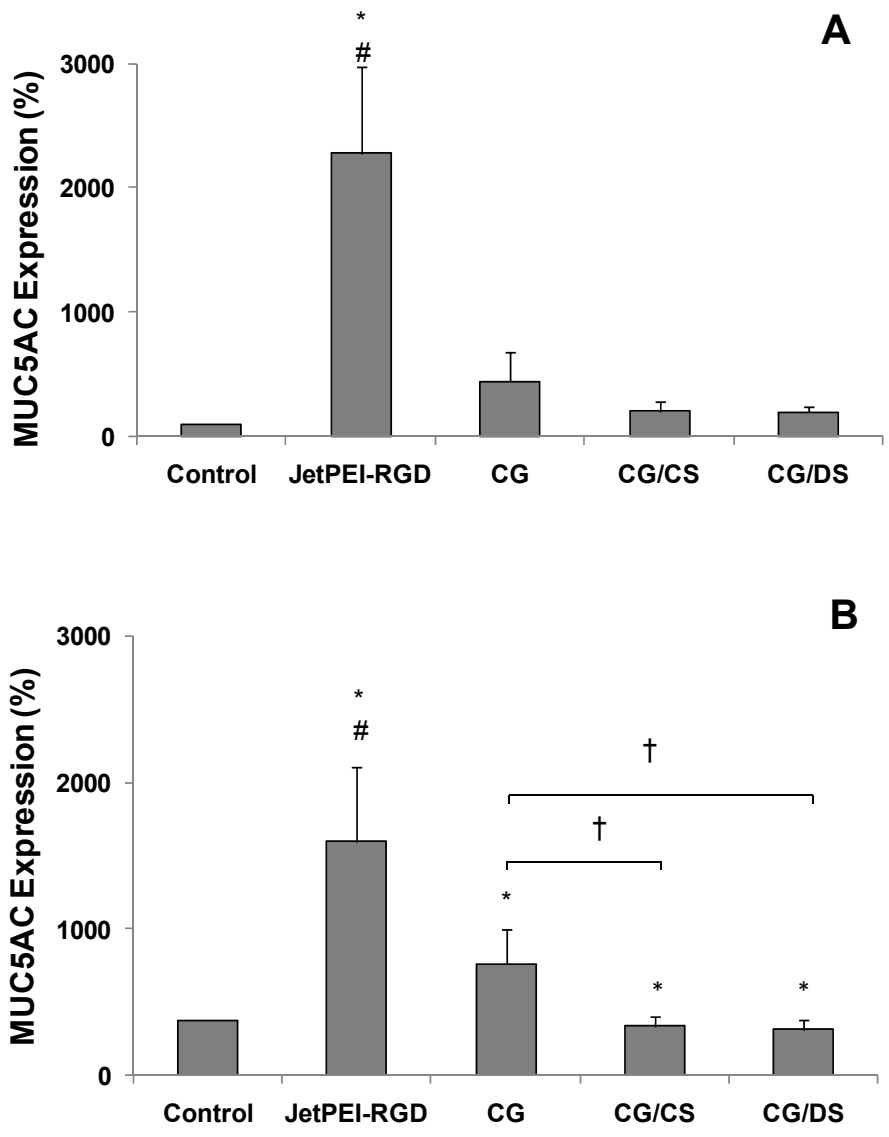


Figure 3. Relative expression of MUC5AC was quantified by ELISA in HCE cells (A) and in IOBA-NHC cells (B) 72h post transfection. Untreated cells (Control) and pMUC5AC-JetPEI™-RGD (JetPEI-RGD) were used as a control. CG NPs (CG), CG/CS NPs (CG/CS), and CG/DS NPs (CG/DS) were used to transfect the cells. n=3 independent experiments in triplicate, data are mean±SEM. * $p<0.05$ vs. control, # $p<0.05$ vs all formulations, † $p<0.05$ between formulations

In vivo transfection study. An *in vivo* assay was performed to evaluate the effect of pMUC5AC-loaded NPs transfection on the expression of MUC5AC on the eye surface tissues. CG NPs, the system with the best response in terms of protein expression in conjunctiva cells, was applied onto the eyes of New Zealand rabbits. To facilitate the administration, the NPs were 10-fold concentrated, but no changes in the physicochemical properties were noted.

The macroscopic evaluation showed that pMUC5AC-loaded NPs are well tolerated. No animal showed ocular discomfort, irritation or swelling. Only a mild nasal discharge and increased blinking immediately after instillations were observed in few rabbits. In addition, there were no macroscopic alterations of the ocular surface structures after pMUC5AC-loaded NPs exposure. No edema, redness, or corneal vascularization was observed in treated eyes.

The naked plasmid did not induce MUC5AC expression in any tissue (Figure 4). In contrast, treatment with the cationized gelatin NPs resulted in significant MUC5AC expression in the conjunctiva (122±3%; $p<0.05$), but not in the cornea.

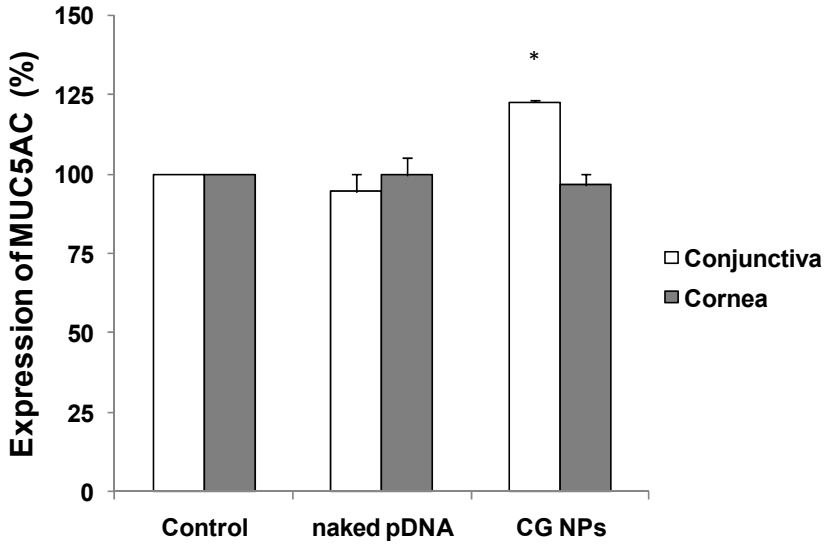


Figure 4. Expression of MUC5AC in rabbit cornea (white bars) and conjunctiva (grey bars) 4 days after transfection with cationized gelatin nanoparticles (CG NPs) or naked pDNA. n=3 independent experiments in triplicate, data are mean±SEM. * $p<0.05$.

Discussion

In this work, we studied the *in vitro* and *in vivo* tolerance and transfection efficiency in ocular surface structures, of a new plasmid encoding a modified human MUC5AC protein associated to hybrid NPs based on cationized gelatin and CS or DS.

Although these NPs were described previously (ref), they associated with a model plasmid (pEGFP) very different than pMUC5AC. Usually, different plasmids with similar sizes do not influence the physicochemical parameters of the NPs [18], but in this case the length of pMUC5AC is more than 2-fold higher than that of pEGFP (13.1 Kb vs. 5.3 Kb). Despite of the huge difference in the plasmid size, no difference in the NPs size were observed, probably due to the capacity of spermine to condense the plasmid.[19] The NPs used in this study were compact structures of nanometric size (less than 150

nm), markedly positive zeta potential and high pDNA association efficiency. Many authors have described the ideal size of a nanocarrier as <180 nm in order to facilitate ingress into the cells.[20] The positive zeta obtained for all the systems is important for increasing the stability of the system[21] and improving the interaction between NPs and the eye surface, increasing the transfection efficiency.[22] The high association efficiency can also be attributed to the high interaction between DNA and the spermine linked to the gelatin backbone.[23-25]

The viability of NP-exposed HCE and IOBA-NHC cells was totally preserved (Figure 1). This is in agreement with previous in vitro works for these nanosystems,[9] indicating an adequate safety level for their application in the ocular surface.

MUC5AC is naturally produced in the conjunctiva by a specialized type of cell, goblet cells, and no expression has been reported in corneal structures thus far. Even though the over-expression of MUC5AC has been reported in other organs and tissues, such as the prostate and airways, it is generally associated with pathological conditions.[26, 27] However, the pMUC5AC-loaded NPs reported here are the first therapy aimed at specifically increasing MUC5AC levels in the eye. This increase in MUC5AC concentrations would be of great importance to improve the lacrimal fluid quality in dry eye therapy. It is important to emphasize that reduced MUC5AC mRNA expression has been related to dry eye conditions, such as Sjögren´s syndrome[6]. Our in vitro transfecttion studies showed an increase in MUC5AC mRNA and protein levels in IOBA-NHC and HCE cells exposed to NPs. The increase in the protein expression detected in the IOBA-NHC cells represents an significant advance once, thus far, the only way to induce the over-expression of MUC5AC was by using pro-inflammatory cytokines and other stimulating factors that cannot be used as therapeutic agents for their several biological undesired responses.[26, 27] Among the different ways to evaluate the transfection efficiency of pMUC5AC-loaded NPs carried out,

just the indirect qualitative evaluation of GFP expression failed. Some previously studies have used GFP expression constructs in their systems and also failed to detect fluorescence. This failure could be due to numerous reasons, including GFP expression below the limit of detection and the failure in forming the chromophore.[28] Notably, the mRNA and protein expression data are pictures of two different and sequential events analyzed at the same time (day 3 post-transfection). One explanation for the difference detected between mRNA and protein production in IOBA-NHC cells is that CG/CS NPs induce longer and lasting expression. So, the possible reason why the mRNA levels at day 3 are higher but CG NPs have a burst effect on expression is that a higher level of MUC5AC is expressed, though just for a few days. Expression of the protein was achieved in HCE cells when using the commercial transfection agent, but not with any of the formulations; thus, possible reasons for the lack of expression in this cell line may be related with the fact that the level of synthesized mRNA was not enough to produce detectable amounts of protein in 72h.[29, 30]

The *in vivo* study showed that pMUC5AC-loaded CG NPs were well tolerated by the ocular surface structures. No macroscopic alterations, edema, redness or corneal vascularization were observed in treated eyes. Only slight explicable signs of ocular discomfort were observed in few rabbits. Nasal discharge was probably because of certain drugs capability of traveling to nasal mucosa after being drained from the ocular surface of treated eyes. Increased blinking could be related with the known irritative effect caused by the instillation, a response that is also observed after a simple buffer solution instillation.[31] Therefore, pMUC5AC-loaded GC NPs seem to be nonhazardous and have good in vivo tolerance.

In addition, treatment with the cationized gelatin NPs resulted in significant MUC5AC expression in the conjunctiva. The inefficiency of the naked plasmid in transfect conjunctiva or cornea highlights the need for an efficient vehicle to deliver the plasmid (Figure 4). It is important to point out that the

expression of the protein MUC5AC only took place in conjunctiva, being the result similar to that obtained in vitro for IOBA-NHC cells. The expression only in conjunctiva is desired because it is the natural target for MUC5AC expression, once no natural expression of MUC5AC has been reported in corneal structures, thus far.

Conclusion

The elevation of MUC5AC levels was achieved for the first time using a new plasmid and cationized gelatin nanoparticles as vehicles. We showed that the nanocarriers successfully transfected rabbit conjunctiva in vivo and were essential for the expression of the mucin from the associated plasmid. Therefore, these gelatin nanoparticles associated with the pMUC5AC plasmid are promising new medicines for restoring MUC5AC levels, creating new possibilities for treating several pathological conditions in which the restoration of these levels is benefical, such as dry eye.

Acknowledgements

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Artículo V

Treatment of dry eye using hybrid cationized gelatin and chondroitin sulfate nanoparticles loaded with MUC5AC plasmid

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Abstract

Dry eye syndrome is accompanied by decreased MUC5AC expression, one of the possible causes of abnormal tear behavior in this disease. We previously developed hybrid nanoparticles comprised of chondroitin sulfate and cationized gelatin that are capable of transfecting conjunctiva cells *in vitro* with a plasmid encoding MUC5AC. We demonstrate the clinical utility of these new nanoparticles in an animal study. The work was divided into two parts. First, we applied the nanoparticles to healthy mouse eyes to evaluate tolerance to the formulation. Secondly, we used the nanoparticles to transfect the eyes of mice submitted to desiccation stress, an animal model of dry eye. Macroscopic evaluation of the eyes and fluorescein staining to evaluate the integrity of the corneal barrier found that the nanoparticles did not exhibit significant toxicity and were well tolerated by the healthy animals after being applied to the eyes. In addition, these hybrid nanoparticles expressed MUC5AC in the conjunctiva of healthy mice. Treating the mice submitted to desiccation stress with pMUC5AC-loaded nanoparticles restored tear production to normal levels with the same intensity as the commercially available formulation for treating dry eye (Restasis®; 0.05% cyclosporine A) and restored the integrity of the corneal barrier to some extent. These results not only present the chondroitin sulfate cationized gelatin nanoparticles as new effective vehicles for ocular gene delivery, but they also show that a therapy based on re-establishing MUC5AC levels can improve important clinical signs in the physiopathology of dry eye while avoiding the undesirable side effects associated with the currently available formulations for this pathology.

Keywords: MUC5AC, dry eye, nanoparticles, gene therapy.

1. Introduction

Dry eye is recognized as a disturbance of the lacrimal functional unit (LFU), an integrated system comprising the lacrimal glands, ocular surface (cornea, conjunctiva, and meibomian glands), lids, and the sensory and motor nerves that connect them (1, 2). Dysfunction in any LFU component can lead to dry eye as a result of alterations in the volume, composition, distribution, and/or clearance of the tear film. Two mutually reinforcing global mechanisms, tear hyperosmolarity and tear film instability, have been identified as the most important mechanisms in dry eye pathology (3). Hyperosmolar tears can damage the epithelium of the ocular surface by activating an inflammatory cascade, releasing inflammatory mediators into the tears (4). The acute inflammation may initially be accompanied by increased reflex tearing and blinking, and chronic inflammation may result in reduced corneal sensation and decreased reflex activity, leading to increased evaporation and tear film instability (5, 6). Inflammation can also result in goblet cell loss and decreased mucin production, which further contributes to tear film instability. The only currently available treatment capable of increasing tear production and decreasing the signs of inflammation is based on cyclosporine A (Restasis®; 0.05% cyclosporine A emulsion). However, this therapy could lead to serious undesirable side effects when systemic absorption occurs.

MUC5AC plays an important role in tear homeostasis (7). MUC5AC is a highly glycosylated gel-forming mucin secreted by specialized cells of the conjunctiva, the goblet cells (8). Dry eye is accompanied by decreased MUC5AC expression and exhibits an abnormal pattern of protein glycosylation in goblet cells (9-11).

Ocular gene therapy holds great promise for restoring protein levels in disease; a “corrected” gene can be inserted into the genome to replace an “abnormal” disease-causing gene (12). However, despite several efforts by pharmaceutical scientists, efficient gene therapy in the eye surface still remains a challenge (13).

We recently described the use of hybrid nanoparticles comprised of chondroitin sulfate (CS) and cationized gelatin that associate a new plasmid encodes human MUC5AC (pMUC5AC) in transfection (14). The objective of the present work was to evaluate the use of the hybrid nanoparticles loaded with pMUC5AC in the treatment of a mouse model of dry eye and compare its efficiency with the only eye drops approved to increase tear production in dry eye patients (Restasis®).

2. Materials and methods

2.1 Materials

Type A gelatin (137 KDa) was kindly gifted from Nitta Gelatin (Canada). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), tripolyphosphate (TPP), and spermine hydrochloride (SPM) were purchased from Sigma (Spain). Chondroitin sulfate was purchased from Calbiochem (United States). Restasis® was supplied by Allergan Inc. (United States).

2.2 Cationized gelatin synthesis

The gelatin was cationized as described previously (15) and prepared as a 1% (w/v) polymer solution in 0.1 M phosphate buffer (pH 5.3). For each mole of gelatin carboxyl groups, 3 mol of EDC and 50 mol of SPM were added. The final pH was adjusted to 5.0 and the solution allowed to react for 18 h in a warm bath at 37ºC. After the reaction, the solution was dialyzed for 48 h, followed by lyophilization.

