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Tesis Doctoral

NUEVOS NANOSISTEMAS A BASE DE PROTAMINA PARA LA LIBERACIÓN DE ANTÍGENOS

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INFORMAN:

Que la presente Memoria Experimental titulada: "Nuevos nanosistemas a base de protamina para la liberación de antígenos", elaborada por José Vicente González Aramundiz, 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.

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Fdo. María José Alonso Fdo. Noémi Csaba

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RESUMEN/ABSTRACT

RESUMEN

En la presente tesis doctoral se plantea el diseño, desarrollo y optimización de sistemas coloidales nanométricos a base de protamina para la vehiculización y liberación de antígenos. En particular los sistemas desarrollados se dividen en (i) nanopartículas, sistemas de tipo matricial que se compone de un polisacárido como ácido hialurónico o alginato entrecruzado con protamina y (ii) nanocápsulas, sistemas de tipo reservorio, constituidas por un núcleo líquido oleoso (Miglyol®, escualeno o α tocoferol) y una cubierta de protamina. Dependiendo del tipo de estructura, se han asociado distintos antígenos modelo como son el antígeno recombinante de la hepatitis B y el antígeno de influenza H1N1. Los sistemas presentan una adecuada estabilidad en suspensión y en polvo seco a temperatura ambiente. Estudios *in vitro* han demostrado que ambos tipos de sistemas son eficazmente internalizados por células inmunocompetentes y estimulan la secreción de diversos tipos de citocinas. Estudios *in vivo* han demostrado la efectiva acción de los distintos nanosistemas con el antígeno asociado generando una respuesta inmune adecuada y prolongada. Por otra parte, se ha evaluado la posibilidad de que los diferentes sistemas sean formulaciones administradas libres de agujas, a través de la vía nasal, obteniendo respuestas inmunes protectoras frente al antígeno asociado y pudiendo modular el tipo de respuesta obtenida (humoral o celular), dependiendo de la vía de administración.

Todos estos resultados ponen de manifiesto el potencial que poseen las estructuras desarrolladas a base de protamina para la vehiculización y liberación de antígenos, consiguiendo avanzar en el desarrollo de nuevos adyuvantes así como en la termo-estabilidad de las diversas formulaciones.

ABSTRACT

The main goal of this thesis has been the design, development and optimization of protamine-based colloidal systems for antigen delivery. In particular, the developed nanosystems can be divided in (i) nanoparticles, a matrix-type system consisting in a polysaccharide such as hyaluronic acid or alginate, cross-linked with protamine and (ii) nanocapsules, composed by a liquid oily core (Miglyol® 812, squalene or α tocopherol) and a surrounding protamine shell. Depending on the type of nanostructures developed, we have associated different antigens such as the recombinant hepatitis B surface antigen and the haemagglutinin of influenza virus H1N1. The systems present adequate stability in suspension and at room temperature in a freeze-dried form. *In vitro* studies have shown that both systems are efficiently internalized by immune cells and stimulate the secretion of different cytokines. *In vivo* studies have shown an effective action of the different antigen-loaded nanosystems, achieving high and prolonged immune responses. Moreover, the developed nanosystems have been evaluated for a needle-free vaccination approach by nasal route, achieving a protective immune response against the associated antigen and evidencing the ability to modulate the immune response (humoral or cellular) depending on the administration route chosen.

Globally, with these results we highlight the potential of the developed protamine-based nanosystems for antigen delivery and their contribution to the development of new vaccine adjuvants as well as improving their thermostability.

INTRODUCCIÓN

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La implementación de las vacunas en la medicina moderna ha sido la intervención más exitosa tanto en términos de salud pública como con respecto al aumento de la esperanza de vida de la población. Ya desde el siglo XVIII, cuando Edward Jenner observó que la viruela de vaca era capaz de inmunizar contra la viruela humana, múltiples han sido los avances que se ha realizado en este campo [1].

Respecto a los grandes logros de la vacunación, es posible mencionar la significativa reducción de algunas enfermedades como lo son paperas, sarampión, polio, tétanos, difteria, tos ferina, fiebre amarilla, entre otras, y la gran proeza de la erradicación de la viruela [2].

Estos logros han sido alcanzados en su mayoría por patógenos vivos atenuados, inactivados o toxinas inactivadas. Éstos son altamente inmunogénicos y generan una protección contra la infección de larga duración. Sin embargo, el uso de este tipo de microorganismos puede ir acompañado de reacciones anafilácticas, reactividad del patógeno atenuado e inestabilidad intrínseca del producto entre otros. Por estos motivos, múltiples esfuerzos se han enfocado en aumentar el perfil de seguridad de las vacunas, para ello se han estudiado otros tipos de antígenos haciendo uso de ADN recombinante y subunidades purificadas de antígenos [3].

Las vacunas de ADN contienen los genes necesarios para codificar un antígeno o una proteína. Muchas de ellas se encuentran en la actualidad en diversas fases de ensayos clínicos [4, 5]. Las vacunas de subunidades utilizan una parte del patógeno que puede desencadenar una respuesta inmune adecuada [6]. Si bien estos antígenos son mucho más seguros, poseen el problema de que son menos inmunogénicos, por lo que se hace necesario el uso de adyuvantes y/o sistema de liberación, para su administración e intensificación de la respuesta

inmune generada. En esta introducción, se pretende revisar los aspectos básicos para la generación de la respuesta inmune, el uso de adyuvantes y sistemas de liberación de antígenos, principalmente con el uso de la nanotecnología.

El sistema inmune y la generación de la inmunidad

El sistema inmune es el conjunto de estructuras y procesos biológicos capaces de defender la integridad biológica frente a sustancias o materiales ajenos, provenientes del exterior así como del propio organismo. La capacidad de defensa, adquirida antes de nacer y madurada en los primeros años de vida, se puede dividir en dos: inmunidad inespecífica y especifica o adquirida. Si bien, esta separación es para poder mejorar su comprensión, el sistema inmune responde como conjunto y dependiendo de los factores implicados predomina una u otro tipo de respuesta.

La inmunidad innata es una respuesta rápida de primera defensa y no requiere sensibilización previa. Es mediada a través de células fagocíticas como neutrófilos, macrófagos, células dendríticas entre otras, y componentes humorales. La respuesta específica o adquirida, es una respuesta altamente especializada, mediada por un complejo sistema de células (principalmente B y T) y procesos que tienen como fin desarrollar una respuesta inmune específica frente a un antígeno dado. Una de las ventajas de este tipo de respuesta es que consigue una memoria inmunológica, la cual es la base de la vacunación.

Cuando entra un antígeno al organismo, las células presentadoras de antígenos (APC) como macrófagos y especialmente las células dendríticas captan las moléculas extrañas e inician el proceso denominado presentación del antígeno [7], característico de la inmunidad específica. Como se muestra en la **Figura 1**, las células dendríticas de forma inmadura, son capaces de fagocitar el antígeno y junto con él migrar hacia los nódulos linfáticos y madurar. Es ahí donde

presentan al antígeno en su membrana en conjunto con el complejo mayor de histocompatibilidad I ó II (MHC I o II), además de secretar moléculas co-estimulatorias, que serán llamadas “segundas señales” para la activación de las células T [3].

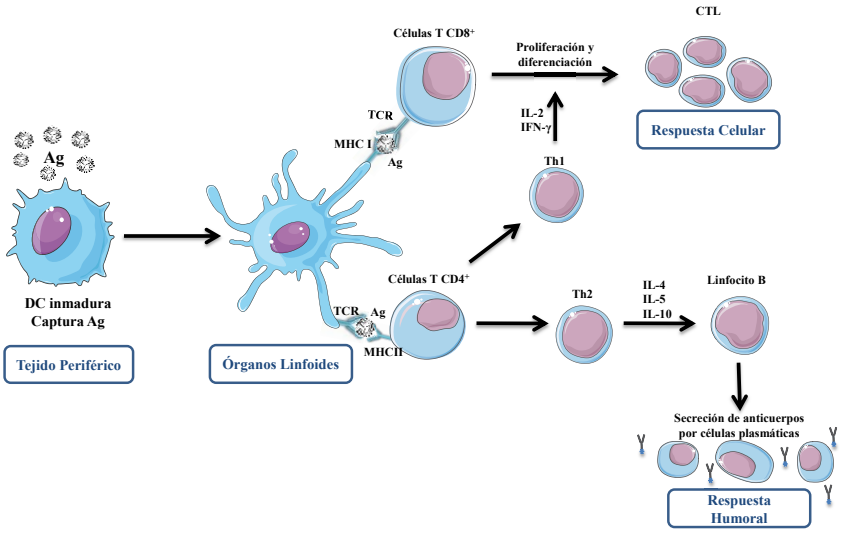

Figura 1: Esquema general de la presentación de antígeno (Ag) y generación de la respuesta inmune. El Ag es captado por las células dendríticas (DC) inmaduras en el tejido periférico. A continuación, migra hacía los órganos linfoides haciendo que la célula madure. Es ahí donde se presenta el Ag por medio del complejo mayor de histocompatibilidad (MHC) I ó II activando las células T $CD8^+$ o $CD4^+$ y se origina una respuesta celular o humoral respectivamente. Adaptado de [3].

De forma general, antígenos extracelulares o péptidos derivados de proteínas provenientes de un medio extracelular (por ejemplo vacunas proteicas) se presentan en MHC II, mientras que péptidos sintetizados de manera endógena se presentan en MHC de tipo I. Sin embargo existe la evidencia que algunos antígenos exógenos pueden ser presentados por medio de MHC I, lo cual abre una atractiva forma de que antígenos exógenos produzcan una respuesta celular, como veremos a continuación.

El complejo de antígeno - MHC I o II presente en las membranas de las APC, son reconocidos por los receptores de células T (TCR). El complejo antígeno - MHC I es reconocido por las células de tipo T $CD8^+$ o citotóxicas, generando una respuesta celular y diversas citoquinas como interferón gama (INF-γ) y factor de necrosis tumoral alfa (TNF-α). Por otra parte, el reconocimiento de MHC II es a través de los receptores de células T $CD4^+$ o colaboradoras (T helper), las cuales al ser activado puede diferenciarse en células de tipo Th1 o Th2.

El tipo de diferenciación se caracteriza por el tipo de citoquinas que producen, por ejemplo Th1 produce INF-γ e interleucina 2 (IL-2), mientras que Th2 secreta interleucinas 4, 5 y 10. Una respuesta Th1 permite una inmunidad celular, en cambio una respuesta Th2, permite la activación de linfocitos B, lo que genera una respuesta humoral mediada a través de anticuerpos.

Los anticuerpos también conocidos como inmunoglobulinas, pueden ser de cinco clases: M (IgM), A (IgA), G (IgG), D (IgD) y E (IgE). Todas ellas tienen la propiedad de unirse específicamente al anticuerpo que indujo su formación y favorecer su destrucción.

Como se puede apreciar, todo el largo proceso de generación de la inmunidad, se inicia por el reconocimiento del antígeno por una APC, por ello es indispensable que el antígeno sea reconocido por el sistema inmune. Lamentablemente, los nuevos antígenos, aunque son más seguros, no son fácilmente captados por el sistema inmune por su falta de inmunogenicidad. Esto ha obligado al estudio y al uso de un adyuvante y/o un sistema de liberación que sea capaz de favorecer el reconocimiento del antígeno y la activación y maduración de las APC.

Adyuvantes y Vacunas

Como mencionamos previamente, el uso de antígenos más seguros trae consigo la necesidad de emplear un adyuvante en la administración de vacunas.

En términos generales un adyuvante es un compuesto que puede incrementar y/o modular la inmunogenicidad intrínseca de un antígeno [8]. Los únicos adyuvantes universalmente aprobados para su uso en humanos son las sales insolubles de aluminio, más conocidas como álum [9]. Su uso en la precipitación de proteínas data de 1926 por Glenny *y col*. Aunque el efecto adyuvante de estas sales fue descrito en la década de los 70s, hasta el día de hoy se estudia su mecanismo de acción exacto [10, 11]. El álum ha presentado una buena acción por vía parenteral sin embargo, posee algunos inconvenientes entre los que destacan: (i) reacciones de hipersensibilidad, hinchazón y/o eritemas tras ser administrado (ii) inestabilidad tras la congelación, debido a la destrucción de la estructura tipo gel que forma, disminuyendo o dejando inactiva la vacuna (iii) es un adyuvante que por otras vías distintas a la parenteral no presenta actividad adyuvante y por último (iv) su perfil de respuesta inmune está asociado principalmente a una respuesta humoral, relacionada con la producción de linfocitos Th2.

Por ello, los esfuerzos de investigación se han concentrado en la búsqueda de nuevos adyuvantes que sean capaces de mejorar aquellos aspectos en los cuales el álum falla. Algunos de estos nuevos adyuvantes se resumen en la **Tabla 1**, en donde destacan el MF 59^{TM}, ASO4^{TM} y ASO3^{TM} aprobados por la Agencia Europea de Medicamentos (EMA) para su uso en humanos.

La nanomedicina y la búsqueda de nuevos adyuvantes

La nanomedicina es un área de la nanotecnología que abarca diferentes aplicaciones de la biotecnología medicina, química, tecnología farmacéutica, entre otras en la prevención, tratamiento, monitorización y diagnóstico de enfermedades utilizando materiales y estructuras de escala nanométrica (1-1000 nm) [12, 13]. Si bien, es en cáncer donde se han utilizado los mayores recursos y se pueden observar los mayores avances, otras áreas como la

inmunización están siendo un campo de la nanomedicina que está en pleno auge.