2.3 Preparation and characterization of nanoparticles

The nanoparticles were formed using the ionic gelation technique (16). Cationized gelatin (CG_{137spm}) was dissolved in water at a concentration of 1 mg/mL, CS at a concentration of 0.125 mg/mL, and TPP at a concentration of 0.125 mg/mL. All solutions were prepared in water and sterilized by filtration (0.22 μm, Millex®GV, Millipore, Ireland). The plasmid and CS solution

were incorporated into the TPP solution. Nanoparticles were obtained by adding the resulting mix to the CG_{137spm} solution with magnetic stirring at room temperature and isolated by centrifugation (Beckman CR412, Beckman Coulter, US) at 10,000 rcf for 30 minutes at 4ºC with 0.1% (v/v) glycerol. The nanoparticles were then resuspended in water at a final plasmid concentration of 0.2 µg/µL. The mean particle size was determined by photon correlation spectroscopy (PCS). The samples were diluted to the appropriate concentration. Each analysis was carried out at 25ºC with a detection angle of 173º. The zeta potential was obtained by laser Doppler anemometry (LDA) measuring the mean electrophoretic mobility. The samples were diluted with a millimolar solution of KCl. The PCS and LDA analyses were performed with a Zetasizer 3000HS (Malvern, UK).

2.4 *In vivo* assay

To evaluate the *in vivo* expression of MUC5AC in mice under desiccation stress and healthy animals, conscious animals were placed in a restraint box and 5 µL of the nanoparticle dispersion (0.2 µg/µL) topically applied to the cul-de-sac, four times a day for 5 days, to a total of 20 µg of plasmid (4 µg/day). Transfection efficiency was evaluated 48 h after the last application when animals were sacrificed in a CO_2 chamber and the ocular tissues collected.

2.5 Desiccation stress model

Dry eye was induced in mice by subcutaneously injecting scopolamine (1 mg in 0.2 mL) using a 30G needle in the flank three times a day (in alternative flank: right, left, right) for 5 days. The cages housing the mice were placed 6 inches in front of a fan directed to blow across the wired screen side of the cage 24 h a day in a controlled humidity hood (less than 40%). The induction of dry eye started 5 days after the first application until the last application in a total of 10 days.

2.6 Macroscopic evaluation

The macroscopic evaluation of ocular structures and clinical signs, such as ocular discomfort, presence of corneal and/or conjunctival alterations, mucous discharge, and lid swelling, was performed at different times after each application using a grading scale of 0 – 2 as described in Table 1.

Table 1. Grading scale for several parameters analyzed in the macroscopic evaluation of the eyes before and after any intervention.

Grade	Discomfort	Cornea	Conjunctiva	Discharge	Lids
0	No reaction	No alterations	No alterations	No discharge	No swelling
1	Blinking	Mild opacity	Mild hyperemia/ Mild edema	Mild discharge without moistened hair	Mild swelling
2	Enhanced blinking/ Intense tearing/ Vocalizations	Intense opacity	Intense hyperemia/ Intense edema/ Hemorrhage	Intense discharge with moistened hair	Obvious swelling

2.7 Tear production

Tear production was measured using the Phenol Red Thread (PRT) with sterile cotton thread (ZoneQuick Thread, Oasis Medical, US). The threads, which turn red when tears are absorbed, were held with jeweler forceps in the inferior conjunctival sac for 30 seconds while the mice were manually restrained. After 30 seconds, the thread was removed and the distance measured in millimeters.

2.8 Evaluation of corneal barrier integrity

Corneal fluorescein staining was employed to evaluate the integrity of the corneal barrier. The eyes of the mice were photographed using a slit lamp biomicroscope (Leica, GmB) and a cobalt blue light 1 minute after the application of 1 μL sodium fluorescein (10 mg/mL).

2.9 RT-PCR

To quantify the relative expression of MUC5A mRNA, RT-PCR was performed using the expression of the S80 gene as a reference. MUC5AC mRNA levels were expressed with respect to the mRNA levels of S80. Total mRNA was extracted using Trizol and purified using the RNeasy Mini Kit (Qiagen, Germany). The mRNA was quantified using a spectrophotometer (Molecular Probes, US). The cDNA was obtained using the SuperScript®Villo cDNA Synthesis Kit (Invitrogen, US) according to the manufacturer's directions. RT-PCR was performed in a 7500 RT-PCR System (Applied Biosystems, US) using the following cycles: 50ºC for 2 minutes, 95ºC for 10 minutes, and 59ºC for 1 minute.

2.10 Statistical analysis

The results were expressed as mean ± standard error of mean (SEM) of two independent experiments and statistically analyzed by ANOVA, followed by the Tukey test.

3. Results and Discussion

The decrease in MUC5AC expression observed in several conditions known as dry eye has been extensively documented. This protein seems to play an important role in the stability of the lacrimal fluid and, thus, to be a potential target for a new therapy for dry eye. We recently developed nanoparticles capable of transfecting cells with a plasmid encoding MUC5AC, inducing the expression of MUC5AC *in vitro* in human conjunctiva cells, in terms of both mRNA and protein, and exhibiting a low toxicity profile (14, 16). We described for the first time a therapy focused on specifically increasing MUC5AC levels. Concerning the potential of these systems as carriers in gene therapy in the eye surface, these nanoparticles were prepared to transfect conjunctiva cells with pMUC5AC.

Large and negatively charged molecules, such as DNA plasmids, face several problems in being internalized by cells. However, the association of the plasmid with the nanoparticles compacted the genetic material and neutralized its charge (Table 2). The small size of the nanoparticles (<150 nm) could favor its uptake by the cells (17), and the positive zeta potential (~+30 mV) may increase interactions with the epithelium (18). The properties of the nanoparticles are mainly due to the presence of spermine attached to the backbone of the gelatin. Spermine is a small polyamine capable of interacting with the DNA, compacting and protecting it (19).

To evaluate the efficacy of such nanoparticles in transfecting pMUC5AC for the treatment of dry eye, we performed studies in healthy animals and mice submitted to desiccation stress, an animal model capable of reproducing the physiopathology of dry eye.

Table 2. Physicochemical properties of the nanostructures

	Size (nm)	ζ Potential (mV)
pMUC5AC	570.9 ± 45.7	-10.5 ± 1.6
Blank NPs	142.9 ± 17.3	+33.6 ± 4.2
pMUC5AC-NPs	127.5 ± 10.5	+37.0 ± 0.7

NP, nanoparticle

Healthy animal studies

The first studies were performed in healthy animals in order to detect possible signs of toxicity and to certify that the dose is able to induce MUC5AC expression in the ocular surface. The eyes of healthy animals were transfected with the hybrid nanoparticles. A solution of free plasmid, blank nanoparticles dispersion, and untreated animals were used as controls. Macroscopic evaluation of the eyes found no clinical alterations after the

different treatments, and all signs were scored as 0 for the cornea, conjunctiva, and lids alterations, and for discomfort or discharge.

In order to evaluate possible damage of the ocular epithelium caused by the nanoparticles, fluorescein staining was performed before and after treatment. Fluorescein is a water-soluble dye enhanced by blue light that has been used in animal models of dry eye for ocular surface staining, corneal uptake measurements, and clearance tests. Fluorescein staining has been described as the result of uptake due to a disruption of corneal epithelial cell–cell junctions and/or damaged corneal epithelial cells (20). We applied fluorescein solution into the inferior conjunctival sac using drops, and the staining was examined using a slit lamp microscope with a cobalt blue filter. None of the treatments were able to disrupt the integrity of the corneal epithelium that would result in tissue staining (Figure 1). This result was expected as each administration was below the toxic *in vitro* concentration attributed to these nanoparticles according to studies in human corneal epithelium cells.

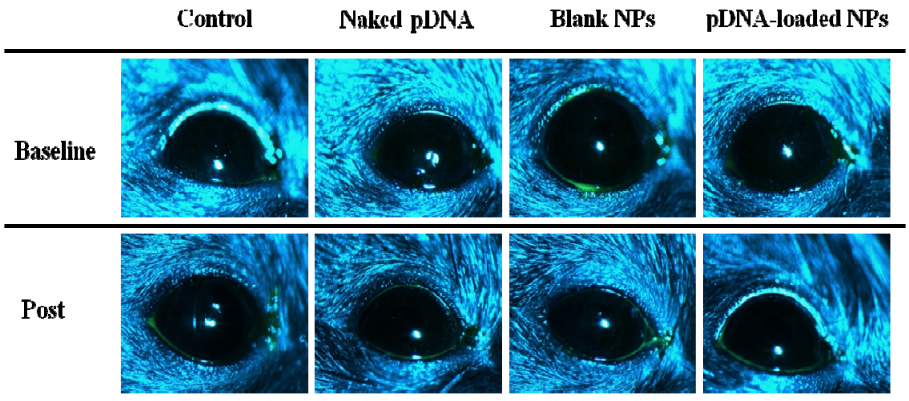

Figure 1. Slit lamp images of fluorescein staining of the ocular surface at baseline and 48 h post-treatment. Four different interventions are shown: untreated control (Control) and eyes treated with naked pMUC5AC (Naked pDNA), blank nanoparticles (blank NPs), or pMUC5AC-loaded nanoparticles (pDNA-loaded NPs).

After confirming that the nanoparticles were well tolerated and did not induce any alteration in the ocular surface, transfection efficiency was analyzed by qRT-PCR. The quantitative evaluation of mRNA synthesis is a widely used technique for evaluating transfection and protein expression. MUC5AC mRNA was expressed in mouse conjunctiva, but the same response was not observed in the cornea (Figure 2). The untreated control, naked plasmid, and blank nanoparticle controls did not express mRNA as well as the nanoparticle-treated group. Transfection of these same nanoparticles *in vitro* led to the synthesis of MUC5AC mRNA in human corneal cells, but not MUC5AC protein. Previous work found that the transfection of this same plasmid *in vivo* in rabbit eyes also only resulted in protein expression in the conjunctiva and not in the cornea (14). Therefore, MUC5AC (protein or mRNA) probably is not expressed in the cornea *in vivo*. Thus far the only part of the eye recognized to produce MUC5AC is the conjunctiva.

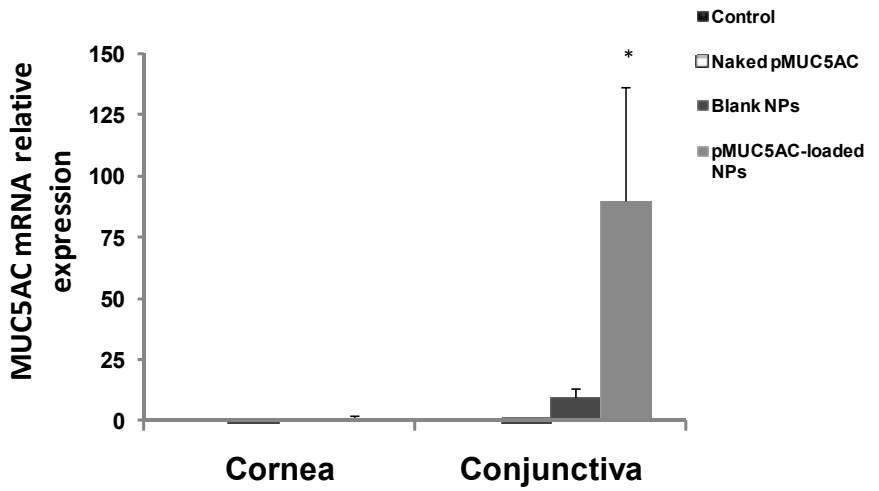

Figure 2. Expression of MUC5AC mRNA in mouse cornea and conjunctiva after different treatments: (i) untreated controls, (ii) naked plasmid, (iii) blank nanoparticles, and (iv) pMUC5AC-loaded nanoparticles. n=2; data are mean±SEM. *$p<0.01$.

The clinical response of the treatments was evaluated in terms of tear production. The PRT tear test is one of the best techniques for analyzing a patient's lacrimal system. The test was developed to overcome the disadvantages of the Schirmer tear test, including variable results, poor repeatability, low sensitivity for detecting dry eyes, and failure to measure basal secretion, even when used with anesthesia (21). The phenol red threads showed that the pMUC5AC-loaded nanoparticles were not able to increase tear production (Figure 3). No significant differences were detected between treated mice and untreated mice, blank nanoparticle application, or free plasmid solution ($p<0.01$). However, tear production tended to increase after administration of the nanoparticles. Blank nanoparticles and the plasmid solution did not induce tear production.

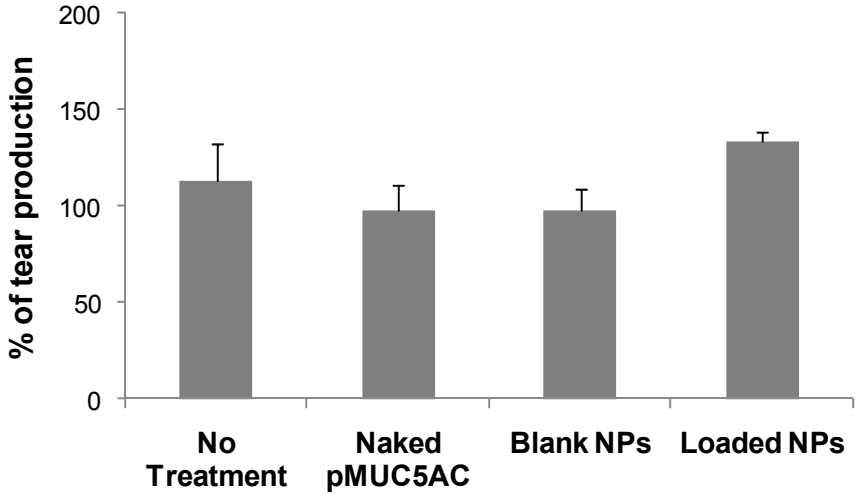

Figure 3. Percentage of tear production 48 h post-transfection compared to initial and final levels. n=10 of 2 independent experiments; data are mean±SEM.

Desiccation stress model studies

To determine whether this new treatment leads to an improvement in the clinical signs of dry eye, the nanoparticles were applied to a mouse model of dry eye and compared to a commercial formulation (Restasis®). The desiccation stress model is an animal model capable of inducing dry eye, resulting in changes in the ocular surface epithelium, with features that mimic human keratoconjunctivitis sicca. Such similarities are characterized by altered function of the corneal epithelial barrier, reduced conjunctival goblet cell density, increased conjunctival epithelial proliferation, and production of inflammatory cytokines, among others (22). In this model, animals are submitted to a blower from fans, low humidity, and the administration of the anticholinergic drug scopolamine (23). After the induction of dry eye, the mice received four different treatments: (i) free pMUC5AC, (ii) blank nanoparticles, (iii) pMUC5AC-loaded nanoparticles, and (iv) a commercial formulation (Restasis®; 0.05% cyclosporine A). Macroscopic evaluation of the eyes found no clinical alterations after the different treatments, and all signs were scored 0 for the cornea, conjunctiva, and lids alterations, and for discomfort or discharge.

Fluorescein staining is an important tool for evaluating the establishment of dry eye and treatment progress. Minimal scattered punctate staining or no staining is usually observed in the corneas of control mice after applying the fluorescein dye. After submitting the eyes to desiccation stress, patches of punctate and diffuse corneal fluorescein staining is observed (24). This evaluation is important as the damaged epithelium could be more susceptible to the possible toxic effects of the nanoparticles, and because the objective of MUC5AC expression is improved tear quality and eye protection. Treatment with pDNA-loaded nanoparticles decreased the corneal permeability to fluorescein compared to the basal levels measured before treatment (Figure 4). As reported in other studies, the commercial emulsion was also able to revert the ocular surface alterations after the treatment.

The clinical response was evaluated by changes in tear production, which was measured at three different times: baseline before desiccation stress, after desiccation stress, and after treatment. After desiccation stress, tear production significantly decreased (Figure 5). The only two treatments that increased tear production in these mice were the pMUC5AC-loaded hybrid nanoparticles and the commercial formulation. This elevation was similar to the basal level of tear production measured in healthy mice. No significant difference was observed between the effective treatments with respect to elevated tear production. The fact that blank nanoparticles and free plasmid were not able to increase tear production corroborate that the increase observed for the loaded nanoparticles was due to elevated MUC5AC levels and not irritation of the eye. No evidence indicates how MUC5AC increased tear production in the eye, but it could probably be due to increased tear quality, decreasing evaporation. This mechanism is the most probable as no anti-inflammatory or immunosuppression effect is attributed to MUC5AC.

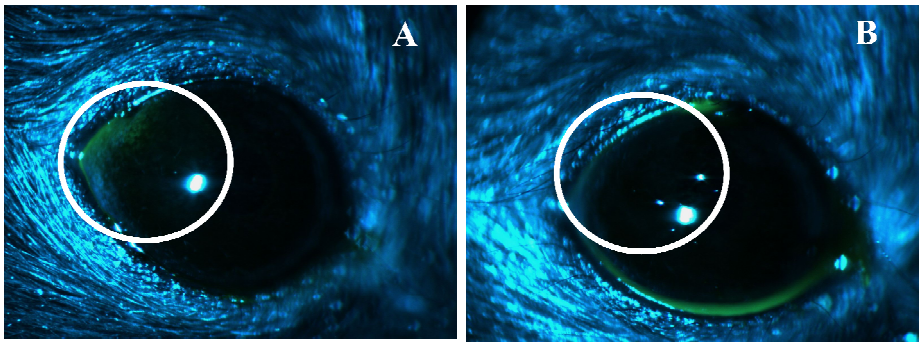

Figure 4. Slit lamp images of fluorescein staining of the ocular surface of mouse eyes submitted to desiccation stress (A) before treatment and (B) after treatment with the pMUC5AC-loaded hybrid nanoparticles.

Basically, the two advantages of using the nanoparticles to restore tear production are the absence of systemic side effects and the spectrum of application. The current therapy for dry eye is based on the palliative use of

artificial tears or the use of immunosuppressant drugs or corticosteroids. This approach can avoid the undesirable side effects associated with the systemic absorption of these classical drugs used in the management of dry eye. In addition, the therapy with pMUC5AC-loaded nanoparticles could be useful in other non-autoimmune dry eye sub-types in which T lymphocyte activation plays a secondary role and the use of immunomodulatory drugs is questionable.

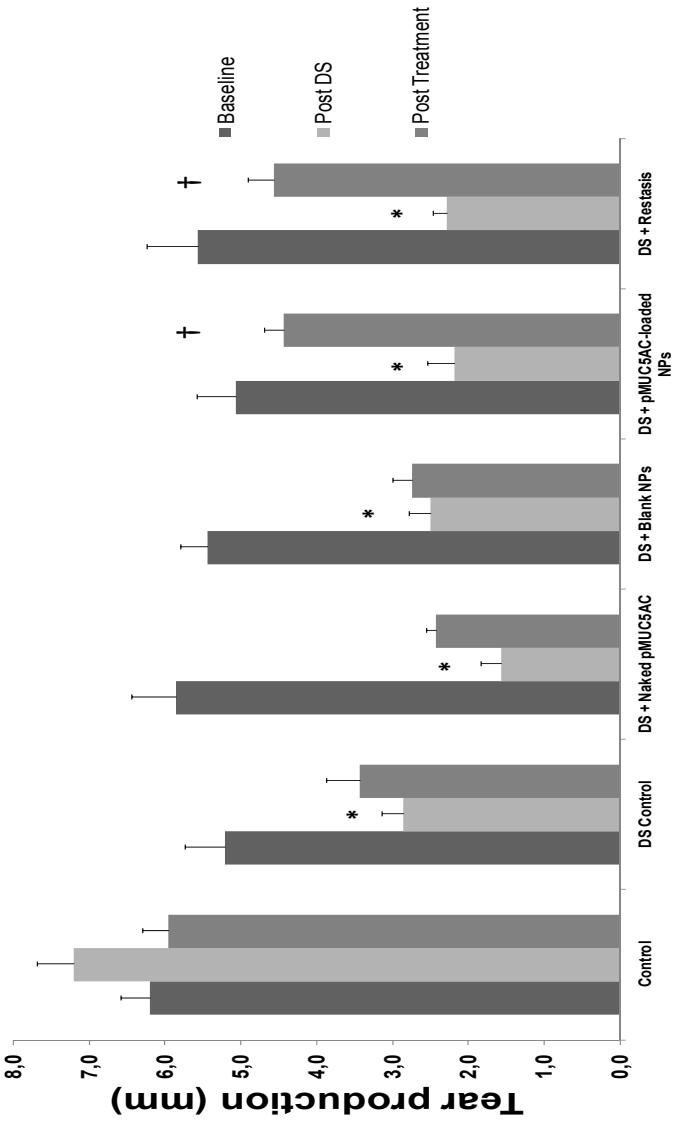

Figure 5. Tear production after different treatments (mm). The columns represent the different stages of treatment: baseline (white), post-desiccation stress (DS; grey), and post-treatment (black). Six different interventions are shown: untreated normal eyes, untreated dry eyes, and dry eyes treated with naked pMUC5AC, blank nanoparticles, pMUC5AC-loaded nanoparticles, or Restasis. n=8 of 2 independent experiments; data are mean±SEM. *$p<0.01$ vs. baseline, †$p<0.01$ vs. DS.

4. Conclusions

The elevation of MUC5AC levels in the conjunctiva restored tear production in mice submitted to desiccation stress and improved the integrity of the corneal barrier. The therapy increasing MUC5AC levels had the same efficiency as the currently available formulation, but without the associated side effects. These improvements were achieved by using hybrid nanoparticles of cationized gelatin and chondroitin sulfate capable of transfecting the mouse conjunctiva with a plasmid encoding MUC5AC. The results are a proof-of-concept of the efficacy of these nanoparticles, and a therapy focused on restoring MUC5AC levels can treat dry eye syndrome.

5. Acknowledgements

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DISCUSIÓN GENERAL

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La necesidad de buscar nuevos materiales adecuados para la terapia génica, unida a las características ventajosas de los vectores nanoestructurados como transportadores de material genético, es lo que nos han llevado a investigar el potencial de nuevas nanopartículas poliméricas, elaboradas a base de proteínas cationizadas, como vehículos para la administración de ADN plasmídico.

Inicialmente, nos hemos centrado en el estudio y caracterización del potencial de una serie de proteínas, cationizadas utilizando diferentes aminas de bajo peso molecular, como nuevos biomateriales de interés para la elaboración de nanopartículas que puedan servir para la administración de ADN plasmídico en la superficie ocular. Las proteínas seleccionadas fueron la gelatina (de diferentes pesos moleculares y puntos isoeléctricos), la albúmina y el atelocolágeno; habiéndose utilizado para su cationización dos tipos de aminas de bajo peso molecular: la etiléndiamina (una diamina) y la espermina (una tetramina). A partir de estos materiales, se han preparado nanopartículas por gelificación ionotrópica mediante reticulación por medio de un contraión. A continuación, para la optimización del nanosistema más prometedor, se ha recurrido a la incorporación de un polianión, el sulfato de condroitino (CS) o el sulfato de dextrano (DS), en la composición de dicho sistema, tratando de mejorar el perfil de toxicidad y capacidad de transfección de los vehículos.

Seguidamente, los diferentes sistemas desarrollados han sido ensayados *in vitro* para lo cual se ha utilizando un plásmido modelo que codifica la proteína verde fluorescente (GFP).

Por último, los sistemas con los que se ha obtenido una respuesta mejor han sido ensayados incorporando un plásmido, especialmente diseñado, que codifica la proteína MUC5AC. Esta proteína es una mucina fundamental para la homeostasis del fluido lacrimal y está involucrada en la patología del ojo seco. En este caso, los ensayos se realizaron tanto *in vitro* cuanto *in vivo*, en diferentes especies de animales; a fin de caracterizar mejor el potencial de aplicación que puede tener el nuevo tratamiento propuesto.

Teniendo en cuenta todo ello, la discusión de los resultados de esta tesis doctoral ha sido dividida en dos partes:

Parte I: Desarrollo y caracterización de los sistemas basados en proteínas cationizadas para la administración de ADN plasmídico en la superficie ocular, obtenidos por un procedimiento de gelificación ionotrópica.

Parte II: Desarrollo y caracterización de un nuevo tratamiento para el ojo seco basado en la terapia génica con nanopartículas polimérica, a las que asocia un plásmido que codifica la proteína MUC5AC.

Parte I: Desarrollo y caracterización de los sistemas basados em proteínas cationizadas para la administración de ADN plasmídico en la superficie ocular.

Para el éxito de la terapia génica en la práctica clínica, en este momento se puede afirmar que el desarrollo de nuevos vectores capaces de vehiculizar el material genético es necesario o incluso imprescindible. En las dos últimas décadas, los polímeros cationizados surgieron como biomateriales para el desarrollo de este tipo de vehículos. Muchas aplicaciones así como diferentes estructuras han sido propuestos para esos

polímeros, entre los cuales se encuentran: complejos[1], hidrogeles[2], micropartículas[3] y nanopartículas[4]. No obstante, hasta el momento, todos los sistemas presentan algunas limitaciones, como son por ejemplo la baja eficacia de transfección o la potencial toxicidad. Estas limitaciones no necesariamente deben ser atribuidas a los polímeros cationizados, pero pueden tener su origen en la metodología de preparación del sistema en cuestión, como ocurre en el caso de las nanopartículas que se obtienen por coacervación, en donde se hace uso tanto de disolventes orgánicos como de reticulantes químicos muy problemáticos, como es el caso del glutaraldehído[5,6].

Al igual que los lípidos[7,8] y polisacáridos catiónicos[9,10], las proteínas cationizadas presentan gran interés farmacéutico como posibles materiales para la terapia génica. En la presente memoria, la elección de las proteínas objetos de estudio para la elaboración de nanopartículas se ha basado en el

1 Aoyama T *et al.* Enhanced expression of plasmid DNA–cationized gelatin complex by ultrasound in murine muscle. *Journal of Controlled Release* 2002; **80:** 345–356.
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8 del Pozo-Rodriguez A *et al.* Solid lipid nanoparticles for retinal gene therapy: Transfection and intracellular trafficking in RPE cells. *International Journal of Pharmaceutics* 2008; **360:** 177-183.
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10 Paolicelli P *et al.* Chitosan nanoparticles for drug delivery to the eye. *Expert Opinion on Drug Delivery* 2009; **6:** 239-253.

éxito previo obtenido con algunas de ellas. Así, entre las proteínas, la gelatina cationizada es la más estudiada principalmente debido al hecho de que el producto de partida, la gelatina, es un material GRAS (*Generally Recognized As Safe*) ampliamente utilizado tanto en el campo farmacéutico como alimentario[11]. En contrapartida, otras proteínas como la albúmina y el atelocolágeno han despertado nuestro interés por su comprobada utilidad como vehículos para una serie de moléculas. Así, el atelocolágeno se ha comercializado como agente de transfección de siRNA (AteloGene™, Koken, Japan), mientras que la albúmina forma parte del primer sistema nanoparticular aprobado por el FDA (*Food and Drugs Administration*) (Abraxane®, Celgene, United States) para su uso como medicamento para el tratamiento del cancer[12]. Especificamente para este trabajo, se ha empleado HSA recombinante (rHSA), obtenida por biotecnología, por ser una materia prima con menor capacidad imunogénica y más pura. Por todo ello, las tres que han sido seleccionadas para ser cationizadas son: gelatina, albúmina y atelocolágeno.

En la Tabla 1, aparecen los diferentes productos cationizados así como sus características, que han sido utilizados en este trabajo para el desarrollo de nuevas nanopartículas por gelificación ionotrópica.

La cationización, realizada a través de una reacción de tipo carbodiimina, permitió incrementar el punto isoeléctrico de las proteínas, pasando de un valor de 5 hasta uno próximo a 11 en algunos casos, como se puede observar en la Tabla 1. Muchas aminas, todas ellas de bajo peso molecular, han sido empleadas para la cationización de proteínas como la hexalamina, colamina, putrescina, espermidina y sobre todo, la diamina etiléndiamina y la tetramina espermina. En este caso, para la cationización de

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[12] http://www.atelocollagen.com/atelogene/index.html/ en 15.02.2010

las proteínas, han sido empleadas la etiléndiamina (ED) y la espermina (SPM)[13,14]. Esta última, además, es una tetramina endógena que posee una conocida capacidad para interaccionar y también proteger el material genético[15]. Estructuras en las que se incluye la SPM han sido propuestas recientemente como alternativas menos toxicas para la terapia génica, frente a otros polímeros ya consagrados como es el caso de la polietilénimina (PEI)[16].

Tabla 1. Características de las proteínas utilizadas en el estudio.

Polímero	**PM (kDa)**	**Amina**	**$IEP_{inicial}$**	**IEP_{final}**	**Producto Final**
Gelatina	19	ED	5.0	9.80	CG_{19ed}
Gelatina	19	SPM	5.0	10.33	CG_{19spm}
Gelatina	137	ED	9.0	10.59	CG_{137ed}
Gelatina	137	SPM	9.0	11.03	CG_{137spm}
Gelatina	238	ED	9.0	10.67	CG_{238ed}
Gelatina	238	SPM	9.0	10.89	CG_{238spm}
Albúmina	66	ED	5.0	9.35	$rHSA_{ed}$
Albúmina	66	SPM	5.0	10.11	$rHSA_{spm}$
Atelocolágeno	300	ED	7.0	9.87	$Atelo_{ed}$
Atelocolágeno	300	SPM	7.0	9.81	$Atelo_{spm}$

PM, peso molecular; IEP, punto isoeléctrico; ED, etiléndiamina; SPM, espermina; CG gelatina cationizada; rHSA, albumina sérica humana recombinante; Atelo, atelocolágeno.

[13] Hori K *et al.* Controlled-release of epidermal growth factor from cationized gelatin hydrogel enhances corneal epithelial wound healing. *Journal of Controlled Release* 2007; **118:** 169–176.

[14] Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of the Progress of Disseminated Pancreatic Cancer Cells by NK4 Plasmid DNA Released from Cationized Gelatin Microspheres. 2004; **21:** 1109-1118.

[15] Vijayanathan V *et al.* Formation of DNA nanoparticles in the presence of novel polyamine analogues: a laser light scattering and atomic force microscopic study. *Nucleic Acids Research* 2004; **32:** 127-134.

[16] Thakor D, Spigelman I, Tabata Y, Nishimura I. Subcutaneous Peripheral Injection of Cationized Gelatin/DNA Polyplexes As a Platform for Non-viral Gene Transfer to Sensory Neurons. *Molecular Therapy* 2007; **15:** 2124-2131.

Puesto que en este trabajo se recurre a la síntesis de nuevos compuestos que se utilizan para el diseño de nuevas estructuras para la vectorización genética (en este caso, nanopartículas), la realización de algunos ensayos se puede considerar fundamental. En particular, no solo la evaluación de la posible toxicidad, sino también de su eficacia como agente de transfección o de la capacidad real para formar las nanopartículas por la técnica de gelificación ionotrópica partiendo de estos nuevos materiales modificados, han sido evaluadas en esta primera parte del trabajo.

Toxicidad celular

Para determinar la posible toxicidad de los nuevos biomateriales sintetizados, se ha llevado a cabo un estudio de viabilidad celular utilizando células inmortalizadas del epitelio corneal (HCE), que han sido incubadas en presencia de cantidades crecientes de los polímeros modificados. Además, se ha realizado también un estudio de hemolisis de glóbulos rojos, a fin de calibrar mejor las posibles implicaciones que, a la hora de su administración *in vivo*, pueden plantear estos nuevos polímeros.

Los factores que determinan mayoritariamente la toxicidad de los polímeros modificados, así como también de las nanopartículas, son el peso molecular y la amina utilizada para la cationización (Figura 1). Algunos autores han descrito previamente la influencia de estos mismos factores en las propiedades de otros sistemas[17]. La influencia del tipo de amina empleada en el proceso de cationización se relaciona principalmente con el número de grupos aminos cationizados y/o cationizables. A modo de ejemplo, la Figura

[17] Song Y *et al.* Effect of Molecular Weight and Degree of Substitution of Quaternized Cellulose on the Efficiency of Gene Transfection. *Bioconjugate Chemistry* 2010; **21:** 1271–1279.

1A ilustra el efecto del tipo de amina en el perfil de toxicidad de la albúmina cationizada.