Tabla 1: Diversos adyuvantes utilizados en la administración de antígenos. MPL: monofosforil lípido A, QS21: saponina purificada de quillaja A, GSK: GlaxoSmithKline.

Nombre	Compañía	Componentes	Enfermedad	Ref.
Álum	Varias	($AlPO_4$) ó ($Al(OH)_3$)	Diversas: DTP (difteria, pertussis y tétanos), Neumococo, Hepatitis B, etc.	[9]
MF 59	Novartis	Emulsión O/W de: escualeno, tween® 80 y sorbitan triolato)	Influenza	[14]
ASO1	GSK	Liposomas con MPL y QS21	Malaria	[15]
ASO2		Emulsión O/W de: MPL y QS21	Malaria	[16]
ASO3		Emulsión O/W de: escualeno, α tocoferol y tween® 80	Influenza	[17]
ASO4		MPL y álum	Hepatitis B	[18]
AFO3	Sanofi-Pasteur	Emulsión O/W de: escualeno, oleato de sorbitan, polioxietileno cetoestearil y manitol	Influenza	[19]

En términos generales, la nanomedicina en inmunización se ha enfocado en el diseño y elaboración de sistemas de liberación de antígenos. Se pretende que estas estructuras de tamaño nanométrico puedan mejorar la inmunogenicidad

del antígeno asociado, permitir una administración libre de agujas, favorecer la obtención de una formulación termoestable, ayudando con ello a una mejora consistente en la cobertura inmunológica [20, 21].

Ya desde la década de los 70s el profesor Peter Speiser, utilizó nanopartículas de poliacrilamida para la liberación controlada de vacunas [22]. Este hallazgo abrió un nuevo campo de investigación, donde diferentes tipos de estructuras y con diversos tipos de materiales comenzaron a diseñarse y desarrollarse, con una asociación eficaz de antígenos, para su aplicación en vacunas [23].

Diferentes sistemas nanoparticulados han sido estudiado para su aplicación en vacunas, entre ellos se pueden encontrar vectores virales, virosomas, liposomas, complejos inmunoestimulantes, nanopartículas de calcio, nanopartículas orgánicas e inorgánicas no biodegradables (ej: oro, látex, sílica, poliéster, etc) y nanopartículas poliméricas biodegradables, entre otras.

El uso de nanosistemas para la liberación de antígenos es una interesante estrategia para el desarrollo de vacunas. Entre sus ventajas se encuentra el proteger al antígeno de su degradación y el poder controlar su liberación. Esta última característica es la que más destaca en el uso de nanosistemas en vacunas, ya que la manera eficiente de liberar los antígenos a las APC, especialmente a las células dendríticas, además de promover la internalización del antígeno y la posterior maduración de la APC determinará el desarrollo de una respuesta inmune protectora [24]. Otra ventaja es la posibilidad de co-encapsular múltiples epítopes de antígenos en un solo vehículo [25], abriendo la posibilidad de vacunas multivalentes.

Los diversos sistemas de liberación de antígenos de tamaño nanométrico pueden ser internalizados por las APC de diversas maneras: a través de receptores (de forma selectiva), fagocitosis, macropinocitosis y endocitosis (ya sea mediado por clatrina, caveolina o independiente de ambos) [26]. El que un

nanosistema se internalice de una u otra manera dependerá principalmente de su tamaño, forma, propiedades y ligandos que tenga en la superficie [27].

En los últimos años, el estudio de los receptores presentes en las APCs y las diferentes vías de reconocimiento e internalización de sistemas y patógenos ha revolucionado la forma de diseñar vacunas. Diferentes receptores que reconocen señales de peligro (PRRs, del inglés "pattern-recognition receptors"), han sido identificados en donde destacan: TLR (del inglés: Toll Like Receptor), tanto de superficie como endosomal, NLR (del inglés: Nucleotide-binding oligomerization domain Receptors), CLR (del inglés C-type lectin receptor) RLH (del inglés RIG-like helicases) y Scavenger [28].

De todos estos, los que han tenido mayor impacto han sido los TLR. Diferentes moléculas han sido reconocidas como agonistas de estos receptores con interés tanto para el diseño de vacunas (principalmente como adyuvante), como para diferentes enfermedades infecciosas, cáncer, alergias y enfermedades autoinmune entre otras [29, 30].

Con estos nuevos hallazgos el uso de nanopartículas como sistemas de liberación de antígenos no podía quedar indiferente. Es por ello que se han utilizado diferentes materiales, que sean capaces de imitar señales de peligro para el sistema inmune, muchos de ellos agonistas de los receptores anteriormente mencionados y que sean capaces de modular la respuesta inmune [31]. Dentro de éstos, los biomateriales se presentan como buenos candidatos para cumplir estas funciones ya que pueden liberar el antígeno selectivamente a las células dendríticas, facilitando su presentación y por medio de ello, las células dendríticas son capaces de activarse, madurar y desencadenar una respuesta inmune eficiente [32].

Dentro de los biomateriales, los polímeros han sido ampliamente estudiados para la elaboración de nanosistemas en el área de inmunización. Las

nanopartículas poliméricas han tenido una gran relevancia con el precedente de las micropartículas de PLGA (ácido poli-láctico-glicólico). Este material biodegradable (aprobado por la FDA en 1969 para su uso clínico) en la década de los 80s fue recomendado por la organización mundial de la salud (OMS) para su uso en vacunas monodosis y así mejorar la cobertura de inmunización [33]. La OMS sugirió como antígeno el toxoide tetánico por la elevada incidencia del tétanus neonatal producto de la escasa inmunización de las mujeres en países del tercer mundo. Es así como a partir de esta evidencia, este material en conjunto con el ácido poli-láctico (PLA), han sido dos de los polímeros más estudiado para su uso en sistemas de liberación de vacunas.

Otro de los polímeros ampliamente estudiado en el ámbito de vehiculización de antígenos y también de fármacos es el quitosano. Este material, obtenido tras la desacetilación de la quitina, ha demostrado que por su alta capacidad de formar estructuras nanométricas y fácil manejo, es un material ideal para la vehiculización de macromoléculas [34]. Por otra parte, nanoestructuras de quitosano, han sido capaces de asociar eficientemente macromoléculas y antígenos y poder vehiculizarlos por vías mucosas [35].

Con la previa experiencia de partículas en quitosano cargadas con el toxoide tetánico [35, 36], recientemente nuestro grupo de investigación ha publicado el desarrollo de sistemas a base de quitosano estructurado en forma de nanopartículas (nanoesferas) [37] o nanocápsulas [38], en las que se ha demostrado su efectividad para transportar el antígeno recombinante de superficie de la Hepatitis B (rHBsAg) por vía nasal o a través de una sola dosis por vía parenteral, logrando inmunidad frente a esta enfermedad.

Otros biomateriales que han comenzado a ser ampliamente estudiado en el campo de la vehiculización de fármacos y antígenos son los polipéptidos y poliaminoácidos. Si bien su uso es menos extendido que los polímeros, estudios recientes han demostrado su potencial como material para formar diferentes

micro- y nanosistemas. Una interesante revisión de este tema se encuentra recogido en los anexos de la presente memoria.

Polipéptidos y poliaminoácidos y su uso en liberación de antígenos

Los polipéptidos (PP) y poliaminoácidos (PAA) son moléculas formadas por monómeros de aminoácidos a través de enlaces amidas siendo generalmente biocompatibles y no tóxicos; lo que los hacen un interesante material para su uso en liberación de fármacos y antígenos [39].

Uno de los poliaminoácidos utilizados en el desarrollo de nanopartículas para la liberación de antígeno es el ácido poli-glutámico. Este biomaterial ha demostrado que es capaz de producir una mayor liberación a las APC de la proteína vehiculizada, ayudando a activar la maduración de las células dendríticas [40]. Otro de los poliaminoácidos ampliamente estudiado en el desarrollo de nanosistemas es la polilisina. Este material, por su carga positiva a pH neutro, ha sido empleado especialmente como agente complejante en la vehiculización de material genético. En el área de inmunización se ha demostrado que nanopartículas recubiertas de este material actuarían como adyuvante en vacunas ADN [41].

Un grupo especial de péptidos, que es necesario mencionar son los CPP (del inglés: Cell-penetrating peptides). De manera general, son péptidos de no más de 30 aminoácidos, anfipáticos y poseen una carga neta positiva [42]. Tiene la propiedad de penetrar las membranas celulares y translocar diferentes moléculas dentro de las células de forma independiente de receptores y energía [43]. Por esta propiedad en las APC, es posible considerarlo en inmunización y en el diseño de nuevas vacunas como una alternativa a los adyuvantes convencionales, asociado a su fácil manufactura, sencilla manipulación y baja toxicidad [44].

Dentro de los CPPs, uno que se ha sido ampliamente estudiado en cuanto a su actividad adyuvante es la poliarginina (pARG). Una nueva vacuna (IC41) ha sido reportada en diversos estudios clínicos, en donde la pARG junto con 5 péptidos sintéticos, son la base de una vacuna terapéutica contra la hepatitis C [45, 46].

Otro péptido con propiedades CPP es la protamina. Éste nombre refiere a un conjunto de péptidos catiónicos ricos en arginina (**Figura 2**). De forma natural es sintetizada en la última etapa de la espermátide de muchos animales y plantas, siendo su rol fisiológico la condensacion del ADN [47]. Si bien su estudio comienza en el año 1868 por Friedrich Miescher, desde aquello muchos son los trabajos que se han realizado para caracterizar a este conjunto de péptidos alifáticos, fuertemente básicos, de peso molecular aproximado entre los 4 – 10 kDa [48].

La protamina es utilizada actualmente como excipiente farmacéutico siendo su principal aplicación en la formulación de liberación sostenida de insulina: NPH (del ingles: Neutral Protamine Hagedorn), aparte de tener registro sanitario como principio activo por ser el antagonista de la heparina.

Debido a su alta afinidad por el material genético la protamina ha sido ampliamente estudiada en la liberación controlada de material genético (ADN, ARN y siARN). De hecho, su mezcla con oligonucleótidos forma nanopartículas de manera espontánea llamadas “Proticles”, las cuales aumentan considerablemente la internalización celular del material genético con baja toxicidad [49, 50].

Este polipéptido ha sido estudiado para diversas patologías y vías de administración, como por ejemplo formando parte de nanoparticulas por vía nasal para su administración a cerebro [51], en conjunto con liposomas y ADN (denominados LPD, del inglés liposome-polication-DNA) [52, 53], para la

vehiculización de moléculas delicadas como el péptido vasoactivo intestinal [54], utilizando una probable sinergia en actividad antimicrobiana [55], etc.

Nº A.A.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Péptido 1	P	R	R	R	R	R	S	S	S	R	P	I	R	R
Péptido 2	P	R	R	R	R	-	S	S	R	R	P	V	R	R
Péptido 3	P	R	R	R	R	-	S	S	S	R	P	V	R	R
Péptido 4	P	R	R	R	R	-	A	S	R	R	-	I	R	R

Nº A.A.	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
Péptido 1	R	R	R	P	R	A	S	R	R	R	R
Péptido 2	R	R	R	P	R	V	S	R	R	R	R
Péptido 3	R	R	R	P	R	V	S	R	R	R	R
Péptido 4	R	R	R	P	R	V	S	R	R	R	R

Nº A.A.	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**Total AA**
Péptido 1	R	-	G	G	R	R	R	R	32 (21 R)
Péptido 2	R	R	G	G	R	R	R	R	32 (21 R)
Péptido 3	R	R	G	G	R	R	R	R	32 (20 R)
Péptido 4	R	-	G	G	R	R	R	R	30 (21 R)

Figura 2: Comparación de la estructura primaria de los 4 péptidos principales de la protamina. En gris las diferencias aminoacídicas (AA). P: Prolina, R: Arginina, S: Serina, V: Valina, G: Glycina, I: Isoleucina y A: Alanina. Adaptado de [56].

En el área de la inmunización, estudios preliminares han demostrado que la protamina incrementa los efectos inmumoestimulante de oligonucleotidos CpG [57] y mRNA [58, 59]. Además, micropartículas de PLGA recubiertas con protamina para su uso en vacunas para alergia, demostraron un efecto adyuvante con el uso de este polipéptido [60, 61]. Por último, liposomas LPD cargados con el antígeno ántrax, administrado por vía nasal, evidenciaron de que nanosistemas a base de protamina puede ser una plataforma válida para la vacunación a través de mucosas [62].

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CAPÍTULO 2

CAPÍTULO 2

NANOVACCINES: NANOCARRIERS FOR ANTIGEN DELIVERY

Adapted from: “Nanovaccine: Nanocarriers for antigen delivery”

González-Aramundiz JV, Cordeiro AS, Csaba N, De la Fuente M Alonso MJ. Biologie Aujourd`hui, 2012 206 (4): 249-261.