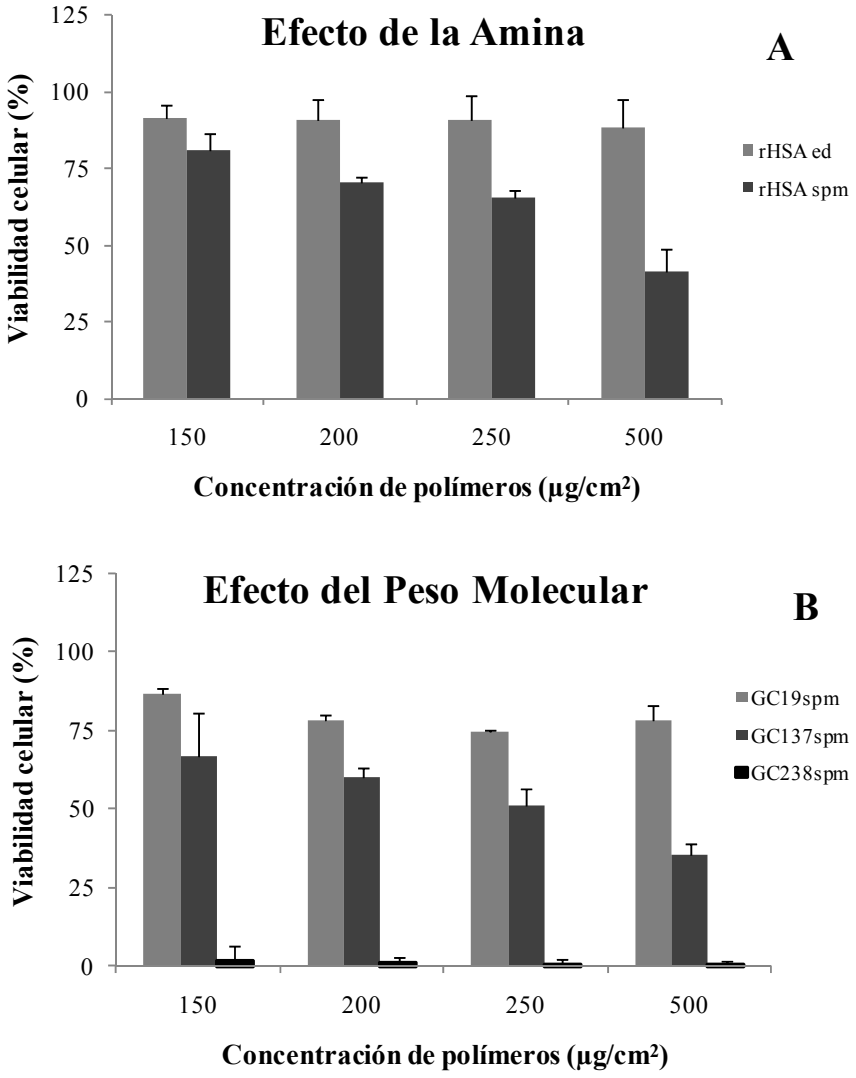


Figura 1. Influencia de la amina empleada en la cationización (A) y del peso molecular (B) de los polímeros en la viabilidad de células HCE incubadas durante 3h con diferentes concentraciones de polímeros.

Es bien conocido el efecto que esos grupos amino disponibles tienen sobre la reducción de la viabilidad celular. Concretamente, para cada uno de los polímeros sintetizados, cuando se compara el mismo material cationizado, bien con ED o bien SPM, la diferencia en el perfil de toxicidad celular es significativa. Este comportamiento puede atribuirse a las diferencias estructurales entre ambas aminas: ED es una diamina mientras que la SPM es una tetramina. En estudios realizados con implantes musculares de hidrogeles elaborados con gelatinas cationizadas con ED y SPM, se ha comprobado que los hidrogeles que incluyen SPM inducen una respuesta inflamatoria en el tejido, mientras que los hidrogeles con ED son relativamente bien tolerados[18]. La presencia de un mayor número de grupos amino en la SPM se puede considerar por tanto la responsable de ese incremento de la toxicidad.

De la misma manera, un peso molecular elevado es generalmente reconocido como potencial inductor de toxicidad. Este efecto queda patente en el gráfico de la Figura 1B, que refleja la viabilidad de las células HCE en presencia de gelatinas de diferentes pesos moleculares, todas ellas cationizadas con SPM. Como se puede apreciar, se produce un incremento de la citotoxicidad de los polímeros con el incremento de su peso molecular. Este comportamiento se ha observado también para otros polímeros como PEI[19], dextrano-DEAE[20] o la celulosa cationizada[21]. A diferencia de lo que ocurre con otros polímeros como los metacrilatos con aminas primarias, la toxicidad de estas proteínas cationizadas que se evalúan en este trabajo no

[18] Kushibiki T *et al.* Controlled release of plasmid DNA from hydrogels prepared from gelatin cationized by different amine compounds. *Journal of Controlled Release* 2006; **112:** 249-256.
[19] Fischer D *et al.* A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharmaceutical Research* 1999; **16:** 1273-1279.
[20] Fischer D *et al.* In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* 2003; **24:** 1121–1131.
[21] Song Y *et al.* Effect of Molecular Weight and Degree of Substitution of Quaternized Cellulose on the Efficiency of Gene Transfection. *Bioconjugate Chemistry* 2010; **21:** 1271–1279.

está relacionada con la ruptura de la membrana celular, ya que ninguna de ellas indujo tal efecto, el cual ha sido evaluado mediante el test de test de hemólisis[22].

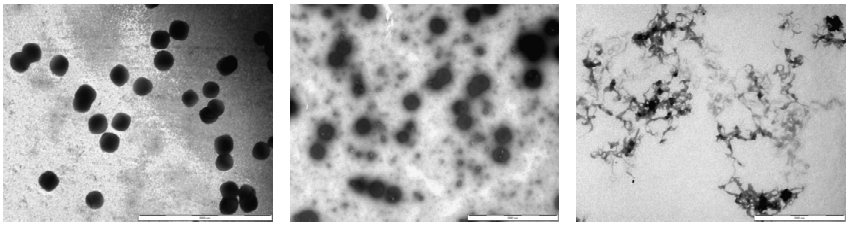
Formación de las nanopartículas por gelificación ionotrópica

Tan importante para el desarrollo del presente trabajo como la evaluación de la toxicidad de las proteínas cationizadas, es la investigación acerca de su aplicabilidad real para el objetivo principal propuesto en el mismo, que no que es otro que la formación de nanopartículas capaces de vehiculizar material genético, por un proceso de gelificación ionotrópica. Es importante destacar una vez más que esta técnica resulta muy adecuada cuando lo que se pretende es la incorporación a la estructura de las partículas, de moléculas de carácter hidrofílico y que además sean inestables, ya que permite formar sistemas con elevada eficacia de asociación, sin el inconveniente de trabajar con disolventes orgánicos u otros reactivos de elevada toxicidad[23].

Para llevar a cabo esta investigación, se ha utilizado un plásmido modelo que codifica la proteína verde fluorescente (pEGFP), el cual ha sido incorporado a la estructura de los sistemas. Así, con respecto a la capacidad para formar las partículas, se han identificado tres factores que parecen ser fundamentales. En primer lugar, la naturaleza del polímero y, una vez más, su peso molecular y la amina que ha sido empleada en la cationización del material, resultan determinantes. Es de destacar la dificultad observada para la formación de las nanopartículas tanto a partir del $Atelo_{spm}$ como del

[22] Sovadinova I *et al.* Mechanism of Polymer-Induced Hemolysis: Nanosized Pore Formation and Osmotic Lysis. *Biomacromolecules* 2011; **12:** 260–268.
[23] Nagarwal RC *et al.* Polymeric nanoparticulate system: a potential approach for ocular drug delivery. *Journal of Controlled Release* 2009; **136:** 2 -13.

$Atelo_{ed}$, que podría atribuirse a la estructura triple hélice poco flexible que es propia de la molécula de atelocolágeno y que se traduce en una dificultad del material para compactarse, al interaccionar con el agente reticulante tripolifosfato (TPP). Así, las partículas obtenidas a partir de atelocolágeno cationizado no presenta una forma definida y tiene un tamaño elevado, tal y como se ha observado en los análisis de dispersión de luz dinámica y de microscopía electrónica de transmisión. Cuando comparada la morfología de las partículas de albúmina y gelatina cationizadas con las de atelocolágeno, que se muestran en la Figura 2 por las nanopartículas elaboradas con CG_{19spm}, $rHSA_{spm}$ y $Atelo_{spm}$, respectivamente.



CG_{19spm} NPs $rHSA_{spm}$ NPs $Atelo_{spm}$ NPs

Figura 2. Imágenes de microscopía electrónica de transmisión de nanopartículas elaboradas con CG_{19spm}, $rHSA_{spm}$ y $Atelo_{spm}$.

La albúmina cationizada, la cual presenta una estructura globular, también puede dar lugar a nanopartículas por gelificación en un rango muy estrecho (con respecto a la relación polímero/reticulante utilizados), si bien la morfología y el tamaño de las partículas es muy semejante a la de las nanopartículas de gelatina cationizada obtenidas por la técnica de gelificación ionotrópica, como también se puede observar en la imagen que se recoge en la Figura 2.

Protección del material genético

La presencia de endonucleasas en el fluido lacrimal, supone una amenaza para el éxito de cualquier tratamiento basado en la terapia génica que vaya a ser instilado sobre la superficie ocular, puesto que es posible que el sistema desarrollado no sea capaz de mantener la integridad del material genético hasta que éste alcance su lugar de acción. En consecuencia, para evaluar la capacidad de las nuevas nanopartículas para proteger el ADN plasmídico, se ha realizado un ensayo de resistencia a DNasa, más específicamente frente a DNasa I.

Con excepción de la nanopartículas obtenidas a partir de atelocolágeno, todas las demás nanoestructuras desarrolladas han sido capaces de proteger el ADN plasmídico frente a la degradación por DNasa (específicamente frente a la DNasa I). Este efecto es mucho más pronunciado cuando la estructura del sistema se forma partiendo de polímeros cationizados con SPM, lo que demuestra que la amina empleada en el proceso de cationización es determinante para la protección del material genético. Tal efecto ya fue observado en estudios realizados anteriormente, en los que, utilizando bromuro de etídio, se comprobó que la gelatina cationizada con SPM interacciona más fuertemente con el material genético[24].

La explicación del porqué las nanopartículas de atelocolágeno cationizados resultaron ineficaces para la protección del plásmido, es la misma que justifica por qué este material tiene una capacidad limitada para la formación de las nanopartículas, es decir su estructura en forma de triple

[24] Kushibiki T *et al.* In vitro transfection of plasmid DNA by cationized gelatin prepared from different amine compounds. *Journal of Biomaterials Science-Polymer Edition* 2006; **17:** 645-658.

hélice. En virtud de esa estructura, el atelocolágeno no es capaz de enrollarse suficientemente para proteger la estructura del ADN plasmídico[25]. Por el contrario, este material ha tenido el éxito en lo que se refiere a la protección de siRNA[26], lo que debe ser atribuido al hecho que el siRNA presenta una forma rígida de pequeños bastones que pueden alinearse con la estructura del polímero, de forma muy diferente a lo que ocurre en el caso de la estructura superenrollada de un plásmido[27].

Eficacia en transfección in vitro

La capacidad del plásmido modelo pEGFP para transfectar *in vitro* células inmortalizadas del epitelio corneal humano (HCE) depende principalmente del tipo de amina empleada en la cationización del material constitutivo del vehículo. Como se visualiza en la Figura 3, las nanopartículas obtenidas a partir de los polímeros en los que se emplea SPM presentan una mayor capacidad para transfectar las células HCE, lo que nuevamente puede ser atribuido a la mayor capacidad para proteger el ADN plasmídico ofrecida por esas partículas, de acuerdo con lo anteriormente comentado. Es importante destacar que esa diferencia en el comportamiento no se observa cuando el vehículo utilizado son hidrogeles, los cuales han sido administrados *in vivo*. En este caso, los hidrogeles preparados con gelatina

25 Sano A *et al.* Atelocollagen for protein and gene delivery. *Advanced Drug Delivery Review* 2003; **55:** 1651-1677.
26 Kawakami E *et al.* Atelocollagen-mediated systemic administration of myostatin-targeting siRNA improves muscular atrophy in caveolin-3-deficient mice. *Development, Growth & Differentiation* 2011; **53:** 48-54.
27 Gary DJ, Puri N, Won Y. Polymer-based siRNA delivery: Perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. *Journal of Controlled Release* 2007; **121:** 64–73.

cationizada con ED o con SPM, exhiben la misma capacidad para transfectar *in vivo* células del tejido muscular [28] o renal[29] de ratones.

En nuestro estudio, también se ha puesto de manifiesto la mayor capacidad superior de las nanopartículas elaboradas con gelatina para transfectar las células HCE, sobre todo si se trata de gelatinas de mayor peso molecular. Así, independentemente del tipo de amina empleada para la cationización, las gelatinas de 137kDa y 238kDa muestran una actividad biológica superior a la que corresponde a los demás materiales estudiados (Figura 3). Además, a diferencia de lo que ocurre con nanopartículas híbridas en las que están presentes oligómeros de quitosano[30], que es el polímero catiónico natural que más se utiliza como agente de transfección, cuando se utilizan oligómeros de gelatina cationizada (CG_{19ed} y CG_{19spm}) no se observa incremento de la capacidad de la partículas para transfectar las células HCE.

En lo que respecta a la capacidad de transfección de las nanopartículas de rHSA cationizada evaluadas en este trabajo, los resultados se asemejan mucho a los anteriormente descritos para complejos de HSA cationizada con hexalamina, en las que se observó una baja capacidad de transfección presentan baja capacidad (< 1%) en fibroblastos[31].

[28] Kushibiki T *et al.* Controlled release of plasmid DNA from hydrogels prepared from gelatin cationized by different amine compounds. *Journal of Controlled Release* 2006; **112:** 249-256.
[29] Kushibiki T *et al.* Targeting of Plasmid DNA to Renal Interstitial Fibroblasts by Cationized Gelatin. *Biological & Pharmaceutical Bulletin* 2005; **28:** 20017-22010.
30 de la Fuente M, Seijo B, Alonso MJ. Bioadhesive hyaluronan-chitosan nanoparticles can transport genes across the ocular mucosa and transfect ocular tissue. *Gene Therapy* 2008; **15:** 668-676.
31 Fischer D *et al.* Cationized human serum albumin as a non-viral vector system for gene delivery? Characterization of complex formation with plasmid DNA and transfection efficiency. *International Journal of Pharmaceutics* 2001; **225:** 97–111.

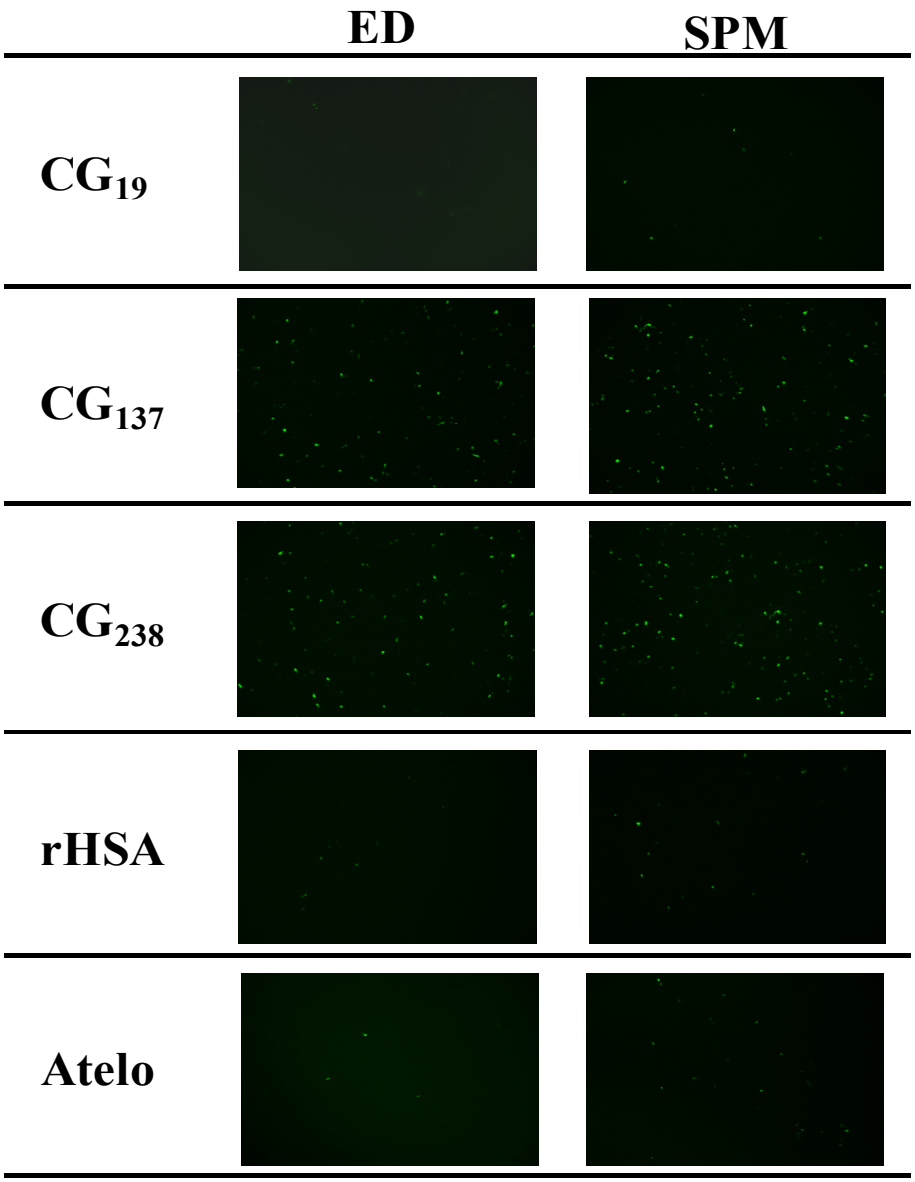


Figure 3. Expresión de la proteína verde fluorescente en células HCE, tras su incubación en presencia de nanopartículas elaboradas a partir de los diferentes polímeros cationizados con etiléndiamina (ED) o espermina (SPM). El tiempo de incubación 3h (1µg pEGFP/pocillo) y examen al microscopio de fluorescencia 48h postransfección.