ABSTRACT

Vaccination has become one of the most important health interventions of our times, revolutionizing health care, and improving the quality of life and life expectancy of millions all over the world. In spite of this, vaccine research remains a vast field for innovation and improvement. Indeed, the shift towards the use of sub-unit antigens, much safer but less immunogenic, and the recognized need to facilitate the access to vaccines in the global framework is currently stimulating the search of safe and efficient adjuvants and delivery technologies. Within this context, nanocarriers have gained particular attention over the last years and appear as one of the most promising strategies for antigen delivery. A number of biomaterials and technologies can be used to design nanovaccines that fulfill the requirements of new vaccination approaches, such as single-dose and transmucosal immunization, critical for achieving a widespread coverage while reducing the overall costs in relation to traditional forms of vaccination. Here we present an overview of the current state of nanocarriers for antigen delivery, developed with the perspective of contributing to the global vaccination goal.

Framework and technological needs

The first scientific attempt to control an infectious disease by the deliberate use of vaccination was brought forth by Edward Jenner more than 200 years ago. Jenner's work turned the scientific community attention towards the development of vaccines and their potential for prevention and eradication of life-threatening diseases, such as smallpox, polio, diphtheria or tetanus [1]. The implementation of vaccination has increased over the time, being nowadays recognized as a greatest milestone in health protection. Furthermore, vaccination remains an interesting and vast field for innovation [2-4].

A major goal in vaccination is to achieve worldwide coverage against highly infectious diseases, such as tuberculosis, polio, diphtheria, tetanus, cholera, pertussis and to focus research on emerging or re-emerging diseases such as HIV or malaria. Currently, the population with access to the existing vaccines is extremely reduced and some infectious diseases exhibit complex pathogenesis mechanisms, which introduce great difficulties in the development of efficacious vaccines. The benefits of achieving an effective immunization program go beyond the life-saving objective, particularly in developing countries where vaccination allows the reduction of the sickness burden, thus contributing to their global development. This crucial observation has led several public and private institutions, such as the World Health Organization (through the Global Alliance for Vaccines and Immunization) and the Bill & Melinda Gates Foundation, to commit with the challenge of improving current vaccination programs [5, 6].

Traditionally developed vaccines consist of either live attenuated or inactivated pathogenic agents. Live attenuated vaccines stand out for their unique capacity to enhance strong innate and long-lasting immune responses without needing additional adjuvant components. However, their instability and potential to revert to the virulent form represents a serious risk for the patient health. To

overcome this problem, by the end of the 19th century, researchers developed inactivated whole organism vaccines as well as toxoid vaccines. These vaccines were shown to be safer than the previous ones, despite the limitation of yielding weaker immunity levels, thus requiring the use of an adjuvant, i.e. alum, and multiple-dose vaccination programs. More recently, vaccine research has been oriented towards the development of purer, safer and easier to produce antigens, namely 1) sub-unit vaccines in which a unique fraction of the pathogenic agent is used as antigen, 2) DNA vaccines and 3) conjugate vaccines, which consist of the presentation of the antigen covalently linked to a protein or membrane complex with stronger immunogenicity [2, 4]. Regardless of the promising features of these forms of vaccination, their use in a global perspective is still limited, essentially because of their low immunogenicity, thus making the search for new adjuvants a critical need for improving vaccination coverage [7, 8].

Besides the development of new adjuvants the current technological challenges in vaccination are aimed at improving the stability of the commercially available vaccines and decreasing the number of doses needed for an efficient immunization, altogether reducing the costs of this type of health intervention. Indeed, it is known that the assumed commitment to a cold chain protocol for the worldwide distribution of vaccines is frequently infringed with the subsequent risk of irreversible antigen damage [9, 10]. On the other hand, current schedules typically require the parenteral administration of several vaccine doses in order to reach adequate levels of protection, a fact that represents a real challenge in developing countries [9, 11].

The goals are therefore set for progress and innovation in the vaccination field with the final aim of reaching universal accessibility to vaccines that are simultaneously effective, affordable and safe. The most remarkable advances in

vaccination reported to date, and the role of nanotechnology are reviewed in the following sections and summarized in **Figure 1**.

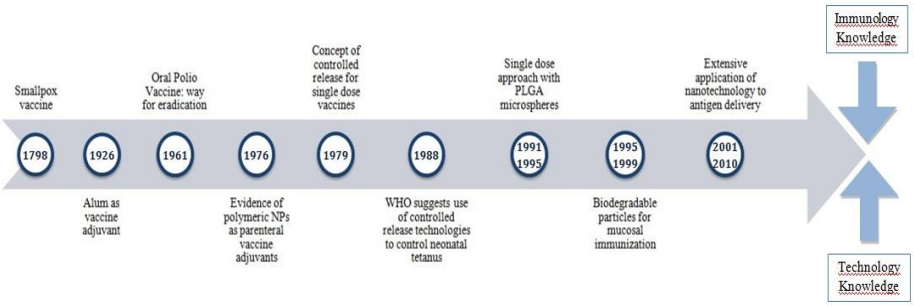

Figure 1: Milestones in vaccination and evolution of vaccines towards the development of single-dose and needle-free approaches. NPs: nanoparticles; WHO: World Health Organization; PLGA: poly(D,L-lactic-co-glycolic acid).

The essential role of adjuvants in vaccination

The term adjuvant encloses a heterogeneous group of compounds with different activities and functions, intended to enhance the quality, length and extent of a specific immune response [12]. The discovery and rational design of new compounds with adjuvant properties is becoming a key point in vaccination research that benefits from the knowledge gathered in the field of immunology. Indeed, advances in the study of activation mechanisms of antigen presenting cells (APCs) and the discovery of pattern recognition receptors for highly conserved structures of pathogens, such as the toll-like receptors (TLRs), are having a major impact in the development of new adjuvants [13].

The first significant adjuvants developed for human vaccines are the aluminum compounds depicted for the first time in 1926 and generally referred to as *alum.* Despite its favorable safety profile, alum is not adequate for the recently developed sub-unit vaccines, due to the probable loss of antigenicity of these antigens when adsorbed to this adjuvant [14]. Another concern of alum is its sensitivity to damage upon freezing [10].

Emulsion technologies represent a second approach to the development of new adjuvants. The first attempt involved the use of water-in-oil emulsions, known as Complete and Incomplete Freund's Adjuvant (CFA and IFA). Since then, toxicity concerns have led to the development of new oil-in-water emulsions made from highly purified emulsifiers, which have successfully led to some marketed vaccines for influenza (Fluad®, Focetria® and Prepandrix®) and human papilloma virus (HPV) (Cervarix®) [15, 16].

In parallel to the development of emulsions, vaccine research has focused on exploring alternative lipid-based antigen delivery technologies. Liposomes are the main class of delivery systems explored for drug delivery applications, and consequently, they have been widely investigated as antigen delivery systems in vaccination. In this field, it is worth highlighting the therapeutic tuberculosis vaccine RUTI® which is now undergoing clinical development [4, 15-17].

The integration of functional viral envelope glycoproteins into liposomes has led to an interesting type of antigen adjuvants named virosomes. Importantly, they retain the cell binding and membrane fusion properties of the native virus, therefore manifesting an improved capture by the APCs and antigen processing. Vaccines against influenza (Inflexal V®) and hepatitis A (Epaxal®) have already reached the market, and several are undergoing clinical trials [18].

Colloidal structures constituted by a combination of phospholipids, cholesterol and saponines, named immunostimulating complexes (ISCOMs), have been explored for their ability to associate hydrophobic and hydrophilic antigens, their good stability, and ability to induce both humoral and cellular immune responses. However, their potential use in human vaccines is hampered by toxicity concerns attributed to certain saponines as Quil A and QS-21 [19].

Other advanced lipidic formulations are the synthetic biomimetic supra molecular Biovector™ (SMBV) particles, consisting in a polysaccharide core of

positive or negative particles, surrounded by a phospholipid layer, which allows the incorporation of distinct active compounds. These structures are particularly studied in the area of mucosal vaccine delivery and have reached the clinical development status [20].

Regardless of the interesting features of lipid-based adjuvants, there is a trend to explore the potential of biocompatible polymeric nanostructures for antigen delivery. These delivery systems present several advantages and are promising adjuvants in vaccination, as highlighted in the next sections.

The potential of polymeric nanostructures as adjuvants in vaccination: nanovaccine

In 1976, Kreuter and Speiser reported for the first time the potential use of polyacrilic nanoparticles as an adjuvant for an influenza vaccine [21]. Following this pioneering work there has been a significant number of reports on the development of micro- and nano-sized particulate delivery systems [4, 22, 23].

Throughout the last years, different studies have been carried out to understand the importance of particle size in vaccination. Recent works have made clear that small and large nanoparticles reach the lymph nodes by different mechanisms and interact differently with APCs. While nanoparticles with a size inferior to 100 nm have shown an improved ability to drain to the interstitial flow and be transported to the lymph nodes for antigen presentation to resident DCs, larger particles typically reach the lymph nodes in a cell-associated manner [17, 24]. Similarly, the internalization mechanisms by APCs are dependent on the size of the particle engulfed: small nanoparticles are usually taken up by DCs through receptor-mediated endocytosis into clathrin-coated pits or through caveolae, while larger particles are generally phagocytosed specially by macrophages [25, 26]. In this regard, the route of internalization is gaining interest for the understanding of immune responses,

and receptor-mediated endocytosis has been related to cross-presentation processes and induction of combined cellular and humoral responses [27-29].

For the engineering of nanovaccines, the forming materials must be biocompatible and biodegradable and posses a good safety record. Biodegradable polymeric delivery systems exhibit a number of advantages as vaccine adjuvants: (i) they reproduce the natural particulate form of pathogenic agents, passively targeted to APCs; (ii) they can be engineered to specifically interact with certain cell populations as microfold-cells (M-cells) and dendritic cells (DCs) [30]; (iii) they can accommodate immunopotentiators as TLR agonists for an increased response; (iv) they can control the release of the antigen and prolong the exposure and duration of the immune response; (v) they can be administered by alternative non-invasive transmucosal routes; (vi) in the case of dry powder formulations generated upon freeze-drying, these adjuvants are known stabilizers of the associated antigens [9, 31-33].

Among the materials studied, the polyesters poly(D,L-lactide) (PLA) and poly(D,L-lactic-co-glycolic acid) (PLGA), as well as their PEGylated derivatives, have been widely explored, first for the preparation of microparticles, and few years later for the development of smaller nanosized particles [34-36]. Other biomaterials, such as oils, polyethylenoxides, and cationic polymers, have subsequently been incorporated to PLA/PLGA formulations in the search for vaccines with improved properties [37-40]. Further optimization of the adjuvant properties can be achieved by modification of their surface properties with immunostimulatory components such as the TLRs agonists CpG oligonucleotides [41].

Among the natural polymers that have been disclosed for the preparation of nanovaccines, special mention should be given to the biodegradable polysaccharide chitosan (CS), which has either been used as a polymeric coating or as the core forming material of nanovaccines [42-45]. Nanovaccines based

on CS are promising systems for the development of vaccines, in particular for transmucosal vaccination due to its mucoadhesive character, as discussed in the section rntitled “the promise of nanovaccine for transmucosal vaccination”. Other CS derivatives as PEGylated and quaternized CSs have similarly been explored for this application [45, 46].

As stated above, nanoparticulate delivery systems can be prepared from a variety of biomaterials with defined properties and are promising technologies in the search for efficient adjuvants for labile and low-immunogenic sub-unit antigens. Interesting results concerning the application of polymeric nanoparticles for single-dose and needle-free vaccination that have been reported to date are disclosed in the next sections and summarized in **Table 1**.

Table 1: Relevant examples of nanosystems for single dose and needle-free vaccination: immunization results in animal models (results obtained with model antigens are omitted).

Type of Nanostructure	Immunization route	Antigen	Dose	Immunization scheme	Key observations	Ref.
PLA/PLGA nanoparticles	Intramuscular	TT	30 µg	Single dose	Antibody titres generated by the NP lasted for over 5 months, an improved immune response as compared to that of a saline solution of the antigen.	[47]
CS nanocapsules		HBsAg	10 µg	Single dose Weeks 0, 4	A single-dose approach: protective IgG levels comparable to those obtained for the alum-adsorbed antigen in a two-dose administration schedule.	[48]
PGA nanoparticles	Intraperitoneal	JE vaccine BIKEN	1 µg	Single dose	Effective protection against JE virus in levels similar to those obtained with the conventional vaccine administered with alum.	[28]
PLA-PEG / PLA nanoparticles	Nasal	TT	40 µg	Single dose	PLA-PEG NP facilitated antigen transport through nasal route in comparison with PLA NP.	[34]
CS nanoparticles		TT	10 µg 30 µg	Days 1, 8, 15	IgA titres generated by the NP in saliva and broncho-alveolar/intestinal lavages, were much higher than those obtained with the antigen in solution.	[44, 49]
		HBsAg	10 µg/20 µg	Days 1, 28	Increasing anti-HBsAg IgG levels (seroprotective) over time.	[42]
CS-coated nanocapsules		HBsAg	10 µg	Days 0, 28	Increasing IgG levels over time for up to 112 days. Significantly increased response when the immunostimulant imiquimod was incorporated into the oily core.	[50]
PEG-CS / CS nanoparticles	Nasal	DT	10 µg	Days 0, 7, 14	Both formulations achieved mucosal and systemic immune responses, although the PEG coating allowed higher antibody titers.	[51]

Type of Nanostructure	Immunization route	Antigen	Dose	Immunization scheme	Key observations	Ref.
TMC / MCC / CS nanoparticles	Oral	TT	5 Lf TT/mice	Days 0, 22	Positively charged TMC and CS NP showed better results than the negatively charged MCC NP.	[52]
PLA-PEG / PLA nanoparticles		TT	498 µg	Single dose	The PEG coating was found to be crucial for the stabilization of the nanoparticles in the gastrointestinal environment, explaining the improved absorption of the antigen after oral administration to rats.	[53]
Lectin-decorated PLGA nanoparticles		HBsAg	10 µg	Week 0, 2	Lectinized NP provided higher anti-HBsAg titres than the plain PLGA NP, probably due to selective targeting to the M-cells. IgG titres elicited were comparable between orally administered lectinized NP and intramuscularly administered alum-HBsAg.	[54]

Abbreviations: NP: nanoparticle; PLA: poly(lactid acid); PLGA: poly(lactic-co-glycolic acid); CS: chitosan; PGA: poly(γ-glutamic acid); PEG: polyethylene glycol; PEO: polyethylene oxide; TMC: *N*-trimethyl chitosan; MCC: mono-*N*-carboxymethyl chitosan; PCL: poly(ε-caprolactone); Lf: limit of flocculation; TT: tetanus toxoid; HBsAg: recombinant Hepatitis B surface antigen; DT: diphtheria toxoid; OVA: ovalbumin; JE: Japanese encephalitis.