Optimización de los sistemas con polianiones

Un sistema ideal para terapia génica se puede considerar aquel que cumple los 4 pre-requisitos siguientes: (i) fácil producción; (ii) capacidad de protección del material genético; (iii) eficacia en la transfección; (iv) ausencia de toxicidad. Teniendo en cuenta todo ello, los sistemas que han resultado más prometedores en la primera parte de este trabajo han sido tres formulaciones de gelatina cationizada, concretamente las nanopartículas de $CG_{137ed,}$ CG_{137spm} y CG_{238ed}. Para continuar los estudios, se ha decidido optimizar uno de los tres, en concreto las nanopartículas de CG_{137spm} , por ser las que han dado lugar a una mayor expresión de la proteína verde fluorescente en células HCE.

La estrategia que se ha diseñado para mejorar las propiedades de estas nanopartículas obtenidas a base de polímeros catiónicos, consiste en introducir en su nanoestructura un polímero de carga negativa. De esta manera es posible combinar en el mismo sistema, las adecuadas propiedades de transfección y protección del ADN plasmídico que presentan los polímeros positivos, con algunas propiedades interesantes de los polímeros negativos, como es su reducida toxicidad y, en casos específicos, su capacidad para interaccionar con receptores específicos que facilitan la internalización celular. En consecuencia, se han elegido los polímeros aniónicos sulfato de condroitino y sulfato de dextrano para la optimización de las nanopartículas de gelatina cationizada CG_{137spm}. Ambos, son polímeros naturales, biodegradables y biocompatibles, capaces de interaccionar con los receptores CD44 y HARE (*Hyaluronic Acid Receptor for Endocytosis*)[32,33].

[32] Harris EN, Weigel PH. The ligand-binding profile of HARE: hyaluronan and chondroitin sulfates A, C, and D bind to overlapping sites distinct from the sites for heparin, acetylated low-density lipoprotein, dermatan sulfate, and CS-E. *Glycobiology* 2008; **18:** 638-648.

Además, pueden tener otras muchas aplicaciones en el campo biomédico, que van desde ingeniería de tejidos hasta su utilización como estabilizantes en diversas formulaciones. Así, en este trabajo se han desarrollado las nanopartículas híbridas de gelatina cationizada con espermina y sulfato de condroitino (GC_{137spm}/CS) y de gelatina cationizada con espermina y sulfato de dextarno (GC_{137spm}/DS), que han sido evaluadas y comparadas con las nanopartículas constituidas únicamente por gelatina $GC_{137spm.}$ A todas ellas se ha asociado el plásmido modelo que codifica la proteína verde fluorescente GFP.

Lo primero que es interesante destacar es que la incorporación de los polímeros con carga negativa, permite una amplia modulación de algunas características fisicoquímicas que son importantes para el comportamiento de las nanopartículas, como son su tamaño y potencial zeta. Tomando como ejemplo las nanopartículas híbridas GC_{137spm}/CS, tal y como se refleja en los resultados recogidos en las Tabla 3 y 4, resulta evidente que es posible obtener partículas con propiedades diferentes, simplemente variando la relación entre los dos polímeros constituyentes de las mismas.

[33] Harris EN, Weigel JA, Weigel PH. Endocytic function, glycosaminoglycan specificity, and antibody sensitivity of the recombinant human 190-kDa hyaluronan receptor for endocytosis (HARE). *Journal of Biological Chemistry* 2004; **279:** 36201-36209.

Tabla 3. Influencia de la cantidad de sulfato de condroitino (CS) y de agente reticulante tripolifosfato (TPP) en el diámetro medio (nm) de nanopartículas de GC_{137spm} cargadas con 5% of pDNA.

		Ratio CG_{137spm}/TPP			
		30/1	12/1	6/1	4/1
Ratio CG_{137spm}/CS	15/1	167 ± 7	218 ± 34	253 ± 12	301 ± 28
	6/1	186 ± 20	258 ± 23	290 ± 20	+
	3/1	253 ± 47	278 ± 32	270± 76	+
	2/1	202 ± 11	197 ± 6.9	+	+
	1.5/1	173 ± 20	+	+	+
	1/1	+	+	+	236 ± 13
	0.75/1	168 ± 2	170 ± 17	155 ± 14	153 ± 14

"+" precipitación del sistema

Tabla 4. Influencia de la cantidad de sulfato de condroitino (CS) y de agente reticulante tripolifosfato (TPP) en el potencial zeta (mV) de nanopartículas de GC_{137spm} cargadas con 5% of pDNA.

		Ratio CG_{137spm}/TPP			
		30/1	12/1	6/1	4/1
Ratio CG_{137spm}/CS	15/1	48.2 ± 1.9	42.4 ± 4.2	34.6 ± 6.9	25.3 ± 2.6
	6/1	39.7 ± 2.1	40.5 ± 4.6	36.7 ± 11	+
	3/1	42.3 ± 2.7	37.5 ± 1.8	34.8 ± 14	+
	2/1	35.6 ± 3.4	28.7 ± 4.5	+	+
	1.5/1	34.9 ± 3.4	+	+	+
	1/1	+	+	+	-10.5 ± 16
	0.75/1	-35.8 ± 0.4	-34.2 ± 7.0	-36.7 ± 2.3	-38.1 ± 2.1

"+" precipitación del sistema

Esta facilidad para modular las propiedades de los nanosistemas resulta extremadamente interesante, puesto que permite adecuar sus características a las necesidades y limitaciones propias de cada vía de administración. Así, mientras que para su utilización en la superficie ocular una carga superficial positiva puede ser importante para aumentar la interacción de las partículas con el epitelio ocular[34], en la administración por otras vías, como por ejemplo la intravitrea[35] en el caso del ojo o la administración parenteral[36], la interacción de las nanopartículas cargadas positivamente con determinados elementos del tejido (humor vítreo o proteínas plasmáticas por ejemplo) resulta indeseada. Así pues, la facilidad para obtener sistemas con propiedades tan diversas haciendo uso de esta estrategia, amplia considerablemente el abanico de las futuras aplicaciones de estas nuevas nanopartículas híbridas.

Por su parte, las características de las nanopartículas de GC_{137spm}/DS, también se mostraron modulables pero con un perfil ligeramente distinto del correspondiente a las nanopartículas de GC_{137spm}/CS. Estas variaciones pueden ser debidas a diferencias estructurales entre el sulfato de condroitino y sulfato de dextrano. Este último, presenta mayor densidad de grupos sulfatos en su estructura que el CS. El impacto de los grupos sulfato en la interacción entre polímeros ya ha sido estudiado por diferentes autores, atribuyéndoles un papel fundamental en el comportamiento de complejos entre polianiones y proteínas[37,38]. Por lo tanto, es de suponer que

[34] Rabinovich-Guilatt L, Couvreur P, Lambert G, Dubernet C. Cationic Vectors in Ocular Drug Delivery. *Journal of Drug Targeting* 2004; **12:** 623–633.
[35] Peeters L *et al.* Vitreous: A barrier to nonviral ocular gene therapy. *Investigative Ophthalmology & Visual Science* 2005; **46:** 3553-3561.
[36] Aggarwal P *et al.* Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy. *Advanced Drug Delivery Reviews* 2009; **61:** 428-437.
[37] Raman R, Sasisekharan V, Sasisekharan R. Structural Insights into Biological Roles of Protein-Glycosaminoglycan Interactions. *Chemistry & Biology* 2005; **12:** 267-277.
[38] Sacco D, Dellacherie E. Interaction of a macromolecular polyanion, dextran sulfate, with human hemoglobin. *FEBS Letters* 1986; **199:** 245-258.

interacciones de magnitudes diferentes también se establezcan en las nanopartículas híbridas en función de que esté presente uno u otro polianión.

La influencia de la incorporación de los polianiones en la formulación no se observa solamente en las propiedades fisicoquímicas de las partículas. La interacción de las mismas con sistemas biológicos también se ve modificada por la presencia del polianión, tal y como se ha comprobado en el ensayo *in vitro* en el que se ha evaluado la toxicidad de las partículas en células HCE, después de 3h de incubación. Los resultados indican que la presencia de los polianiones en las nanopartículas híbridas, reduce significativamente la toxicidad de las mismas. Sin duda, esta reducción se debe a la neutralización, en cierta medida, de las cargas positivas que son responsables de la toxicidad de algunas nanoestructuras. Este mismo comportamiento ya ha sido observado anteriormente en nanopartículas híbridas, obtenidas en nuestro laboratorio a partir de quitosano y ácido hialurónico[39], así como en complejos de PEI y sulfato de dextrano[40]. Además, la disminución de la toxicidad de las nanopartículas híbridas con respecto a las que no incorporan el polímero aniónico, no estuvo acompañado de una disminución en la capacidad de transfección de las partículas, como se puede observar en la Figura 4. Esto demuestra por tanto, la eficacia de la estrategia de optimización propuesta y el interés de los sistemas híbridos desarrollados.

[39] De la Fuente M, Seijo B, Alonso MJ. Novel hyaluronic acid-chitosan nanoparticles for ocular gene therapy. *Investigative Ophthalmology & Visual Science* 2008; **49:** 2016-2024.
[40] Tiyaboonchai W, Woiszwillo J, Middaugh CR. Formulation and characterization of DNA–polyethylenimine–dextran sulfate nanoparticles. *European Journal of Pharmaceutical Sciences* 2003; **19:** 191–202.

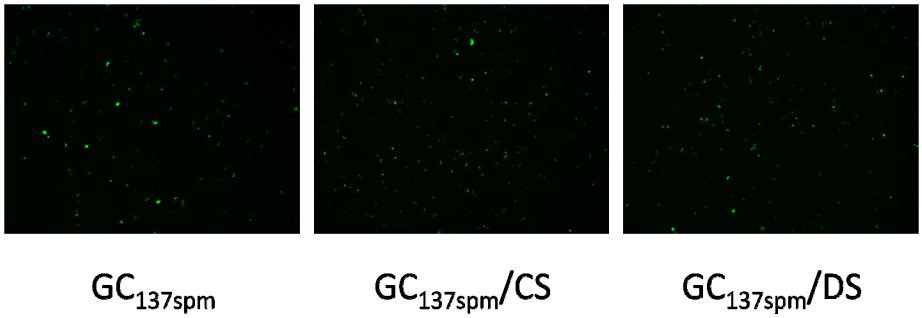


Figura 4. Expresíon de la proteína verde fluorescente en células HCE, 48h después ser incubadas (1 µg pEGFP/pocillo) con diferentesformulaciones de nanopartículas: NP de gelatina cationizada con espermina (GC_{137spm}), NP híbridas que incluyan sulfato de condroitino (GC_{137spm}/CS) o sulfato de dextrano (GC_{137spm}/DS).

Parte II: Desarrollo y caracterización de un nuevo tratamiento para el ojo seco basado en la terapia génica con nanopartículas poliméricas asociando plásmido que codifica la proteína MUC5AC.

Hasta el momento, las alternativas de las que se dispone para tratar la enfermedad conocida como *Síndrome de Ojo Seco* son realmente muy escasas. Las posibles causas que intervienen en el son diversas, pero la reducción de los niveles de la proteína MUC5AC se relaciona constantemente con esta patología. Dado que no existe ningún procedimiento conocido para aumentar específicamente la producción de MUC5AC, en este trabajo nos hemos marcado el reto de desarrollar un nuevo tratamiento basado en la terapia génica, a través de la vehiculización de un plásmido diseñado específicamente para este trabajo, que codifica la expresión de esta proteína. La estructura de ese plásmido es la que se muestra en la Figura 5.

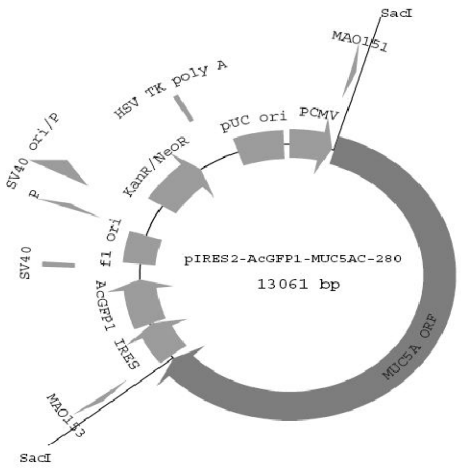


Figura 5. Estructura del plásmido pIRES2-AcGFP1-MUC5AC-280 (pMUC5AC) empleado en los estudios *in vitro*.

Incorporación del plásmido que codifica la MUC5AC a las nanopartículas

Concretamente, se han desarrollado dos plásmidos distintos que codifican la MUC5AC, siendo la diferencia entre ambos que uno de ellos posee una secuencia adicional que codificadora de GFP, con el fin de facilitar la identificación de la expresión de la proteína en los experimentos *in vitro*. El otro plásmido, que no incluye la secuencia codificadora de la GFP, es el que se ha utilizado en los experimentos *in vivo*.

Para evaluar la capacidad de transfección *in vitro* de este nuevo plásmido incorporado en las partículas, se prepararon 3 formulaciones diferentes de nanopartículas: (i) GC_{137spm},, (ii) GC_{137spm}/CS, (iii) GC_{137spm}/DS. Las características de todos estos sistemas se presentan en la Tabla 6.

Tabla 6. Características de las nanopartículas que incorporan el con plásmido pMUC5AC (7.5%): tamaño, potencial ζ y eficacia de asociación (EA).

Formulación	Razón	Tamaño (nm)	PDI	ζ (mV)	EA (%)
pMUC5AC	-	571 ±46	0.4	-10.5 ± 1.6	-
CG_{137spm}:TPP	8:1	262 ±6	0.1	+21.6 ±0.9	99.2±0.5
CG_{137spm}:CS:TPP	12:1:0.5	268±28	0.1	+33.4 ±3.1	97.6±1.3
CG_{137spm}:DS:TPP	15:1:0.5	211 ±8	0.1	+30.6 ±2.0	95.7±3.2

CG_{137spm}, gelatina 137kDa cationizada con espermina; CS, sulfato de codroitino; DS, sulfato de dextrano (promedio ± D.S.; n=3).

Los resultados demuestran que todas las nanopartículas han sido capaces de condensar el ADN plasmídico, una vez que el tamaño de la estructura del plásmido libre pasa de 570 nm a cerca de 200 nm cuando está asociado a las nanopartículas. Además, las partículas han sido capaces de neutralizar la carga negativa del plásmido, dando lugar a sistemas que presentan un valor de potencial zeta positivo. Precisamente, el hecho de que los nanosistemas desarrollados presenten carga positiva, tiene una gran importancia para tratar de incrementar al máximo la interacción entre el nanoestructuras y la superficie del globo ocular. En las tres formulaciones se ha obtenido una elevada eficacia de asociación del plásmido, que se relaciona con la presencia de la SPM en la cadena de gelatina, que es capaz de intercalarse en la estructura doble hebra del ADN, aumentando así la interacción con la estructura de la partícula.

Estudios de transfección in vitro en células del epitelio ocular

La evaluación indirecta de la expresión de la MUC5AC por medio de detección de la fluorescencia de la GFP por microscopia, no resultó exitosa. Como se observa en las Figura 6 y 7, ninguna de las formulaciones y tampoco el control positivo han sido capaces de originar expresión visible en

ninguna de las dos líneas celulares estudiadas, HCE y IOBA-NHC. El único grupo capaz de generar esta expresión de GFP ha sido el que corresponde al control realizado con el plásmido pEGFP (JetPEI-RGD™ + pEGFP) lo que evidentemente no permite extraer ninguna conclusión al respecto de la eficacia del tratamiento. Tal y como ya ha sido documentado en otras ocasiones, no siempre la inclusión adicional de la secuencia de la GFP es eficaz cuando tiene que servir de herramienta para vehículizar nuevos plásmidos. La causa más probable de los resultados obtenidos es que los niveles de producción de la GFP están por debajo del límite de detección de la técnica de microscopía empleada[41].