Controlled release technologies for the development of single-dose vaccines

In the late 70s, Preis and Langer showed that the release of active macromolecules could be extended for periods that exceeded 100 days, upon association to ethylene-vinyl acetate beads. The most remarkable finding was verifying that, as a consequence of this sustained release, the stimulated immune response was comparable to the secondary response induced by the same total dose of antigen emulsified in CFA [55]. This discovery introduced the idea of single-dose vaccination achievable through controlled delivery of the antigen.

In 1988, the WHO proposed the global use of controlled release technologies in the development of single-dose vaccines, a grand challenge in global health as it intends to increase the compliance with the immunization schedule and the effectiveness of vaccination in developing countries. Following this indication, in the next decade, a number of prototypes based on PLGA or PLA microspheres were designed and studied, as can be read next (also see previous section).

The tetanus toxoid (TT) was the first antigen to be considered for a single-dose approach because of the high incidence of neonatal tetanus in developing countries. Provided the first insight on the potential of PLGA microspheres to enhance and prolong the immune response to TT [35]. Despite these promising results, PLGA microspheres presented as main limitation the degradation of the encapsulated antigen and the consequent loss of antigenic activity. This fact was attributed to the harsh technologies applied for their preparation (organic solvents and strong shear forces) and to the acidification of the microenvironment due to the erosion of the polymeric matrix [56, 57]. On view of this, revolutionary solutions were presented. An interesting approach consisted in the isolation of the antigenic protein in oil-based cores

surrounded by outer PLGA shells [58]. Another proposal was to develop microparticles from an intimate blend of PLGA and polyethylene oxide (poloxamer 188), a non-ionic polymeric surfactant, able to block the interaction of PLGA with the antigen and prevent its degradation [39]. On view of these results, the same strategy was later applied for the development of improved PLGA nanostructures [37, 59].

PLGA microparticles have successfully been used for encapsulation of a variety of antigens, including influenza [60], diphtheria toxoid [61] and hepatitis B [62] among others, showing in all cases the possibility to enhance and prolong the release of the encapsulated antigens. PLGA microparticles have also been used for the encapsulation of plasmid DNA (pDNA) designed to express the Hepatitis B surface antigen, HBsAg (DNA vaccination) [63].

In the 90s, PLGA nanoparticles were also developed by our group on view of the promising results achieved with microparticles, and considering the potential advantages of a smaller size [36]. PLA and PLGA nanoparticles showed that the intramuscular immunization of rats with TT-loaded nanoparticles provided anti-TT antibody titers that persisted for more than 5 months, which was significantly better than the immune response elicited by saline solutions of TT [47]. The immune response peaks achieved were higher for nanoparticles than for the corresponding microparticles, leading to the concept that particle size has a major influence in the immune response produced. The same study also concluded that the hydrophobicity of the vehicles is a very important factor, since the PLA nanoparticles provided better immunization results than the PLGA ones, in agreement with previous studies performed with PLA/PLGA microparticles [35]. To study the possibility of improving the results of the immunization with these systems, this group also tested the possibility of administering simultaneously TT-loaded PLGA nanoparticles and alum. The results suggested a synergistic effect between the two adjuvants [47].

Improved PLGA/poloxamer nanosystems previously developed to deliver DNA vaccines [37] were conveniently adapted for the association of more complex antigens, i.e. virus-like particles such as HBsAg, and additionally coated with CS to further improve the presentation of the nanocarrier to immunocompetent cells [38]. CS-coated PLGA/poloxamer nanoparticles delivered HBsAg in a controlled manner for up to 14 days, fully preserving the integrity and antigenicity of the released antigen. The long-lasting delivery properties of these nanostructures evidence their potential for single-dose vaccination, but this possibility has yet to be evaluated in animal models.

Another promising delivery carrier with a potential for single-dose immunization are the so-called CS nanocapsules. In this case, HBsAg was adsorbed onto the nanocapsules' surface, through the electrostatic interactions between the negatively charged antigen and the positively charged CS. These systems were evaluated *in vivo* through intramuscular administration to mice of a 10 μg dose of HBsAg both in a single-dose and two-doses schedule, and compared with the antigen adsorbed in alum. The protective antibody levels induced by the single-dose administration of this system were comparable to the ones elicited by the alum-adsorbed antigen in a two-doses schedule, proving that these CS nanocapsules are a valid prototype for a single-dose vaccination approach [64].

Polyaminoacids have also been studied for their use in drug delivery and vaccine nanocarriers [65]. More specifically, hydrophobically modified polyglutamic acid (PGA) nanoparticles were developed for association of the Japanese encephalitis (JE) vaccine BIKEN (a formalin-inactivated mouse brain-derived vaccine) and treatment of JE. The results showed that, after a single intraperitoneal dose to mice, nanoparticles provided effective protection from lethal JE virus. The level of protection resulted to be comparable to the JE vaccine BIKEN administered with alum [28].

Overall, the above-disclosed information has made evident the potential of micro and nanoparticles as single-dose vaccine formulation approaches. It is however worth noting that the most investigated delivery carriers, PLGA micro- and nanoparticles, have not reached the clinical development status yet, with only a small trial of clinical evaluation [15]. Irrespective of the commercial justifications for the implementation of these technologies, it seems reasonable to conclude on the necessity of making further progress in the development of safe, inexpensive and efficacious polymer particles, which may one day represent single-dose formulation vaccines.

The promise of nanovaccines for transmucosal vaccination

Regardless of the intended achievements in parenteral immunization, needle-free vaccination has been recognized as a great challenge in global health. Needle-free vaccination facilitates compliance with immunization schedules, decreases pain and suffering, requires less healthcare training for vaccination, enables faster vaccine delivery, avoids the risk of incorrect or repeated use of injection devices and allows an eventual cost reduction [11]. This section is focused on mucosal immunization, among the different needle-free delivery approaches, as this is a suitable and already established vaccination protocol for some specific vaccines, i.e. polio, rotavirus, *Salmonella typhi* and adenovirus type 4 and 7 vaccines for oral administration, and influenza vaccine for nasal administration.

The interest in mucosal vaccines relies not only in the induction of systemic immune responses but also of mucosal responses, thus providing additional protection against pathogens even at their site of entry, since most infections start at or affect specifically mucosal surfaces. The use of antigen delivery systems in mucosal vaccination aims at the improvement of the antigen stability and the facilitation of its penetration across the mucosal surface, so that the intact and active antigen could be taken up by APCs and transported

from the mucosal associated lymphoid tissues (MALT) to the lymph nodes or other secondary lymphoid organs [66]. As an example of this process, **Figure 2** represents schematically the pathways involved in intranasal vaccine delivery.

Depending on the organ or site to which it is associated, the MALT may have some differences in structure. For example, in the digestive tract this tissue is often referred to as gut-associated lymphoid tissue (GALT) and is mainly composed of Peyer's patches and M-cells, which are capable of carrying antigens in a particulate formulation from the absorption site until they reach APCs, which migrate to the regional lymph nodes and trigger the adequate immune response. In the human nasal cavity this tissue is known as “diffuse” nasal-associated lymphoid tissue (NALT) and is composed of a collection of isolated subepithelial lymphoid follicles and the lingual, palatine and nasopharyngeal tonsils (adenoids) [32, 66].

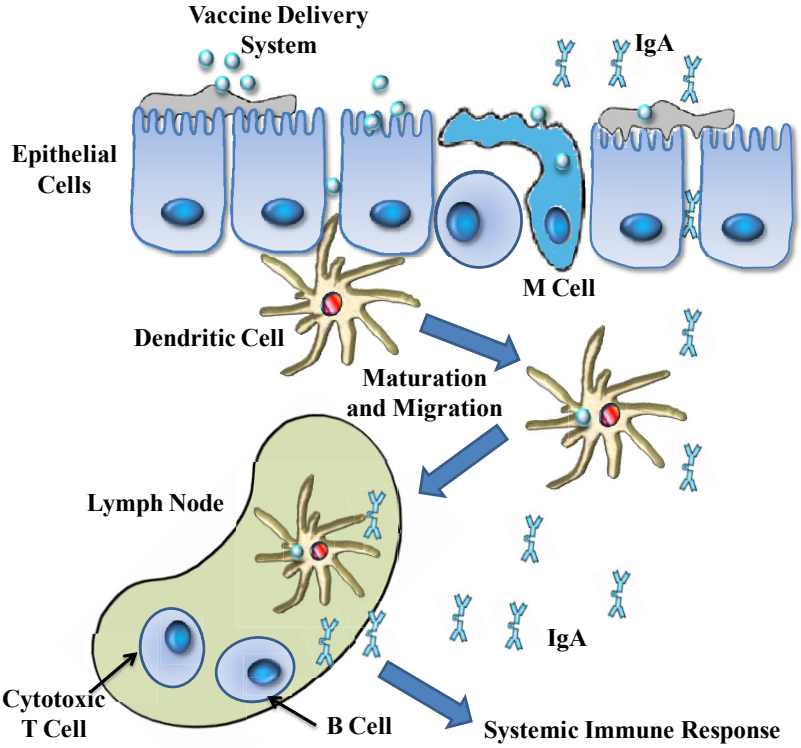

Figure 2: Schematic illustration of the general pathways involved in mucosal immune response following intranasal vaccination.

In all cases, the first barrier to overcome in mucosal vaccination is a dense and dynamic mucus layer that covers and protects the underlying epithelium. For a successful mucosal vaccination, the vehicles should overcome the mucus barrier, adhere or penetrate the epithelium and deliver the immunologically active antigen in a controlled-release fashion. The following sections are intended to analyze information on the most relevant technologies developed for needle-free nasal and oral vaccination as well as the most remarkable immunization results obtained from these vaccination approaches.

Nasal vaccine delivery systems

Some relevant characteristics of the nasal cavity physiology that make it especially attractive for vaccination are its relatively reduced enzymatic activity,

a moderately permeable epithelium, and its high amount of available immune-reactive sites. For the success of needle-free nasal immunization, nanoparticles appear to be an interesting approach, as they are known to increase the residence time upon intranasal deposition and improve the interaction with the mucosal epithelium.

As highlighted in this review, PLGA and PLA have been the most studied polymers for the design of antigen delivery systems, being the first to be evaluated for needle-free nasal vaccination in the early 90s. In 1993, Almeida *et al* demonstrated that PLA microspheres, administrated through the nasal route to guinea pigs, could effectively enhance the immune response of the adsorbed TT, when compared to the soluble antigen [67].

The first nanoparticulate compositions to be evaluated for nasal delivery of antigens were nanoparticles composed by PLA or the PEGylated derivative PLA-PEG. Experiments in rats performed with nasally administered radiolabelled tetanus toxoid ^{125}I-TT have shown that PLA-PEG nanoparticles successfully prolonged the residence time, antigen release and transport to a greater extent than conventional PLA nanoparticles [34]. The hydrophilic coating was proved to be essential for increasing the stability of the nanoparticles upon contact with the mucosal fluids, thereby enhancing their interaction with the epithelium [68]. These results were corroborated in subsequent studies showing that through the careful control of the nanoparticle size and density of PEGylation, it is possible to modulate the interaction of these nanocarriers with the epithelium and hence its mucosal transport. PLA-PEG nanoparticles with an elevated PEG coating density (20-35 %) evidenced a significantly increased transport versus the PLA control nanoparticles [49].

Optimized nanoparticles of PLGA blended with polyoxyethylene derivatives have also been studied for DNA vaccination through the nasal route [37]. These improved nanoparticles could effectively overcome the mucus layer upon

intranasal deposition and enter epithelial cells. Most importantly, immunization studies proved the ability of pDNA-loaded nanoparticles to elicit a fast and strong immune response, with IgG antibody titers against the encoded protein significantly higher than those corresponding to the naked pDNA [37]. Further studies demonstrated the suitability of these nanostructures to associate other types of antigens as proteins and even virus-like particles [38, 59].