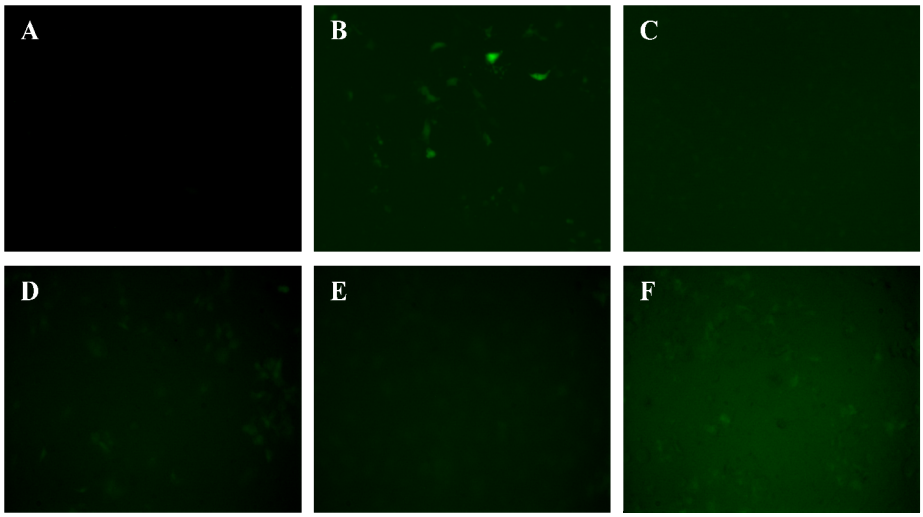


Figura 6. Expresión de proteína verde fluorescente en células HCE después de incubación durante 3h de diferentes nanopartículas (5µg pDNA/pocillo). (A) Control no tratado, (B) JetPEI-RGD™ + pEGFP, (C) JetPEI-RGD™ + pMUC5AC, (D) nanopartículas de CG_{137spm} , (E) nanopartículas de CG_{137spm}/CS , (F) nanopartículas de CG_{137spm}/DS.

[41] Kain SR, Kitts P. Expression and Detection of Green Fluorescent Protein (GFP). *Methods Mol Biol* 1997; **63:** 305-324.

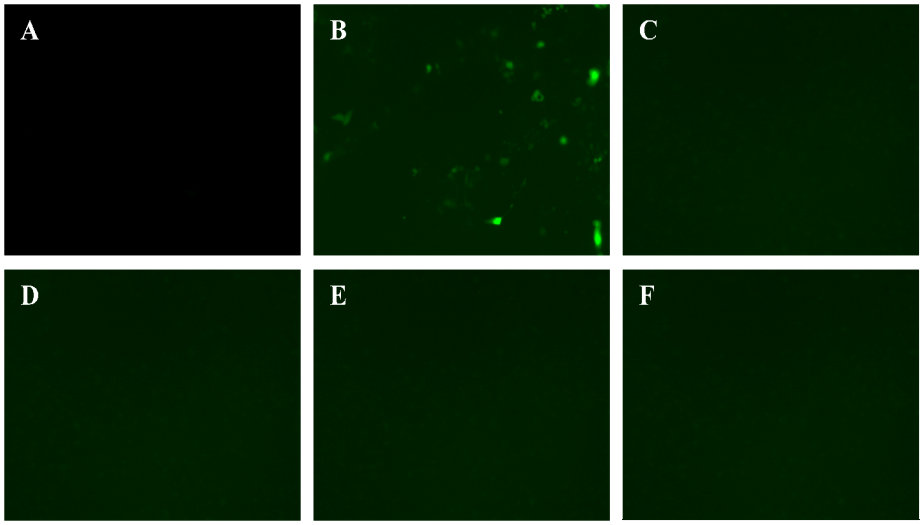


Figura 7. Expresión de proteína verde fluorescente en células IOBA-NHC después de incubación durante 3h de diferentes nanopartículas (5µg pDNA/pocillo). (A) Control no tratado, (B) JetPEI-RGD™ + pEGFP, (C) JetPEI-RGD™ + pMUC5AC, (D) nanopartículas de CG_{137spm} , (E) nanopartículas de CG_{137spm}/CS , (F) nanopartículas de CG_{137spm}/DS.

Tras estos resultados, se ha llevado a cabo la evaluación de la capacidad de transfección en las mismas líneas celulares de cornea (HCE) y de conjuntiva (IOBA-NHC), pero ahora analizando la producción de mRNA por la técnica de RT-PCR (*Real Time-Polymerase Chain Reaction*) (Figura 8), así como también mediante la detección de la proteína por ELISA (*Enzyme Linked Immunosorbent Assay*) (Figura 9).

Las nanopartículas presentaron comportamientos distintos frente a las dos líneas celulares. Así, en lo que se refiere a la expresión de mRNA, las nanopartículas GC_{137spm} fueron más eficaces en cuanto a capacidad para transfectar las células HCE (Figura 8), mientras que las nanopartículas híbridas GC_{137spm}/CS resultaron las más eficientes en las células IOBA-NHC (Figura 8B). En cuanto al comportamiento de las nanopartículas GC_{137spm}/DS, siempre han dado lugar a una respuesta menos eficaz.

Las razones por la cuales el comportamiento de las tres formulaciones estudiadas es diferente todavía no están claras, si bien *a priori* puede ser incluso interesante disponer de sistemas que se puedan comportar de manera distinta en distintos tipos celulares, puesto que de esta manera se podría seleccionar la mejor formulación de cara a lograr una vectorización mucho más eficaz en función del objetivo terapéutico planteado.

Las diferencias en la respuesta de las dos líneas celulares en presencia de las nanopartículas, es todavía más evidente cuando se analiza la expresión de la proteína propiamente dicha; tal y como se puede apreciar en la Figura 9 La cuantificación de la MUC5AC por ELISA reveló que, transcurridas 72h post-incubación de las células con las nanopartículas, la proteína solo se detecta en la línea IOBA-NHC (Figura 9B). Una explicación de este resultado podría estar en que los niveles de mRNA que son necesarios para producir la expresión de la proteína en las células HCE, son superiores a los que se requieren en las células IOBA-NHC. En este sentido, es importante destacar que la producción del mRNA y de la proteína no siempre presenta una clara correlación y que, para determinadas líneas celulares, un número mayor de copias de mRNA es necesario para la traducción de la proteína[42,43].

En cualquier caso, y aunque con las partículas no se ha obtenido un resultado positivo en las células de córnea, lo que es importante destacar es que las células HCE tienen capacidad para producir la MUC5AC después de ser transfectados, como lo demuestra el elevado valor post-transfección que se obtiene tras la administración del plásmido junto con el control positivo (JetPEI-RGD™) (Figura 9A), lo que indica que el plásmido diseñado permite conseguir el objetivo propuesto, en ambas líneas celulares.

[42] Gry M *et al.* Correlations between RNA and protein expression profiles in 23 human cell lines. *BMC Genomics* 2009; **10:** Epub.
[43] Chen G *et al.* Discordant Protein and mRNA Expression in Lung Adenocarcinomas. *Mol Cell Proteomics* 2002; **12**.

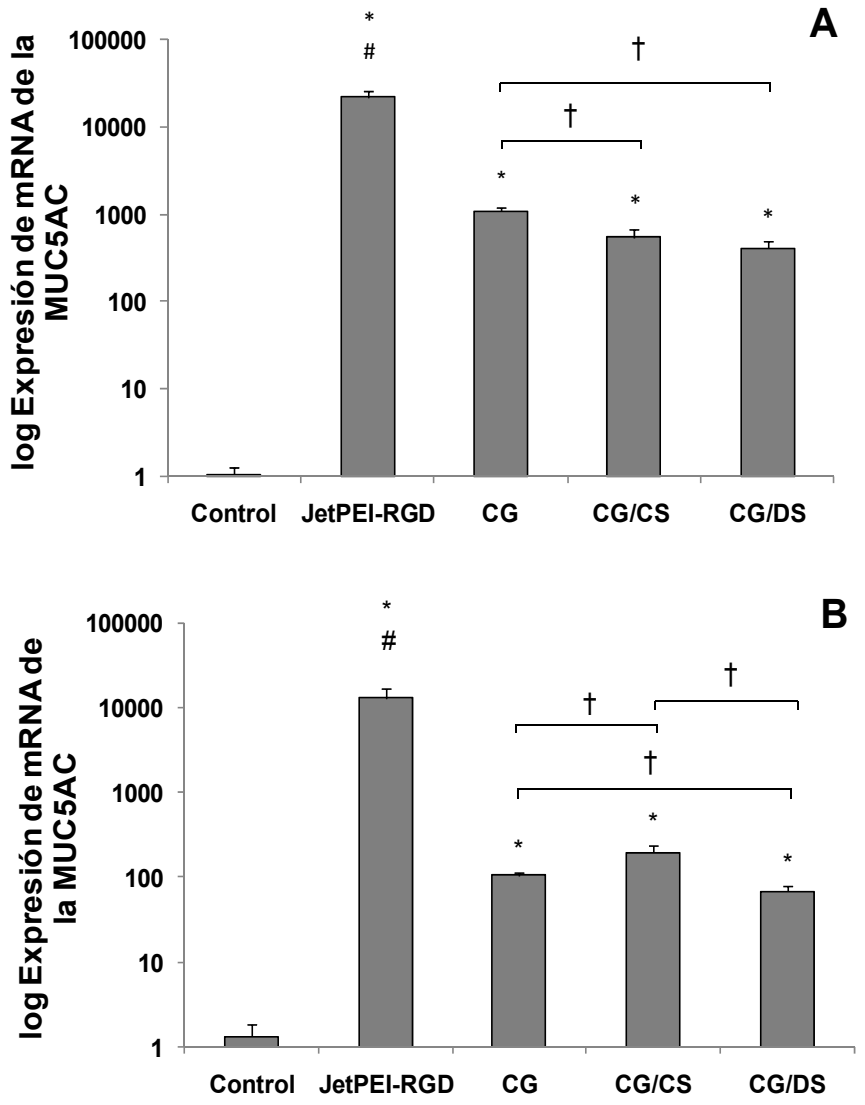


Figura 8. Expresión relativa del mRNA de MUC5AC mRNA, en relación al mRNA de GAPDH, después de 3h de incubación con controles de plásmido-JetPEI y suspensión de las nanopartículas (1µg de plásmido en caso de los controles y 5µg en caso de las formulaciones) en células HCE (A) y IOBA-NHC (B) (n=3 de experimentos independientes en triplicado). * diferencia estadística con respecto al control no tratado ($p<0.05$) † diferencia estadística entre formulaciones ($p<0.05$).

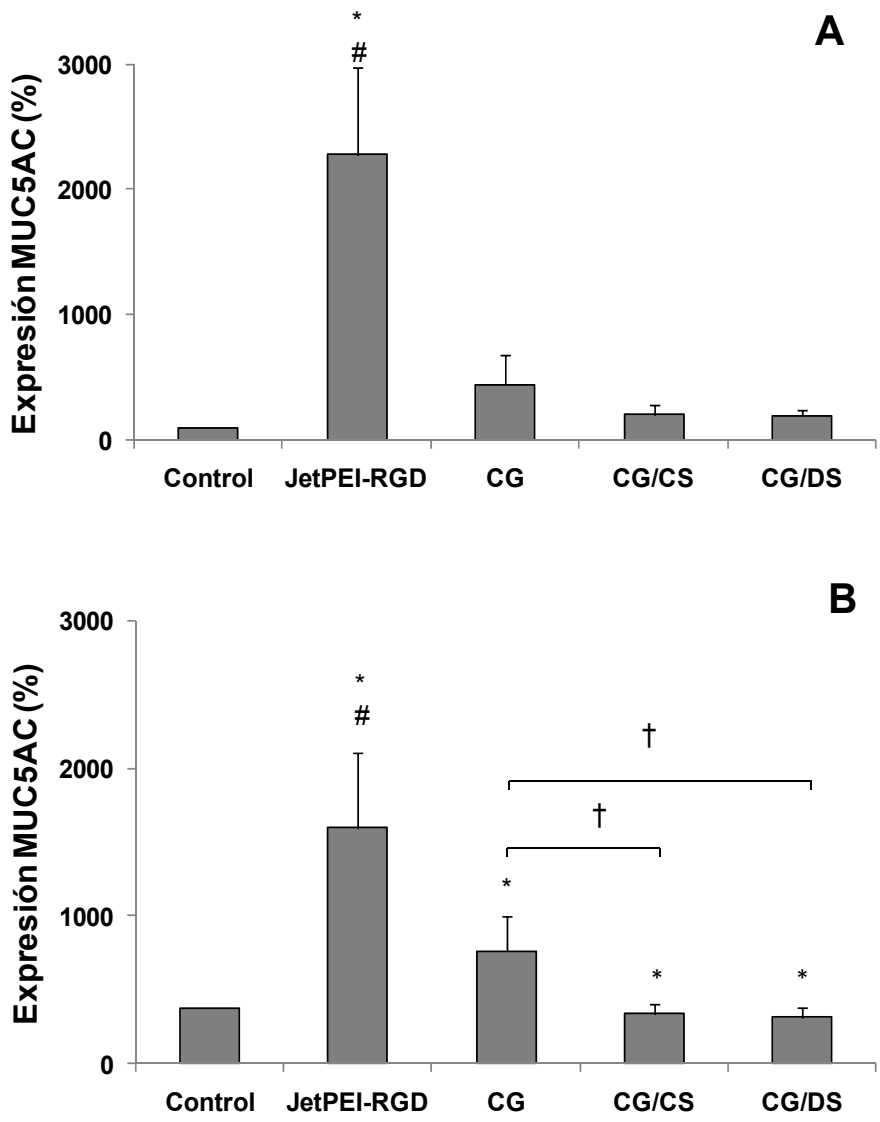


Figura 9. Expresión de MUC5AC en relación al control no tratado después de 3h de incubación con controles de plásmido-JetPEI y de las suspensiones de nanopartículas (1µg de plásmido en caso de los controles y 5µg en caso de las formulaciones) en células HCE (A) y IOBA-NHC (B) (n=3 de experimentos independientes en triplicado). * diferencia estadística con respecto al control no tratado ($p<0.05$) † diferencia estadística entre formulaciones ($p<0.05$).

Experimentos in vivo

Una vez asegurada la capacidad de las nanopartículas desarrolladas para transfectar *in vitro* el plásmido pMUC5AC, la próxima etapa fue la realización de ensayos en animales para comprobar la eficacia real del tratamiento diseñado. Todos los ensayos han sido previamente aprobados y siguieron las normativas del ARVO (*The Association for Research in Vision Ophtalmology*).

Teniendo en cuenta que las nanopartículas híbridas de GC_{137spm}/CS fueron las que produjeron una mejor respuesta en términos de toxicidad y de expresión de MUC5AC en la conjuntiva, tanto en lo que se refiere a la expresión de mRNA como en la capacidad de expresar la proteína MUC5AC, hemos decidido estudiar su potencial de transfección en ratones sanos y en ratones sometidos a proceso de ojo seco inducido, estudio que se ha sido realizado en los Laboratorios Allergan, Inc. en California, que disponen tanto del modelo animal como de las instalaciones adecuadas para llevar a cabo el estudio en las mejores condiciones.

Asimismo, para profundizar un poco más sobre la capacidad de transfección de los otros dos sistemas nanoparticulares elaborados con GC_{137spm} y GC_{137spm}/DS, nos hemos planteado evaluar *in vivo* su comportamiento, pero en ese caso estudiándolos tras su administración tópica ocular en conejos sanos.

Por último se debe señalar que, una vez que las células de los epitelios oculares no han sido capaces de expresar la GFP en los estudios de transfección *in vitro*, se ha optado por retirar la secuencia que la codifica de la construcción plasmídica, con el objetivo de reducir su tamaño y facilitar

así su inclusión en las nanopartículas. La estructura de este plásmido puede ser visualizada en la Figura 10.

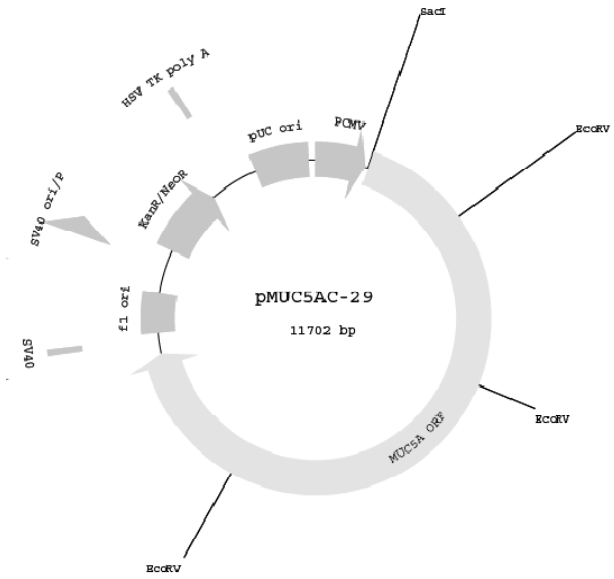


Figura 10. Estructura del plásmido pMUC5AC-29 (pMUC5AC) empleado en los estudios *in vivo*.

Ensayos in vivo en ratones sanos

La formulación de nanopartículas híbridas de GC_{137spm}/CS se ha testado en primer lugar en ratones sanos, a fin de establer cual es el mejor protocolo de transfección en lo que respecta tanto a la capacidad de transfección y como a la tolerancia de los animales sometidos al tratamiento. El protocolo con el que se ha obtenido la respuesta mejor es el que corresponde a la administración de 5 µg de plásmido asociado a las nanopartículas por día, durante cinco días, con un intervalo de 1h entre cada instilación. Como controles, se han utilizado el plásmido libre y las nanopartículas blancas, siendo las propiedades de todas las formulaciones

administradas las que se recogen en la Tabla 7. Para reducir el número de administraciones, todos los sistemas han sido previamente concentrados por centrifugación.

Tabla 7. Características de las distintas formulaciones evaluadas *in vivo*.

	Tamaño (nm)	Potencial Zeta (mV)
pMUC5AC-280	570.9 ± 45.7	-10.5 ± 1.6
NPs-blancas	142.9 ± 17.3	+33.6 ± 4.2
pMUC5AC-280-NPs	127.5 ± 10.5	+37.0 ± 0.7

NPs, nanopartículas

Como se puede apreciar en los valores recogidos en la Tabla 7, con la modificación introducida en la construcción plasmídica, y también como resultado del proceso de centrifugación al que se ha sometido a las formulaciones previo a su administración, se produce una reducción en el tamaño de las partículas sin que esta reducción vaya acompañada de una modificación de su carga superficial.

La evaluación de la tolerancia que ha sido realizada por observación macroscópica del comportamiento de los animales y, también, por el estado de diferentes estructuras ocular como cornea, conjuntiva y parpados; no puso de manifiesto ninguna alteración que pudiera ser indicativa de intolerancia o de toxicidad de la formulación por parte de los animales. Además, se ha procedido a realizar una tinción con fluoresceína de la superficie del globo ocular de los animales, para evaluar posibles efectos nocivos tanto del plásmido y como de las formulaciones de nanopartículas (Figura 11).

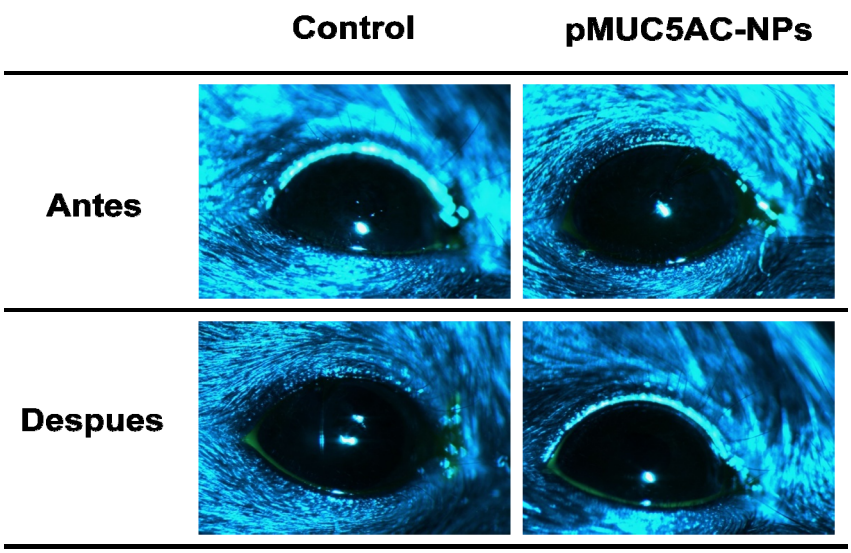


Figura 11. Tinción con fluoresceína de la superficie del globo ocular de los ratones, antes y después de ser tratados con las nanopartículas de GC_{137spm}/CS y el correspondiente ojo control no tratado.

Esta tinción con flouresceína es un método que permite evaluar el progreso de la enfermedad y también su tratamiento. Así, alteraciones en la función de barrera de la cornea se pueden traducir en un teñido de la zona afectada[44]. En este estudio, tal y como se observa en las imágenes de la Figura 11, ninguno de los tratamientos indujo ningún daño o alteración visible en el epitelio corneal, dado que no se visualiza ninguna zona teñida.

Por último, la expresión del plásmido evaluada en términos de expresión relativa de mRNA de MUC5AC ha sido cuantificada por RT-PCR, y los resultados obtenidos son los que se presentan en la Figura 12. Estos resultados demuestran que se produce una expresión significativa y selectiva de la mucina en la conjuntiva de los ratones sanos y verifican que las nanopartículas son capaces de transfectar *in vivo* de manera selectiva la conjuntiva.

[44] Niederkorn JY *et al.* Desiccating stress induces T cell-mediated Sjögren's Syndrome-like lacrimal keratoconjunctivitis. *The Journal of Immunology* 2006; **176:** 3950-3957.

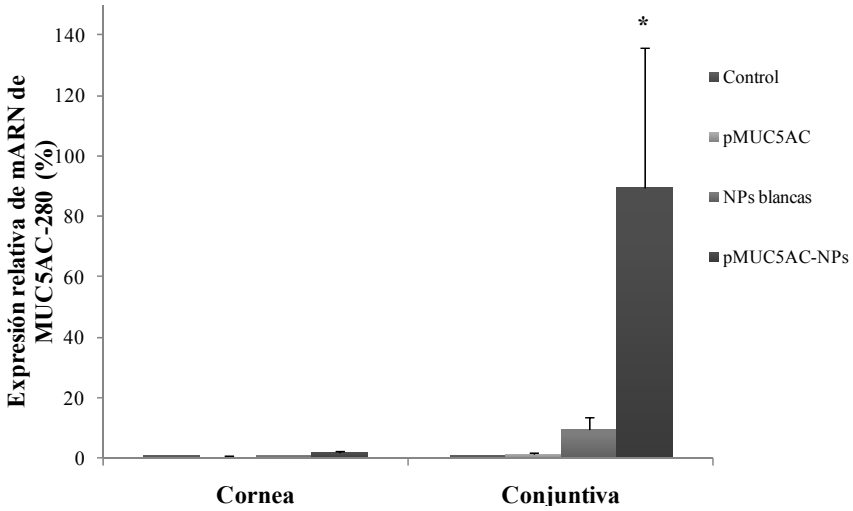


Figura 12. Expresión del mRNA de MUC5AC en cornea y conjunctiva de ratones despues de diferentes tratamientos: (i) controles no tratado, (ii) plásmido desnudo, (iii) nanopartículas blancas, y (iv) nanopartículas cargadas con el plásmido pMUC5AC (n=2). *p<0.01

Estos resultados son extremadamente importantes, una vez que es en la conjuntiva donde se encuentran las células caliciformes productoras de la mucina MUC5AC que va estar presente en la superficie ocular. En este sentido, una vez más, es importante resaltar que en pacientes con ojo seco se produce una disminución significativa de la expresión del mRNA de la MUC5AC en el tejido conjunctival[45]. Por lo tanto, el tratamiento con las nanopartículas de GC_{137spm}/CS que incorporan el plásmido es, hasta el momento, el único tratamiento descrito capaz de incrementar específicamente los niveles de dicho mRNA.

[45] Argüeso P *et al.* Decreased Levels of the Goblet Cell Mucin MUC5AC in Tears of Patients with Sjögren Syndrome. *Investigative Ophthalmology & Visual Science* 2002; **43:** 1004-1011

Ensayos en ratones con el modelo de la enfermedad

En este punto del estudio, se ha considerado crucial comprobar la eficacia del tratamiento, ahora en un modelo animal (el ratón) sometido a estrés por desecación (DS), con el que se simula la enfermedad del ojo seco[46]. Este estrés por desecación consiste en el mantenimiento de los animales en una atmósfera de humedad reducida (30%) y bajo la acción constante de ventiladores, al mismo tiempo que se les administra un fármaco anticolinérgico, la escopolamina. Este modelo tiene capacidad de reproducir muchas de las condiciones observadas en la enfermedad, como son la reducción del número de células caliciformes, incrementos en los niveles de citoquinas pro-inflamatorias, ruptura de la integridad de la barrera corneal o disminución de la producción de lágrimas, entre otros.

Las nanopartículas estudiadas así como el protocolo de administración, fueron los mismos que se utilizarón con los ratones sanos: 5 µg de plásmido asociado a las nanopartículas por día, durante cinco días. En total, el estudio se ha realizado en 6 grupos distintos de animales, a fin de llegar a resultados lo más precisos posible en lo que se refiere a la eficacia del tratamiento. Estos 6 grupos son:

(i) ratones sanos no tratados;
(ii) ratones sometidos al DS no tratados;
(iii) ratones sometidos al DS y tratados con el plásmido libre;
(iv) ratones sometidos al DS y tratados con las nanopartículas blancas;
(v) ratones sometidos al DS y tratados con las nanopartículas asociando el plásmido pMUC5AC;

[46] Siemasko KF *et al.* In Vitro Expanded CD4 CD25 Foxp3 Regulatory T Cells Maintain a Normal Phenotype and Suppress Immune-Mediated Ocular Surface Inflammation. *Investigative Ophthalmology & Visual Science* 2008; **49:** 5434–5440.

(vi) ratones sometidos al DS y tratados con el medicamento comercial de referencia (Restasis®).

El estudio de la tolerancia, evaluada por observación macroscópica del comportamiento de los animales y por el estado de estructuras oculares como cornea, conjuntiva y parpados; no condujo a ninguna observación que pudiera ser indicativa de intolerancia o de toxicidad de la formulación, en ninguno de los grupos evaluados

Como ya se ha comentado, además de ser un método con capacidad para evaluar posibles daños producidos por las formulaciones en la superficie ocular, la tinción con fluoresceína es un método que permite evaluar el progreso de la enfermedad y su tratamiento[47]. En la Figura 13 se muestran las imágenes tras la tinción de la cornea de los ratones sometidos al DS, antes y después del tratamiento con las nanopartículas que contienen el plásmido. Los ratones que han sido sometidos al DS, del mismo modo que ocurre en los individuos que padecen de la enfermedad[48], presentan zonas teñidas en la superficie corneal, tal y como se observa en la Figura 13A. En contrapartida, después de haber sido sometidas al tratamiento diseñado en este trabajo, tales zonas se ven disminuidas o, incluso, han desaparecido, como se visualiza en la Figura 13B.

[47] Dursun D *et al.* A Mouse Model of Keratoconjunctivitis Sicca. *Investigative Ophthalmology & Visual Science* 2002; **43:** 632–638.
[48] Goto E *et al.* The Sparkle of the Eye: The Impact of Ocular Surface Wetness on Corneal Light Reflection. *American Journal of Ophthalmology* 2011**:** In press.

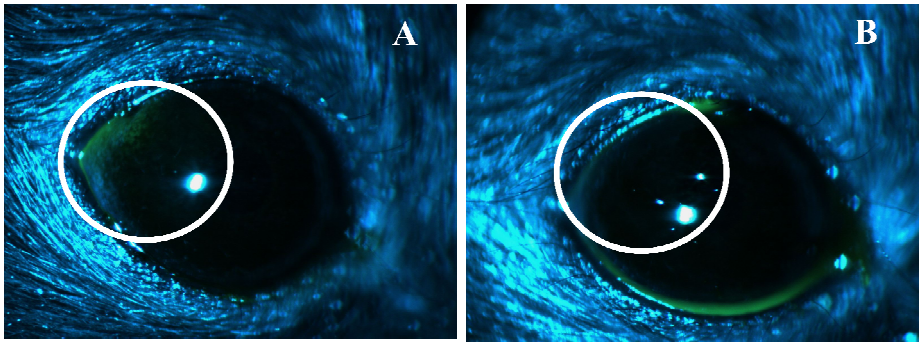


Figura 13. Imágenes tras la tinción con fluoresceína de la superficie ocular de ratones sometidos a estrés por desecación (A) antes del tratamiento y (B) después del tratamiento con las nanopartículas de CG_{137spm}/CS asociando el plásmido pMUC5AC.

Hasta el momento, la única opción disponible para el tratamiento del ojo seco con capacidad para incrementar la producción de lágrimas es la emulsión de ciclosporina A 0,05%, comercializada con el nombre de Restasis®, por Allergan, Inc. Tal y como queda reflejado en el diagrama de barras de la Figura 14, la terapia con las nanopartículas de CG_{137spm}/CS da lugar a una respuesta equivalente a la que se consigue con la formulación referencia de manera que, tras el tratamiento, es posible recuperar los niveles basales de producción de lágrimas. La inexistencia de incrementos en la producción de lágrimas tras la administración de las nanopartículas blancas, revela que este incremento no puede ser debido a una irritación de la mucosa por la presencia de las partículas, por lo que debe ser atribuido únicamente a la eficacia del tratamiento propuesto. Este resultado no sólo es importante en el sentido de representar una alternativa al tratamiento convencional, si no su importancia va mucha más allá dado que, por primera vez, se describe que la reposición de los niveles de MUC5AC es capaz de incrementar la producción de lágrimas. Aunque la MUC5AC se relaciona con la homeostasis de la lágrima, la extensión real del papel que desempeña y su importancia en la terapia de la superficie ocular son poco conocidos y subestimados en muchos casos.

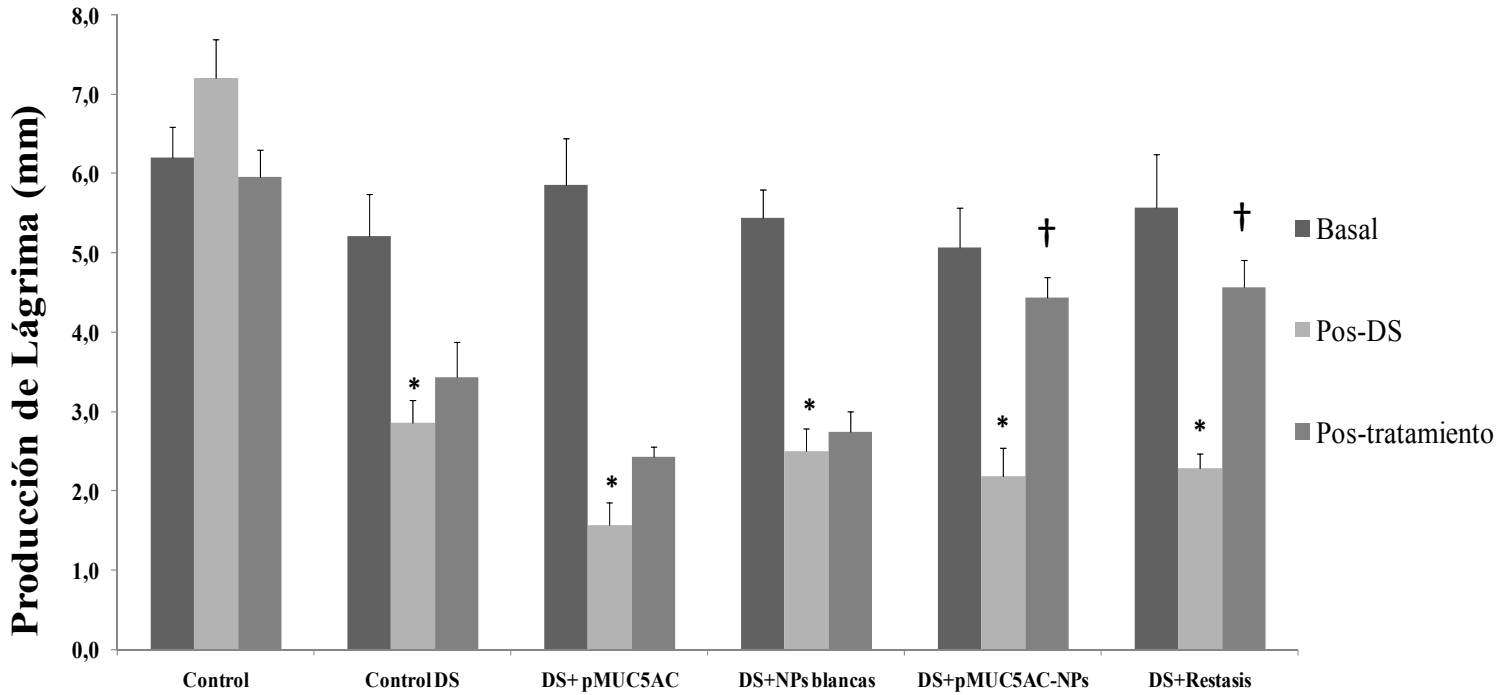


Figura 14. Producción de lágrimas después de diferentes tratamientos (mm). Las columnas representan las diferentes etapas del tratamiento: nivel basal (blanco), pos-estrese por desecación (gris) y pos-tratamiento (negro). Seis diferentes intervenciones son presentadas: animales sanos no-tratados, animales sometido al estres por desecación no-tratados y animales bajo estrese por desecación tratados con plásmido desnudo, nanopartículas blancas, nanopartículas cargadas con pMUC5AC o Restasis ® (n=8 de 2 experimentos independientes). *p<0.01 vs. nivel basal, †p<0.01 vs. DS.