Besides the promise of PLA/PLGA nanoparticles for nasal delivery of antigens, CS -based nanocarriers hold a great potential for this application mainly due to the mucoadhesive properties of this polysaccharide. Vila *et al* reported the first studies performed with CS nanoparticles for nasal immunization and showed the positive role of CS in improving the transport of antigens across the nasal mucosa [44, 69]. With the purpose of exploring how the polysaccharide properties could influence the immunization process, nanoparticles loaded with TT were prepared from CS polymers of different molecular weights (ranging from 23 to 70 kDa). Upon intranasal administration to mice, results showed comparable levels of IgA antibody in saliva, broncho-alveolar and intestinal lavages, irrespective of the CS molecular weight, as highlighted in **Figure 3**. In all cases, the titers were superior to those obtained in animals immunized with the antigen solution [44].

Chitosan nanoparticles also resulted to be suitable for the encapsulation of more complex antigens such as HBsAg, without altering its antigenicity [42]. Intranasal immunization to mice was performed at two different priming HBsAg doses (10 and 20 µg) and a boost dose after 28 days. The anti-HBsAg IgG levels showed a low but increasing immune response over the time, regardless of the dose administered. In both cases, the antibody concentrations were considered to be seroprotective against hepatitis B although the rather low overall response indicates the need for further optimization of the delivery system for this specific antigen.

Stealth PEGylated CS (PEG-CS) nanoparticles have also been studied for intranasal delivery of different antigens and the possible role of PEG in improving the observed immune response was explored [51, 70]. For example, PEG-CS and CS nanoparticles were able to induce systemic and mucosal immune responses against diphteria toxoid (DT) upon intranasal administration to mice. Besides, PEG-CS achieved significantly higher systemic antibody titers in comparison to the ones obtained for CS nanoparticles [51].

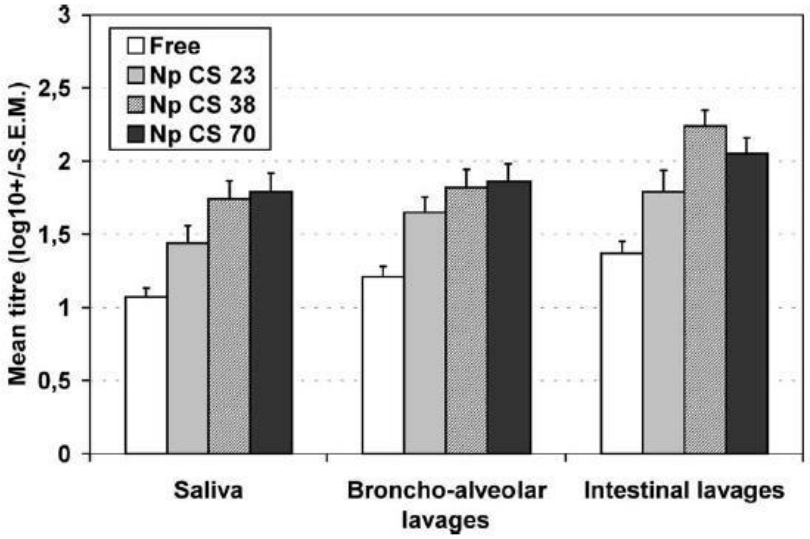

FIGURE 3: IgA responses obtained after intranasal immunization of mice with 10 µg of tetanus toxoid encapsulated into nanoparticles prepared with chitosan polymers that differ in their molecular weight: 23 KDa (Np CS 23), 38 KDa (Np CS 38) and 70 kDa (Np CS 70). As control, the antigen was administered in saline. Titres are presented as the geometric mean titre (GMT) per group. Adapted from [44] with permission.

Other CS derivatives have similarly been explored for antigen delivery purposes. The most recurrent modifications involve the methylation of CS amine groups [71]. Nanoparticles made of oppositely charged methylated CS derivatives (positively charged N-trimethyl CS-TMC and negatively charged mono-N-carboxymethyl CS-MCC) were loaded with TT and intranasally administered to mice. The results have shown that positive CS and TMC

nanoparticles, with a size ranging from 300-400 nm, induced higher serum IgG titers than negative MCC nanoparticles of 90 nm. These results emphasized the assumption that the nature of the nanoparticles surface, as well as the particle size, have a crucial role in obtaining an enhanced immune response [52, 72].

Mangal *et al* have recently reported the superiority of TMC nanoparticles vs. classical CS nanoparticles with regard to their ability to elicit anti-HBsAg antibody titers following intranasal administration to mice. This was attributed to the improved mucoadhesion of TMC nanoparticles, followed by an enhanced antigen uptake [73].

Interestingly, CS-coated nanocapsules, formed by an oily nanocore stabilized with phospholipids and surrounded by a CS shell, as already described in section on Crontrolled release technologies, have also shown a great capacity of association of HBsAg. The most remarkable advantage of this type of improved nanostructure is its versatility, as it is feasible to efficiently incorporate simultaneously lipophilic immunostimulants in the oily core, i.e. imiquimod, and the antigen in the polymer shell. [43]. The results of the *in vivo* experiments performed with this novel formulation have shown a progressive increase of the specific IgG levels over time, achieving seroprotection against the HBsAg for up to 112 days. This response was significantly higher than the one obtained with the nanocapsule formulation in the absence of imiquimod.

Nanoemulsions have similarly been explored for the stabilization and nasal delivery of different molecules, in particular OVA, porcine intestinal alkaline phosphatase (AlkP), and HBsAg [74]. In vivo results show that upon intranasal administration to mice of HBsAg associated to the nanoemulsion, the reported serum anti-HBsAg IgG antibody titers were comparable to the ones obtained for the antigen adsorbed on aluminum hydroxide and given intramuscularly. These results were similarly reported in other animal species (rats and guinea pigs), in a single- or two-doses scheme [75].

Despite the promising examples reported here, there is no doubt about the need to further improve the design and, thus, the efficiency of intranasal delivery vehicles. Nevertheless, the accumulated knowledge and the cross-disciplinary approaches currently underway will hopefully define the way to proceed in the optimization of the design of nanostructures for intranasal antigen delivery.

Oral vaccine delivery

While live-attenuated vectors have shown promising results for oral immunization, problems related to their safety made it necessary to find new solutions for oral vaccination. This route is particularly challenging due to the harsh conditions of the gastrointestinal environment and the need to confront the intestinal mucosa. Nano-sized delivery systems are expected to have several functions in oral vaccine administration: (i) to improve the antigen stability in the gastric environment and increase its bioavailability, (ii) to overcome the mucus layer and interact with the underlying epithelium, (iii) to increase the uptake of these systems by the epithelial and M-cells [76].

The physicochemical properties of particulate vehicles are known to affect the uptake of the antigen along the intestine and by APCs. The smaller size of nanoparticles is considered to be a key parameter influencing the uptake and immunogenicity of these delivery systems, as nanoparticles are better taken up by intestinal cells than microparticles [77-79]. With respect to the surface properties, both the hydrophobicity and the surface charge affect colloidal stability, mucoadhesion properties and absorption of the carriers [77]. In addition, targeting specific receptors on the apical surface of M-cells may enhance the entry of antigens, triggering the immune response and leading to effective protection against mucosal pathogens [30]. Nanoparticles can be conveniently targeted to M-cells by surface modification with selective ligands such as lectins or certain bacterial surface proteins [78, 79].

The importance of the surface properties on the ability of particles to efficiently deliver antigen through the oral route was evidenced by Tobío *et al* [68]. Similarly to what observed when the nanoparticles were administered to the nasal mucosa, the coating of PLA nanoparticles with a hydrophilic and stabilizing PEG coating was found to be crucial in order to avoid nanoparticle aggregation in gastro-intestinal fluids and upon contact with the mucosa. This increased stability has been the explanation for the improved absorption of ^{125}I-labelled TT after oral administration to rats in PLA-PEG nanoparticles [68].

Poly(anhydrides) were also used for the preparation of a specific type of polymer named Gantrez® AN [80]. Nanoparticles prepared with this polymer present interesting bioadhesion characteristics and were therefore studied for oral immunization with ovalbumin (OVA). Nanoparticles provided better immune response than that obtained for the OVA in solution. The survival rate of 100% achieved by the OVA-loaded nanoparticles versus the 40% obtained with the OVA-solution after challenge with an intraperitoneal injection of this allergen also proves that this can be an interesting alternative for oral immunization [81].

Other recent studies have aimed at exploring the possibility to target the M-cells, through the development of carriers that mimic the entry of pathogens. An example of this approach was the study of PLGA nanoparticles decorated with a specific type of lectin (*Lotus tetragonolobus - LTA*). These nanoparticles interacted with Peyer's patches M-cells, as confirmed by confocal microscopy, and provided anti-HBsAg titers, which were higher than those corresponding to the control PLGA nanoparticles. This positive behavior was attributed to the lectin-mediated selective targeting [54].

The RGD peptide (a small sequence composed of L-arginine, glycine, and L-aspartic acid which is involved in cell recognition processes) was also grafted to the PEG residues of the poly(ε-caprolactone-co-ethylene glycol) (PCL–PEG)

copolymer used to prepare nanoparticles. Unfortunately, despite the targeting ability evidenced in vivo, the improvement of the immune response achieved by any of the RGD-grafted formulations was minimal [82]. Thus, these results put in question the interest in targeting the M-cells for achieving adequate immune responses.

Overall, on view of the described works, the evidence of the potential of nanostructures for oral vaccination is still scarce. Besides, the positive role of a specific targeting to M-cells needs to be confirmed. Despite of this, the reported results have paved to way towards the optimized design of nanoparticles intended for oral vaccination. Considering the whole perspective on transmucosal vaccine delivery, it becomes clear that this approach is a valid and interesting pathway for future developments. The research on new delivery vehicles for this type of administration is therefore expected to play a very important role in global health improvement and may represent a new milestone in vaccine research.

CONCLUSIONS

Over the past few decades, the search for new adjuvants capable of enhancing the immunogenic properties of current and developing antigens has driven the attention of the scientific community towards the design of particulate antigen delivery systems. These new adjuvants are not only able to enhance the immunogenicity of safer but poorly effective antigens but may also allow the targeting of these antigens to the adequate immune-competent cells. Beyond this adjuvant capacity polymer-based micro/nanostructures can be now presented as promising single-dose and transmucosal vaccination approaches. Giving room to new routes of administration, nanovehicles are influencing decisively the pathways of vaccinology and gaining an essential place in this field. Hopefully, these progresses in immunization strategies will help to

achieve the universal goal of satisfactory immunization coverage to life-threatening diseases worldwide.

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ANTECEDENTES, HIPÓTESIS Y OBJETIVOS

ANTECEDENTES

1. La nanomedicina se presenta como una estrategia prometedora en el diseño de nuevas vacunas, para aquellas enfermedades que no poseen aún cobertura o para mejorar aquellas que ya se encuentran en el mercado[1]. Estas "nanovacunas" permiten: (i) estabilizar el antígeno, (ii) mejorar la interacción y captación del antígeno asociado por parte de las células inmunocompetentes y (iii) aumentar el transporte del antígeno a través de barreras mucosas, protegiéndolo frente la degradación y vehiculizándolo a las células inmunes[2].

2. La protamina está formada por un conjunto de polipéptidos naturales y es capaz de formar diferentes nanoestructuras mediante procedimientos sencillos, administrarse por diversas vías y vehiculizar moléculas delicadas[3]. Posee un gran potencial como biomaterial para la preparación de nanosistemas por promover la translocación a través de membranas[4], estimulación del sistema inmune[5,6] y un perfil de seguridad aceptable[7]; confiriendo estas propiedades a los sistemas a los que se les ha asociado o en los que forma parte.

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3. Las nanopartículas poliméricas permiten la encapsulación eficaz de diversos antígenos[8] y son vehículos válidos para la administración libre de agujas[9].

4. Las nanocápsulas constituidas por una cubierta polimérica son una estrategia innovadora en la vehiculización de antígenos y en la coadministración de aceites con capacidad adyuvante[10,11].

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HIPÓTESIS

1. Los sistemas constituidos por protamina pueden ser una estrategia adecuada para la asociación, vehiculización y presentación de antígenos.

2. Los sistemas a base de protamina pueden incrementar la captación del antígeno por parte de las células presentadoras de antígeno y potenciar así una respuesta inmune específica.

3. La protamina como componente de los diversos sistemas desarrollados puede favorecer el transporte de antígenos a través de barreras mucosas, induciendo una respuesta inmune eficaz y constituyendo una formulación libre de agujas.

4. Las nanopartículas y nanocápsulas pueden ser transformadas en polvo seco a través de un proceso de liofilización y dicho producto presenta una adecuada estabilidad a temperatura ambiente.

OBJETIVOS

Teniendo en cuenta los antecedentes expuestos y las hipótesis planteadas, el objetivo general de la presente tesis doctoral ha sido el diseño, desarrollo y optimización de nuevos vehículos nanométricos, basados en protamina, para potenciar y modular una respuesta inmune específica frente al antígeno asociado ya sea a través de su administración parenteral y/o por medio de administración nasal. Para lograr este objetivo se han planteado las siguientes etapas:

1. **Desarrollo de nuevos sistemas de nanopartículas constituidos de protamina, evaluación *in vitro* e *in vivo* de su capacidad para vehiculizar antígenos.**

Esta parte de la memoria se ha dirigido al diseño, desarrollo y caracterización de nanopartículas de tipo matricial, que poseen protamina en su estructura. Se ha estudiado su capacidad para asociar un antígeno modelo, su interacción con células inmunes y la capacidad de promover una respuesta inmune eficaz, tanto por vía parenteral como por vía nasal. Estos resultados aparecen recogidos en el capítulo 3 de la presente memoria.

2. **Desarrollo de nuevos sistemas constituidos por nanocápsulas de protamina y evaluación como sistemas transportadores de antígenos**

Los estudios realizados en esta etapa se han dirigido al diseño, desarrollo y optimización de sistemas de tipo nanocapsular con una cubierta de protamina y un núcleo constituido por aceites con capacidad adyuvante. Se ha estudiado su eficacia para asociar antígenos, su estabilidad a temperatura ambiente tras ser liofilizado y su capacidad para interaccionar *in vitro* con el sistema inmune. Por último, ha sido evaluada su capacidad para generar respuestas inmunes sistémicas tras su administración tanto por vía parenteral como nasal. Los

resultados de este apartado se recogen en los capítulos 4 y 5 de la presente memoria.

CAPÍTULO 3

CAPÍTULO 3

PROTAMINE:POLYSACCHARIDE NANOPARTICLES AS ANTIGEN DELIVERY SYSTEMS

Este trabajo ha sido realizado en colaboración con Mercedes Peleteiro[1] y África González-Fernandez[1].

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ABSTRACT

The use of biodegradable nanoparticles as antigen delivery vehicles is an attractive approach towards the design of alum-free vaccines and, thus, to overcome the problems associated to the use of this classical adjuvant. In this work, we present for the first time the potential of protamine-based nanoparticles as novel adjuvants, using recombinant hepatitis B surface antigen (rHBsAg) as a model viral antigen.

The nanoparticles, composed of protamine and a polysaccharide, hyaluronic acid or alginate, were obtained using a mild ionic cross-linking technique. The size and surface charge of the nanoparticles could be modulated by adjusting the ratio between protamine and the polysaccharide. Prototypes with best characteristics and stability were selected for the assessment of the antigen loading capacity, antigen stability during storage and in vitro and in vivo proof-of-concept studies. Results showed that nanoparticles were able to preserve the stability of the associated antigen for at least 3 months upon storage at 4ºC. On the other hand, *in vitro* studies showed that antigen-loaded nanoparticles increased the secretion of cytokines by macrophages, in comparison with the antigen in solution. Finally, *in vivo* studies showed the capacity of these systems to trigger a protective immune response following intramuscular immunization. All these *in vitro* and *in vivo* results suggest that these protamine-polysaccharide nanoparticles may have a great potential as novel adjuvants.

1. INTRODUCTION

Since their implementation in 1926, aluminum salts have had a crucial role in making vaccination the most important human intervention in global health. Despite of its value, alum suffers of important deficiencies, such as (i) insufficient adjuvant effect specially for new antigens such as subunit vaccines and peptides, (ii) lack of effect when administered by other routes than parenteral injection, (iii) associated side effects, i.e. local reactions or hypersensitization of allergic patients and (iv) it is only stable in a low and narrow temperature range [1, 2]. Due to these limitations, significant efforts have been directed towards developing new alternative adjuvants.

In a broad sense, an adjuvant could be defined as a molecule or structure that can increase and/or modulate the immunogenicity of an antigen, allowing it to induce a potent and persistent immune response at low doses [3]. Some authors divided this group in immunostimulants (those interacting with specific receptors) and delivery systems [4]. Particulate delivery systems may act as adjuvants in the sense that they can modify the uptake, trafficking and processing of antigens, resulting in better and more adequate immune responses [3, 5].

In the design of novel “alum-free” vaccine particulate delivery systems, it is necessary to consider the nature of the antigen and its intrinsic immunogenicity, the administration route and the availability of biomaterials with an adequate safety profile.

On the other hand, the importance of the particle size of these delivery systems has been widely discussed over the past years. Nanometric systems have raised expectancy because of their ability to control the release and increase the trans-epithelial transport of antigens, thus being considered as promising strategies for the development of single dose and needle-free vaccines [6, 7].

Different materials have been studied to develop particulate antigen delivery systems, such as lipids, polymers and polysaccharides. In particular, chitosan,

polylactic and polylactic-co-glycolic acid have been the most widely applied polymers in the development of vaccine nanocarriers. [4, 8, 9]. More recently, polyaminoacids and polypeptides have been highlighted for their versatility to form different structures, their safe pharmacokinetic profile and the adjuvant properties presented by some of them [10]. In particular, protamine, an arginine-rich peptide with cell-penetrating properties has shown a synergistic adjuvant effect with other immunomodulatory molecules, i.e. CpG [11]. This effect was also observed when protamine was used to coat PLGA microparticles, the resulting system being able to stimulate the proliferation of antigen-specific T cells and the secretion of IFN-γ [12].

Other materials, such as hyaluronic acid (HA) and alginate (ALG) have also shown immunoadjuvant activity, measured by macrophage and dendritic cell recruitment plus activation and/or induction of cytokine production, when presented in a nanoparticulated form [13-16].

Based on this information, the aim of this work has been the development and characterization of a new nanometric antigen delivery system based on protamine to harness its adjuvants properties in association with a polysaccharide such as alginate or hyaluronic acid. In order to evaluate the ability of these systems to encapsulate and deliver antigens, recombinant hepatitis B surface antigen (rHBsAg) was used as a model antigen. Finally, the *in vitro* and *in vivo* performance of this novel vaccination approach was assessed in macrophage cell cultures and upon administration to mice by either intramuscular or nasal administration.

2. MATERIALS AND METHODS

2.1. Chemicals

Protamine sulfate used in this work was purchased from Yuki Gosei Kogyo, Ltd. (Japan) Hyaluronic acid (HA) of 162 KDa and 29 kDa were provided by Bioiberica (Spain) and by Soliance (France), respectively. Sodium alginate (ALG) (PRONOVA UP VLVG) of <75 kDa was supplied by Novamatrix (Norway). Recombinant hepatitis B surface antigen (rHBsAg) was kindly donated by Shantha Biotechnics Limited (Hyderabad, India). Enzyme linked immunosorbent assay (ELISA) kit (Murex rHBsAg Version 3) was obtained from Diasorin (United Kingdom). Chicken polyclonal antibody to hepatitis B virus surface antigen and rabbit polyclonal to chicken conjugated with horseradish peroxidase were purchased from Abcam pcl (United Kingdom). Rabbit IgG and mouse monoclonal IgG used as controls were purchased from Biokit (Spain) and Acris Antibodies GmbH (Germany), respectively, and secondary Abs conjugated to horseradish peroxidase were from Southern Biotech (USA). 5-TAMRA, 5-Carboxytetramethylrhodamine, Succinimidyl Ester (single isomer) and Alexa Fluor 488-phalloidin were obtained from Invitrogen (United Kingdom). Triton X-100, glucose, trehalose, PBS, hyaluronidase, heparin and aluminum hydroxide gel were obtained from Sigma-Aldrich (Spain). All other chemicals used were of reagent grade or higher purity.

2.2. Preparation of protamine:polysaccharide nanoparticles and loading of rHBsAg

Nanoparticles were prepared by an ionic cross-linking technique as previously described by Calvo et al [17]. Briefly, 1 ml of, either protamine, hyaluronic acid or alginate was kept under magnetic stirring at a concentration of 1 mg/ml. The counter ion was also dissolved in purified water and particles with different protamine:polysaccharide ratios ranging from 1:6 to 6:1 (w/w) were prepared

upon mixing. Nanoparticles were instantaneously obtained by the interaction of the protamine with the polysaccharide. For the encapsulation of rHBsAg, the antigen was mixed with the polysaccharide at a theoretical loading of 2.5 and 5 % (with respect to the total amount of polymers), prior to the production of nanoparticles.

2.3. Nanoparticle characterization

2.3.1. Size and zeta potential

The hydrodynamic diameter and polydispersity index of the particles were determined by photon correlation spectroscopy (PCS) after dilution of the samples with ultrapure water. The zeta potential was measured by laser-Doppler anemometry diluting the samples with KCl 1 mM. (Zetasizer®, NanoZS, Malvern Instruments, Malvern, UK),

2.3.2. Morphological analysis

The morphological examination of the nanoparticles was carried out by transmission electron microscopy (TEM, CM12 Philips, The Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid solution and dried on a copper grid.

2.3.3. Production yield and nanoparticle composition

In order to determine the yield of production, the nanoparticles were isolated by centrifugation for 40 minutes, at 18000 x g and 15ºC (Universal 32R, Hettich Zentrifugen, Germany) and the supernatants were discarded. The sediments of the nanoparticles were freeze-dried (Genesis SQ freeze drier, Virtis, US) and weighed. The yield of production was calculated as follows (equation 1):

$$\text{Production Yield (\%)} = \frac{\text{Nanoparticle weight}}{\text{Theoretical weight (total solids)}} \times 100$$

In order to determine the rate of incorporation of the different components, nanoparticle samples were freeze-dried and analyzed by elemental analysis (FISONS EA 1108, United Kingdom). Protamine and hyaluronic acid content in the nanoparticles samples was analyzed by the nitrogen and carbon content in comparison with protamine and hyaluronic acid as raw materials. As additional controls, nanoparticle supernatants, physical mixtures of the components and non-isolated nanoparticles were also analyzed by the same method.

2.4. Stability of the nanoparticles

The stability of nanoparticles as an aqueous suspension was evaluated under storage conditions (4ºC for three months) and also under physiological conditions (PBS, 37ºC, pH: 7.4 for 7 days), by measuring nanoparticle size at different time points.

2.5. Association efficiency and structural integrity of rHBsAg

Nanoparticles were loaded with the viral antigen rHBsAg as detailed in section 2.2. The efficiency of antigen association was determined by an indirect method, calculating the difference between the total amount of antigen added to the nanoparticle formulation and the amount of rHBsAg remaining in the aqueous external medium. The separation of the antigen-loaded nanoparticles from the aqueous medium was carried by centrifugation for 40 min, at 18000 x g and 15ºC. The amount of non-associated antigen was quantified in the supernatant by ELISA following manufacturer's instructions.

In order to confirm the integrity of the associated antigen, isolated nanoparticles were degraded with a mixture of hyaluronidase and heparin in acetate buffer pH 7.2 during 2 hours at 37ºC. Samples were then centrifuged at 18000 x g for 10 min and analyzed by Western blot as a qualitative tool, following the methods detailed in [18].

2.6 *Freeze-drying studies*

Different concentrations of nanoparticles (0.5; 0.25; 0.125 and 0.0625 mg/ml) loaded with rHBsAg at 2.5%, either alone or in combination with cryoprotectants (trehalose or glucose at 5% w/v), were freeze dried using Labconco Corp (USA). Freezing temperature was -20ºC, and the drying process was maintained for up to 72 hours. The nanoparticle formulations were recovered by manual resuspension by adding ultrapure water to the freeze-dried powders. The reconstituted nanoparticles were characterized with regard to their physico-chemical characteristics (Section 2.3) and the structural integrity of the associated antigen was analyzed by Western blot.

2.6. Cell viability studies

The viability studies were performed in adherent RAW 264.7 murine macrophages (ATCC, USA) by Quick cell counting solution (QCCS) (GenScript Corporation, USA). Cells were cultured in RPMI supplemented with 10% (v/v) heated-inactivated fetal bovine serum (FBS) (PAA, Austria), 2 mM glutamine and 100 U/mL of penicillin/streptomycin, at 37ºC in 5% CO_2 atmosphere. Periodic tests showed that cultures were *mycoplasma* free.

The procedures were adapted from the protocol previously described by T. Lozano *et al* [19]. Briefly, 20.000 cells were plated onto 96-well plates. Cells were then incubated with different concentrations of blank nanoparticles (without antigen) protamine:HA 1:4 and 4:1 (from 3.9 to 250 µg/mL) in 200 µl of RPMI 10 % FBS. After 24 or 48 hours, the plates were centrifuged at 100 x g for 5 min, 100 µl of supernatant were discarded in order to add 50 µl of Quick Cell Counting Solution and mixed gently. Subsequently, plates were incubated for 4 hours, centrifuged again and the supernatants were transferred to clean plates, to avoid possible interference due to the presence of nanoparticles. Finally, the absorbance was measured at 450 nm in an Envision multidetector

(Perkin Elmer Inc, USA). As a positive control, cells were incubated with 5% Triton X-100. As a negative control, RPMI and nanoparticles alone were used. The results were then analyzed through the following equation (equation 2):

$$\%\ \text{Cellular viability} = \frac{\text{Abs Cells} + \text{Nps} - \text{Abs Nps}}{\text{Abs Cells} - \text{Abs RPMI}} \times 100$$

2.7. Cellular uptake studies

For these studies, nanoparticles were prepared with fluorescently labeled protamine (protamine-TAMRA). Briefly, protamine was dissolved in 0.1 M sodium bicarbonate buffer, pH 9.0 at 10 mg/ml concentration. On the other hand, 5-carboxy tetramethyl rhodamine succinimidyl ester (TAMRA) was dissolved in DMSO at 1 mg/ml. An aliquot of 100 µl of this dissolution of TAMRA was slowly added to 1 ml protamine solution under stirring. This solution was incubated for 1 hour at room temperature and finally was dialyzed for 48 h, to remove free TAMRA (Slide- A Lyzer® dialysis cassette 2000 MWCO, Thermo).