Ensayos in vivo: expresión de la MUC5AC en epitelio ocular de conejos

Para el ensayo de las nanopartículas de GC_{137spm} en conejos, fueron instiladas nanopartículas 10 veces concentradas en un total de 75 µg de plásmido, con intervalos de 30 minutos entre cada instilación. La cornea y la conjuntiva han sido extirpadas y homogeneizadas, con subsecuente cuantificándose a continuación los niveles totales de proteínas y niveles de MUC5AC. Los resultados de la expresión de MUC5AC relativa al control aparecen recogidos en la Figura 15.

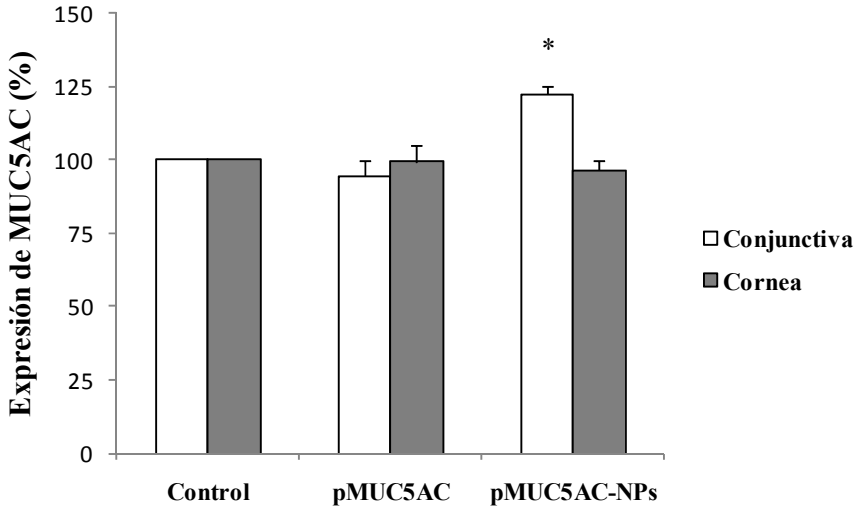


Figura 15. Expresión de MUC5AC en la conjunctiva y en la cornea de conejos (barras blancas y grices respectivamente) 4 días después de la transfección con nanopartículas de gelatina cationizada con espermina (pMUC5AC-NPs) o plásmido desnudo (pMUC5AC) en total de 75 µg de plásmido (n=9 de 3 experimentos independientes). * diferencia estadística significativa ($p<0.05$)

Los resultados indican que las nanopartículas de GC_{137spm} son el vehículo indispensable para que pueda producirse la expresión de la proteína, una vez que el plásmido desnudo no indujo ninguna expresión. Es importante destacar que, al igual que ocurrió en los estudios *in vitro*, la expresión de la proteína MUC5AC fue detectada solamente en la conjuntiva y no en la

cornea. Como estamos hablando de una determinación de la proteína y no de un intermediario implicado en su producción, como es el mRNA, estos resultados pueden significar dos cosas: bien que la cornea necesita más tiempo para expresar la MUC5AC o bien que, sencillamente, este tejido no es capaz de expresarla, sea por una incapacidad intrínseca del propio tejido, o porque las nanopartículas resultan ineficaces para la transfección en este caso.

Por último, en relación a los resultados obtenidos con la formulación de nanopartículas GC_{137spm}, es importante señalar que después de la transfección, algunos animales presentaron ligeros signos de secreción nasal e incremento en el parpadeo. Por ello, antes de pasar a estudiar la siguiente formulación, se han hecho algunos cambios en el protocolo de administración con el objetivo de evitar posibles efectos tóxicos, pero manteniendo el nivel de expresión de la proteína. Estos cambios han sido: empleo de nanopartículas menos concentradas, mayor intervalo entre instilaciones y reducción de la cantidad de plásmido administrado.

En estas condiciones se ha estudiado el comportamiento de las nanopartículas híbridas de GC_{137spm}/DS, también en conejos. Se instiló la suspensión de nanopartículas 4 veces concentradas (en lugar de 10 veces), durante 2 días (lo que supone un total un total de 25 μg de plásmido por día), con intervalos de 1 entre cada instilación. La cornea y la conjuntiva se han extraído y homogeneizado, cuantificándose a continuación los niveles totales de proteína así como los niveles de MU5AC. Los resultados obtenidos se reflejan en el gráfico de la Figura 16.

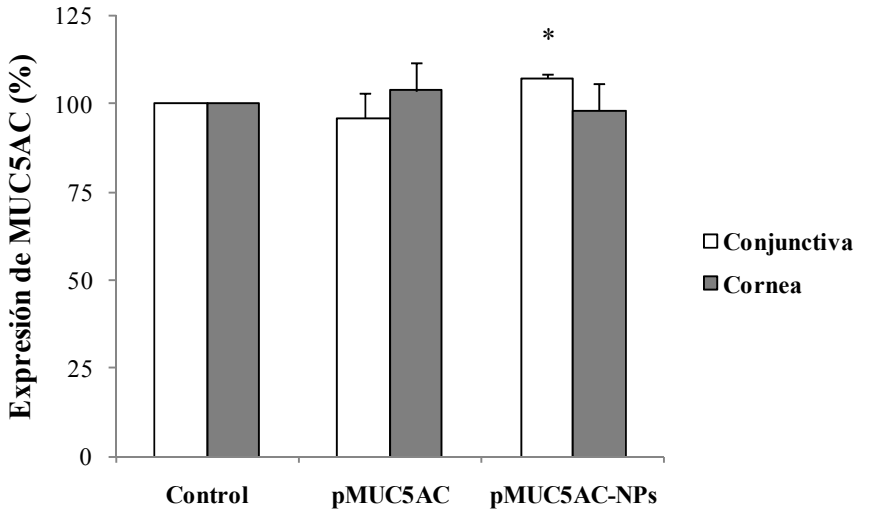


Figura 16. Expresión de MUC5AC en la conjunctiva y en la cornea de conejos (barras blancas y grices respectivamente) 4 días después de la transfección con nanopartículas híbridas de gelatina cationizada con espermina y sulfato de dextrano (pMUC5AC-NPs) o plásmido desnudo (pMUC5AC) en total de 50 µg de plásmido (n=6 de 2 experimentos independientes). * diferencia estadística significativa ($p<0.05$).

Al igual que en el caso de las nanopartículas de CG_{137spm}, las nanopartículas híbridas de CG_{137spm}/DS sólo han sido capaces de transfectar el epitelio conjuntival, si bien en este caso la expresión obtenida ha sido significativamente menor que en el caso de las nanopartículas compuestas exclusivamente por gelatina cationizada. Muy probablemente, estos resultados tegan algo que ver con los cambios introducidos en el protocolo de transfeción, aunque hay que recordar que estas partículas híbridas también han presentado una menor capacidad de transfección en comparación con las GC_{137spm}, en los estudios *in vitro* realizados en las líneas celulares HCE y IOBA-NHC, como ya se ha comentado anteriormente. En contrapartida, en este caso se ha logrado tanto la disminución de la secreción nasal como el incremento en el parpadeo de los animales. Por último, es interesante resaltar que lo mismo que sucedió en los estudios anteriores realizados en ratones, solamente la conjuntiva fue capaz de expresar el plásmido.

CONCLUSIONES

CONCLUSIONES

El trabajo experimental que se recoge en la presente memoria ha sido enfocado al diseño de nuevos sistemas nanoparticulares basados en componentes naturales, como vehículos para la administración de ADN plasmídico en la superficie ocular. Para el diseño de dichos sistemas se ha pensado en la utilización de proteínas cationizadas conjuntamente con polímeros aniónicos de naturaleza polisacarídica. Su desarrollo ha tenido lugar mediante una técnica suave, reproducible y que no requiere el empleo de disolventes orgánicos, la gelificación ionotrópica.

Los resultados obtenidos nos han permitido extraer las siguientes conclusiones:

1. Es posible emplear proteínas cationizadas como biomateriales constitutivos de nanopartículas desarrolladas mediante gelificación ionotrópica, y estas nanopartículas pueden asociar eficazmente y proteger material genético (ADN plasmídico).

2. Estudios realizados en cultivos celulares ha permitido constatar que, de entre las proteínas cationizadas utilizadas, la *gelatina cationizada* con la *espermina* es el biomaterial más prometedor en lo que se refiere su perfil de toxicidad y capacidad de transfección.

3. Ha sido posible incorporar *sulfato de condroitino* y *sulfato de dextrano* en la estructura de las nanopartículas basadas en gelatina cationizada, desarrollando de esta forma nanopartículas híbridas. La incorporación de estos polianiones nos ha permitido modular convenientemente las propiedades físico-químicas de los sistemas, aportando a los mismos, versatilidad y el consiguiente valor añadido

que supone la posibilidad de adaptarlas a requerimientos específicos de determinadas aplicaciones.

4. Estudios realizados en cultivos celulares han evidenciado que, una vez incorporados a las nanopartículas, dichos polianiones tienen la capacidad de reducir significantemente la toxicidad del sistema, sin comprometer su potencial de transfección en células del epitelio ocular.

5. Se ha comprobado la eficacia de los sistemas híbridos para asociar y transfectar ADN plasmídico de interés farmacéutico, concretamente ADN plasmídico que codifica la expresión de la proteína MUC5AC. Asimismo, estudios en cultivos celulares han demostrado que el tratamiento de células conjuntivales humanas con los nanosistemas desarrollados induce una eficaz expresión de MUC5AC.

6. Estudios realizados en dos especies diferentes (conejos y ratones), han permitido corroborar que cuando los animales son tratados con los sistemas nanoparticulares en forma de colirio se produce una eficaz expresión de la proteína MUC5AC en la conjuntiva de dichos animales.

7. Tras someter ratones a estrés por desecación, obteniendo un modelo de síndrome de ojo seco, se ha comprobado que el tratamiento con uno de los nanosistemas desarrollados es capaz de restablecer la producción normal de lágrimas y mejorar capacidad de protección de la superficie ocular en los ratones enfermos. Consecuentemente, podemos afirmar que el empleo de nanopartículas asociando material genético representa una aproximación terapéutica totalmente novedosa al tratamiento del ojo seco.

8. De las conclusiones anteriormente expuestas se desprende, a modo de conclusión global de la presente memoria, que las nanopartículas desarrolladas presentan un gran potencial clínico en la terapia génica de diferentes patologías oculares.

CONCLUSIONS

This experimental work has been focused in the design of new nanoparticulated systems based on natural polymers for gene delivery in the ocular surface. Such systems have been based on cationized proteins and natural polyanions. The development has been made by the ionic gelation technique, mild and reproducible technique that does not require organic solvents.

The results have allowed us to formulate the following conclusion:

1. It is possible to use cationized proteins as biomaterials for nanoparticles made by ionic gelation technique and these systems are able to protect and deliver genetic material (plasmid).

2. In cell culture studies, the gelatin cationized with spermine was the most promising biomaterial among other cationized proteins in which respect it toxicity profile and transfecction efficiency.

3. It has been posible to assemble dextran sulfate and chondroitin sulfate in the structure of nanoparticles base on cationized gelatin. With the introduction of these polyanions it has been possible to conveniently modulate the physicochemical properties of the systems in such way that they can fulfill specific requirements of several applications.

4. Studies performed in cell culture have shown that, once assembled to the nanoparticles, such are able to reduce the toxicity profile of the nanoparticles without compromise the transfection efficiency.

5. The hybrid systems can associate and transfect a plasmid of pharmaceutical interest that codifies the MUC5AC. In cell culture studies in human conjunctival cells the nanosystems con induce the expression of MUC5AC.

6. Studies in two different species (rabbits and mice), have corroborated that animals treated with the eye drops have significant expression of the protein MUC5AC in the conjunctiva.

7. Using an animal model of dry eye in mice, one of the developed systems is able to restore normal tear production and improve the corneal barrier function of the animals. Afterward, we can say that the use of nanoparticles associating genetic material is a new approach to dry eye.

8. The global conclusion of the present work is that the developed nanoparticles present great clinical relevance and are a potential therapy for different eye diseases.

ANEXOS

Lista de Patentes Solicitadas y Concedidas

Patentes Solicitadas:

Párraga Meneses, Jenny; Konat Zorzi, Giovanni; Paolicelli, Patrizia; Seijo Rey, Begoña; Sánchez Barreiro, Alejandro; Contrears Ruiz, Laura; Diebold Luque, Yolanda. **Nanopartículas para la prevención y/o tratamiento de enfermedades de mucosas**. P201031678, Universidad de Santiago de Compostela y Universidad de Valladolid, 2010.

Patentes Concedidas:

Párraga Meneses, Jenny; Konat Zorzi, Giovanni; Paolicelli, Patrizia; Seijo Rey, Begoña; Sánchez Barreiro, Alejandro. **Sistemas nanoparticulares elaborados a base de polímeros aniónicos para administrar moléculas bioactivas para uso cosmético**. ES2345806 (A1), Universidad de Santiago de Compostela, 2010.

Párraga Meneses, Jenny; Konat Zorzi, Giovanni; Seijo Rey, Begoña; Sánchez Barreiro, Alejandro. **Sistemas nanoparticulares elaborados a base de polimeros anionicos** ES2342588 (A1), Universidad de Santiago de Compostela, 2010.

Paolicelli, Patrizia; Konat Zorzi, Giovanni; Seijo Rey, Begoña; Párraga Meneses, Jenny; Sánchez Barreiro, Alejandro. **Nanoparticulas de acido colominico y derivados.** ES2341165 (A1). Universidad de Santiago de Compostela, 2010.

Sánchez Barreiro, Alejandro; Seijo Rey, Begoña; Paolicelli, Patrizia; Konat Zorzi, Giovanni; Párraga Meneses, Jenny. **Nanoparticulated systems prepared from anionic polymers**. WO2010049562 (A1), Universidad de Santiago de Compostela, 2010.

El principal objetivo de la presente memoria ha sido el diseño de nuevos sistemas nanoparticulares capaces de proporcionar una aproximación terapéutica totalmente novedosa al tratamiento del ojo seco. Esta aproximación terapéutica se basa en los principios de la terapia génica, empleando ADN plasmídico que codifica la mucina MUC5AC, una de las principales responsables de la homeostasis del fluido lacrimal. Para el diseño de los sistemas mencionados se ha pensado en la utilización de proteínas cationizadas conjuntamente con polímeros aniónicos de naturaleza polisacarídica. El tratamiento tópico en ratones sometidos a un modelo de síndrome de ojo seco con dichos nanosistemas ha permitido restablecer la producción normal de lágrimas y mejorar capacidad de protección de la superficie ocular en los animales enfermos. Estos resultados suponen la prueba de concepto sobre la aplicación clínica de las nanopartículas diseñadas al desarrollo de la terapia génica de diferentes patologías oculares como el síndrome de ojo seco.