Nanoparticles were then prepared with protamine-TAMRA following the same protocol described in 2.2, using protamine:HA ratios of 1:4 and 4:1.

Raw 264.7 cells (5 x 10^5) were plated onto a 24-well plate (Falcon 3047, BD Biosciences, USA) with 1 ml of RPMI 10% FBS in the presence of labeled nanoparticles (120 µg/ml, 30 min). After washing three times with PBS to remove non internalized nanoparticles, cells were observed under an inverted fluorescent microscope (IX50, Olympus Optical Co GmbH, Germany). For flow cytometry analysis, the adherent cells were washed once with PBS and then detached by incubation with Accutase® (200 µL, 10 min, 37ºC, 5% CO_2.) Finally, cells were washed with complete medium, centrifuged, and the resulting cell suspension was analyzed using a flow cytometer (FC500, Beckman-Coulter, USA).

Internalization of nanoparticles was also examined using a confocal microscope (Leica SP5, Germany). Cells were seeded on a glass coverslip (Menzel-Gläser; Braunschweig, Germany) in a 24-well plate and incubated with nanoparticles as described above. After several washes, cellular cytoskeleton was stained with Alexa Fluor 488-phalloidin (20 min incubation). Finally, the coverslips containing the attached cells were mounted over the slides in the presence of ProLong® Gold Antifade mounting medium (Invitrogen; Eugene, OR, USA) containing DAPI as nucleus stain and were analyzed by confocal microscope.

2.8. Cytokine Secretion Studies

Murine peritoneal macrophages were obtained from female BALB/c mice (6-8 weeks old). Three independent experiments were done using for each experiment a pool of peritoneal macrophages from 3 different mice. In brief, 5 mL of DMEM medium (Gibco; Grand Island, NY) containing 10% FBS were injected into the mouse peritoneal cavity and cells were then collected with a Pasteur pipette. After centrifugation at 100 x g for 5 min at 4°C, the cell pellet was resuspended in complete medium. Then 1×10^5 cells were incubated for 24 hours in the presence or absence of rHBsAg-loaded nanoparticles.. As positive and negative controls, cells were incubated with either 1 µg/mL of lipopolysaccharide (LPS) (InvivoGen, USA) or free medium, respectively. After 24 hours, supernatants were collected, centrifuged at 100 x g for 5 min at 4ºC, and stored at -20ºC before analysis. Levels of different cytokines were determined using the FlowCytomix™ assay (eBioscience, Austria) following the instructions of the manufacturer. First, Ab-coated microspheres, samples (culture supernatants) and biotin-conjugated secondary Abs were incubated for 2 hours at room temperature (RT). After several washes, streptavidin conjugated to phycoerythrin was added and incubated for 1 hour at RT. Finally, beads were studied by flow cytometry.

2.9. Immunization studies

The protocol was adapted to the guidelines of the Spanish regulations (Royal Decree 1201/2005) regarding the use of animals in scientific research and was approved by the ethical committee of the University of Vigo.

Groups of 10 female BALB/c mice with an average weight of 20 g and 4 weeks of age were randomly assigned. The animals were kept conscious during immunization and subsequent sample collection. The groups receiving intramuscular injections of rHBsAg loaded protamine:HA 1:4 and 4:1 nanoparticles and the control groups receiving alum-adsorbed antigen, were immunized with a prime dose of 10 µg of rHBsAg and a boost dose at day 28. The control alum-adsorbed antigen was freshly prepared before immunization as described by Prego *et al* [18]. In parallel, intranasal administration with 10 µg of rHBsAg loaded in Pr:HA 1:4 and 4:1 nanoparticles was also performed, with two boost doses at days 28 and 56.

Serum samples were collected from the mouse maxillary vein at days 42, 126 and 183 (weeks 6, 18 and 26). A pool of sera from mice administered with each formulation and each time point was prepared and IgG endpoint titers against rHBsAg were determined by ELISA. Briefly, rHBsAg at 5µg/mL diluted in Carbonate Buffer (pH 9.6) was incubated overnight at 4ºC in MaxiSorp 96-well plates. Then plates were blocked with PBS-BSA 1% for 1 hour at 37ºC. Serum samples and a mouse monoclonal IgG directed against rHBsAg used as control were serially diluted and incubated for 2 hours at 37ºC. Then, secondary Ab (goat anti-mouse IgG) was added to each well and incubated for 1h at 37ºC. Finally, bound antibodies were revealed with ABTS and the titers were expressed as mIU/ml concentration. For this purpose control rabbit IgG of known concentration (mIU/mL), were also serially diluted and used to transform data from µg/mL to mUI/mL. All serum samples were tested in triplicates.

2.10 Statistical analysis

The analysis of variance (ANOVA) and the Kruskal-Wallis test were performed using Statgraphic centurion XVI (Statpoint Technology). Differences were considered to be significant al a level of $p<0.05$.

3. RESULTS AND DISCUSSION

Our goal in this work has been the development of novel nanoparticulated adjuvants consisting of protamine and polysaccharides, such as HA and ALG, which are known to be safe materials. The nanocarriers developed have been carefully characterized and tested for their *in vitro* and *in vivo* activity as adjuvants, using the rHBsAg as a model antigen.

Characterization of protamine:polysaccharide nanoparticles

These nanosystems were prepared by the ionic cross-linking technique. This technique enables the spontaneous formation of nanoparticles without using high-energy forces [17, 20]. This technique was previously developed in our laboratory for the formation of chitosan nanoparticles using pentasodium tripolyphosphate (TPP) as an ionic cross-linker. Hence, our first explorative attempt was the preparation of protamine nanoparticles using TPP. These nanoparticles had a nanometric size, however, their stability was limited by their easy and rapid dissociation in aqueous media (data not shown). In order to increase the stability of this system and knowing the ability of polysaccharides to undergo a liquid-gel transition upon ionic cross-linking [21, 22], we included a negatively charged polysaccharide in the formulation. With this approach we expected to promote polymer-polymer interactions within the particle matrix and yield protamine nanoparticles with enhanced stability. For this, we explored the utility of two polysaccharides, i.e. alginate and hyaluronic acid (HA), both of them known for their bioadhesive properties and safety profile [23, 24]. In addition, HA was reported to exhibit a capacity to activate macrophages and dendritic cells via Toll-like receptor-4 and to induce of cytokine production (e.g. IL 1β, TNF α, and IL 12) [13-15].

By combining these polysaccharides with protamine, it was possible to obtain nanoparticles with a size in the range of 80-400 nm and low polydispersity (0.1),

which were stable upon storage at 4ºC for at least 3 months. In addition, it was found that their size and surface charge could be easily modulated by adjusting the protamine:polysaccharide ratio.

The additional presence/absence of TPP showed no difference (data not shown), leading finally to the elimination of this material from the formulation. Other polysaccharide-based nanoparticles have been previously prepared without using cross-linking molecules [25, 26]. These polyelectrolyte complexes made with chitosan and hyaluronic acid present high stability and low toxicity. The difference in our case is the size of the cationic polymer. While protamine has a molecular weight of 4 kDa the chitosan used has one of 150 kDa. This makes us infer that protamine may act as an efficient cross-linker in the polysaccharide reticulation and not only as a complexing agent.

In fact, the use of endogenous polyamines such as spermidine, spermine and putrescine have been claimed as cross-linking agents in the preparation of hyaluronic acid, glucomannan and other negatively charged polysaccharidic nanoparticles [27]. In our case, we suggest for the first time the possibility of protamine to act on the same conceptual basis, forming nanoparticles.

As shown in **Figure 1,** protamine:ALG nanoparticles have a size between 80 and 150 nm, depending on the polymer ratios. In contrast, the size of protamine:HA nanoparticles varied between 120 and 500 nm depending on the protamine:HA mass ratio and also on the HA molecular weight. The increase in the particle size observed for the high protamine:HA ratio might indicate that the excess of protamine could link to HA in a disordered form, hindering an efficient crosslinking of polysaccharide, resulting in the formation of less compact nanoparticles with larger size.

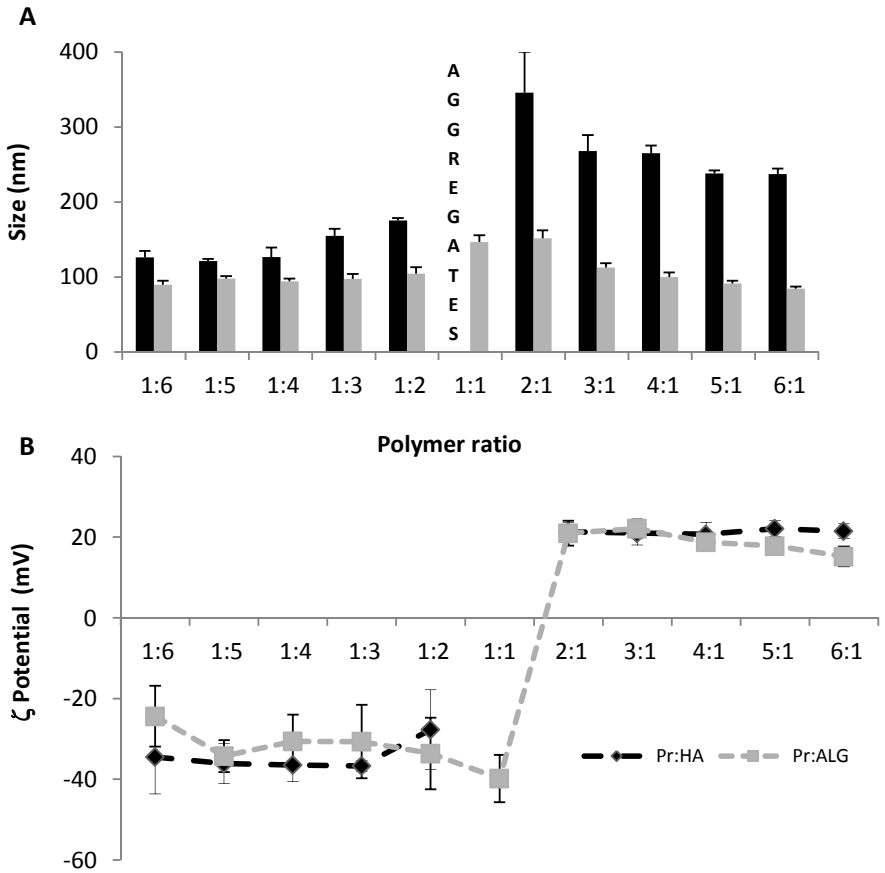

Figure 1: Mean particle size (A) and zeta potential (B) of protamine:hyaluronic acid (■) and protamine:alginate (■) nanoparticle compositions at different polymer ratios. (n>3 ± SD). HA: hialuronic acid. ALG: alginate.

On the other hand, the zeta potential values were negative when the presence of the polysaccharide in the nanoparticles was prevalent as compared to that of protamine. In fact, for the ratio of 1:1 (w/w) it was impossible to obtain nanoparticles, probably due to the balance and neutralization of opposite charges, causing aggregation followed by precipitation of the system.

After this initial screening step, we selected the 1:4 and 4:1 protamine:HA compositions for further studies. Using these two compositions we prepared nanoparticles with two different molecular weights of hyaluronic acid (i.e. 162

kDa and 29 kDa) in order to evaluate the influence of this parameter on the physicochemical characteristics of the systems.

As shown in **Table 1**, independent of the HA molecular weight, nanoparticles with a high amount of hyaluronic acid (protamine:HA 1:4) have constant size, and a negative zeta potential. In contrast, the size of the nanoparticles containing a high amount of protamine (protamine:HA 4:1) is highly dependent on the HA molecular weight. Concretely, the use of high Mw HA leads to the formation of nanoparticles that are smaller than those produced with low Mw HA. This could be due to the fact that high Mw HA has a high number of negatively charged groups, which might help achieving a more efficient cross-linking and, as a result, a smaller particle size. These results show the versatility of the systems and the possibility to modulate particle size and zeta potential simply by properly choosing the adequate HA Mw.

Table 1 also compares the size of the nanoparticles made of protamine and alginate. In this case, the size remains around 100 nm independent of the protamine:ALG ratio. In contrast, as expected, the zeta potential values were determined by the charge of the predominant component of the nanoparticles, being negative when ALG is predominant in the formulation and positive when protamine is the major component of the formulation. For the next studies, we used only hialuronic acid as polysaccharide in order to reduce the number of formulations tested and to evaluate the influence of the nanoparticles physicochemical characteristic in antigen delivery using the same raw materials.

Table 1: Mean particle size, polydispersity index (PDI), zeta potential and production yield of nanoparticles with different hyaluronic acid (HA) or alginate (ALG) molecular weights (n>3 ± SD). Pol.: Polysaccharide

Formulation protamine:pol. mass ratio	Polysaccharide Mw (kDa)	Size (nm)	PDI	ζ potential (mV)	Yield Production (%)
1:4	HA 29	132± 5	0.2	-25 ± 2	49 ± 6
	HA162	126 ± 13	0.2	-36 ± 4	50 ± 11
	ALG <75 kDa	94 ± 4	0.2	-31 ± 7	32 ± 10
4:1	HA29	513 ± 50	0.1	+20 ± 1	56 ± 12
	HA162	265 ± 1	0.1	+21 ± 3	42 ± 9
	ALG <75 kDa	100 ± 6	0.1	+19 ± 2	48 ± 12

The morphology of protamine:HA 4:1 and 1:4 particles was examined by transmission electronic microscopy (TEM). **Figure 2** shows a homogeneous population of nanoparticles with spherical shape regardless of the ratio of their components. These structures are similar to those previously observed for different cyclodextrin:chitosan or hyaluronic acid:chitosan nanoparticles [28, 29], a fact that could be related to the gelating ability of polysaccharides.

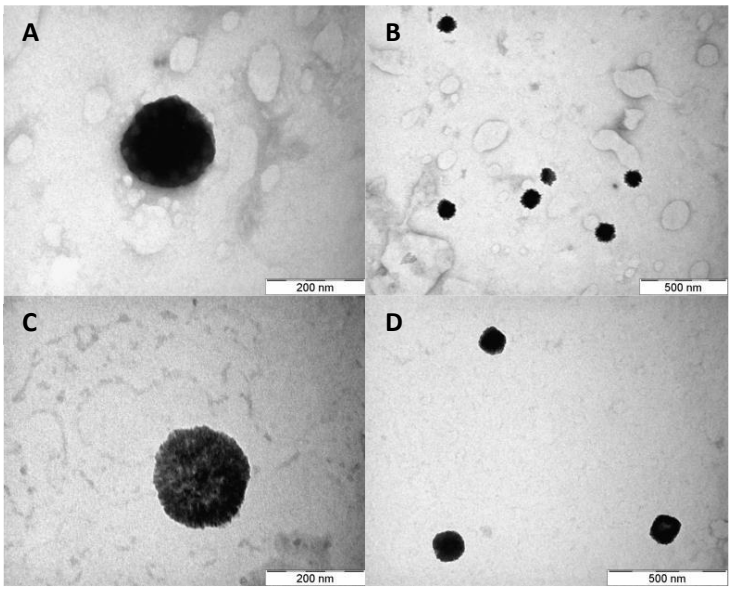

Figure 2: Electron transmission images of different nanoparticle compositions. A-B: protamine:HA 1:4 nanoparticles, C-D: protamine:HA 4:1 nanoparticles. HA: hyaluronic acid.

The final composition of the nanoparticles in terms of protamine:HA ratio, was evaluated by elemental analysis. The results indicate that the percentage of protamine and HA in the nanoparticles is quite similar (i.e. 48:52 % vs 51:49 %) for both theoretical polymer ratios of protamine:HA (1:4 and 4:1) (**Figure 3**). This could be explained by the complete incorporation of the minority component (either protamine or HA) in the nanoparticles until an optimal polymer ratio. However, the excess of the other component is necessary and clearly influences the self-assembly process and the physicochemical characteristics of the resulting nanoparticles. Indeed, we have observed that the use of a 1:1 polymer ratio leads to the formation of macroscopic aggregates, thus corroborating the fact that the excess of a component (i.e. either HA or protamine) is critical for adequate polymer assembling. A similar pattern was observed in the formation process of chitosan-cyclodextrin nanoparticles [28], whereby the use of different ratios of chitosan and cyclodextrin did not affect considerably their final composition.

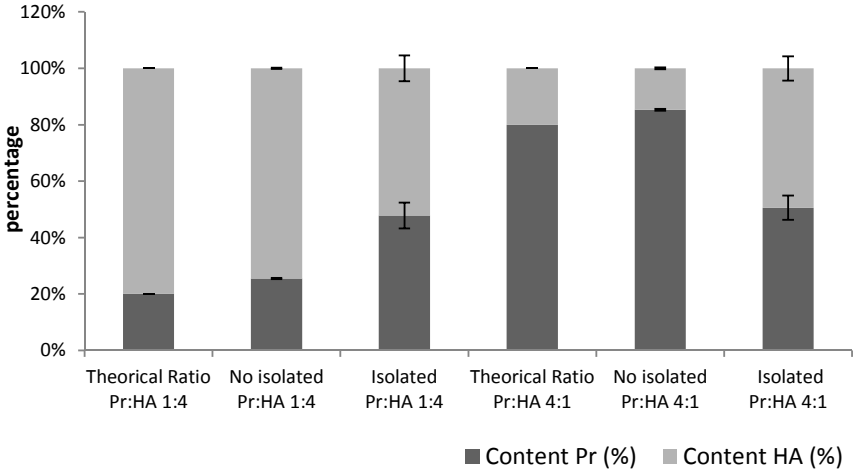

Figure 3: Elemental analysis studies of protamine:HA nanoparticles at 1:4 and 4:1 ratio. (n>6 ± SD). ■: % protamine, ■: % hyaluronic acid: HA.

The nanoparticles yield also confirms the efficient incorporation of both components in the nanosystem and these values are not contradictory with the results obtained by elemental analysis. Additionally, the yield results might indicate that the minor component is completely incorporated in the nanostructure. These results show that it is possible to prepare similar core nanocompositions with sizes and/or different surface characteristics (as shown in **Table 1**).

Stability of nanoparticles

The stability of protamine:HA 4:1 and 1:4 nanoparticles upon incubation in biological media (PBS at 37 ºC) and during storage at 4ºC was monitored by analyzing the evolution of mean particle size with time. The results indicated that the size of the nanoparticles remains constant upon incubation in PBS at 37ºC for up to 7 days. In addition, nanoparticles stored at 4ºC remained stable for at least 3 months. Overall, from these results it maybe concluded that protamine:HA nanoparticles have a very positive stability profile. In particular, the stability profile in PBS is quite exceptional if we take into account that these nanoparticles are formed by a simple ionic cross-linking process. Other

protamine based carriers, such as protamine-albumin-oligonucleotide nanoparticles, have also shown an adequate stability profile upon incubation of high ionic strength media) [30]. However, this is not the case for other nanostructures combining polycations such as polyarginine and HA [31]. This underlines the necessity to properly assess the colloidal stability of nanoparticles under physiologically relevant conditions since this property cannot be effectively predicted.

Antigen encapsulation efficiency

For the entrapment of rHBsAg within the nanoparticles, the antigen was previously mixed with hyaluronic acid at theoretical loadings of 2.5 and 5%, with respect to the total amount of solid material (protamine + hyaluronic acid) and, then, added to protamine solution. As shown in **Table 2**, the encapsulation of rHBsAg inside the nanoparticles is high, irrespective of the protamine:HA polymer ratio. Nevertheless, some differences between the two different NP compositions could be observed. The particle size increased slightly in the case of nanoparticles with a high amount of hyaluronic acid, while in the case of nanoparticles protamine:HA 4:1 e the size, this increase is more remarkable, around 300 nm. This difference could be explained by the distinct structural organization of nanocarriers made of different polymer ratios.

The surface charge was unmodified with respect to the nanoparticles without antigen, thus suggesting that the rHBsAg might be efficiently entrapped within the nanoparticle matrix. In order to confirm that the integrity of the antigen is not affected by the encapsulation process, rHBsAg-loaded protamine:HA 1:4 and 4:1 nanoparticles were degraded with hyaluronidase and the extracted antigen was analyzed by Western blot. Results indicate that the antigenicity of the antigen was preserved during the particle preparation process. Similar results were found for chitosan nanoparticles prepared by ionic cross-linking and the same antigen (rHBsAg) [18]. This protective effect is in fact considered

a key advantage of these nanocarriers with regard to their ability to entrap delicate molecules, i.e. proteins.

Table 2: Physicochemical properties of rHBsAg loaded nanoparticles and their encapsulation efficiency (n>3 ± SD).

Formulation Pr:HA mass ratio	rHBsAg Theoretical Loading (%)	Size (nm)	PDI	ζ potential (mV)	Encap. effic. (%)	rHBsAg Loading (µg/ml)
1:4	-	126 ± 13	0.1	-37 ± 4	-	-
	2.5	146 ± 11	0.1	-31 ± 5	90 ± 3	20.0
	5	197 ± 10	0.1	-31 ± 3	92 ± 1	41.1
4:1	-	265 ± 10	0.2	+21 ± 3	-	-
	2.5	693 ± 29	0.2	+26 ± 1	95 ± 3	21.2
	5	563 ± 38	0.2	+23 ± 2	90 ± 2	40.2

Freeze-drying studies

Despite having no information about the nanoparticles stability at room temperature in aqueous suspension, there is no doubt that preserving this stability would represent a major advance for their use as antigen carriers. Thermostable vaccines would have a particularly great impact in developing countries, where the lack of equipment for vaccine storage at 4ºC and the maintenance of the cold chain still remains a critical limitation.

Taking this into account, in the present work, we chose freeze drying as a strategy to increase the stability of vaccine formulations [32]. Nanoparticle formulations were lyophilized at different concentrations, in presence or absence of cryoprotectors (detailed in 2.6) and, then reconstituted for further particle size analysis. The results indicated that the size of the nanoparticles did not suffer significant changes upon freeze-drying and reconstitution. The fact

that these nanoparticles do not need any cryoprotective agent for their freeze-drying represents a specific advantage of this technology. The ease of freeze-drying of the nanocarriers could be attributed to the arginine rich structure of protamine, since this amino acid is used as a stabilizing excipient to prevent damage to the vaccine during the drying process [33].

In addition to these positive results, it was also possible to confirm by Western blot analysis that the antigenicity of the freeze-dried antigen associated to protamine:HA nanoparticles was preserved. Therefore, we can conclude that the association of rHBsAg to protamine:HA nanoparticles represent a useful strategy for designing a powder-based vaccine formulation.

Cell viability and nanoparticles internalization studies

The toxicity of protamine:HA 1:4 and 4:1 formulations was evaluated in a macrophage cell line (RAW 264.7). For this purpose, RAW 264.7 cells were incubated with blank nanoparticles and the metabolic activity of the cells was monitored by Quick cell counting solution (QCCS). As shown in **Figure 4**, cells maintain their viability upon incubation with nanoparticle concentration within the range of 3.9 – 62.5 µg/mL. Interestingly, despite the presence of nanoparticles, the cell viability increased over the time. This might be due to the fact that after 48-hrs exposure, cells are able to adapt to the presence of the nanoparticles and their viability is no longer affected. These results are similar to those reported for hyaluronic acid:chitosan nanoparticles, the positive features being attributed to the high biocompatibility of the polymers and its potential implications in biological process such as cellular regeneration [34].

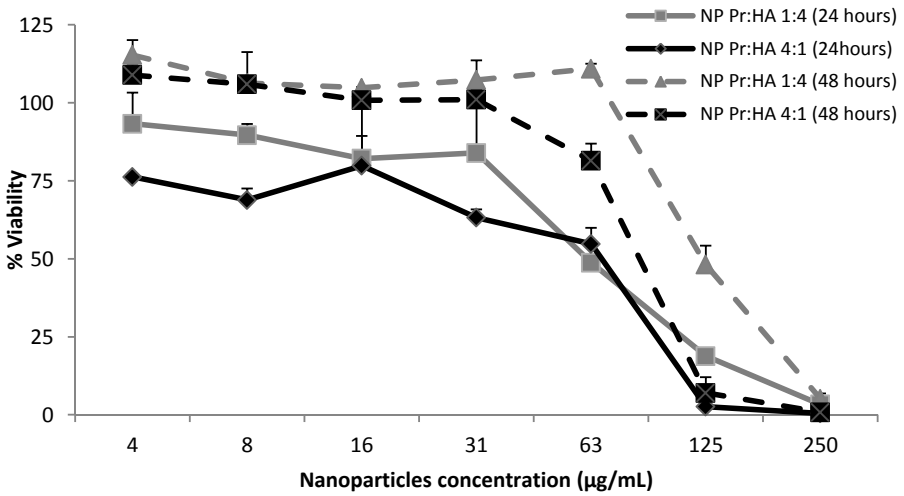

Figure 4: Effect of nanoparticles formulations on the viability of Raw 264.7 cells determined by QCCS assay. (n=3 ± SD)

The process of internalization of the nanostructures in antigen presenting cells is a crucial step to trigger the immune response. In fact, the recognition and consequent phagocytosis of pathogens by antigen presenting cells is the first step during innate immune responses, allowing the development of an adaptive immune response [35]. Therefore, in order to study the possible interaction of the developed formulations with immune cells, we examined their internalization by RAW 264.7 cells using confocal microscopy. For this study, nanoparticles were prepared with TAMRA conjugated fluorescent protamine. As confirmed by PCS and laser-Doppler anemometry analysis, the use of the protamine-TAMRA conjugate did not alter the physicochemical characteristics of formulations. As illustrated in **Figure 5**, at 30 minutes post-incubation the nanoparticles were efficiently internalized by the cells. Complementary cytometry analysis showed that nanoparticles with a positive charge exhibit a higher uptake than those with a negative charge. This can be attributed to a non-specific uptake mediated by the electrostatic interaction between the positively charged nanoparticles and the negatively charged cell membranes [36] as well as to the inherent penetration properties of protamine [37].

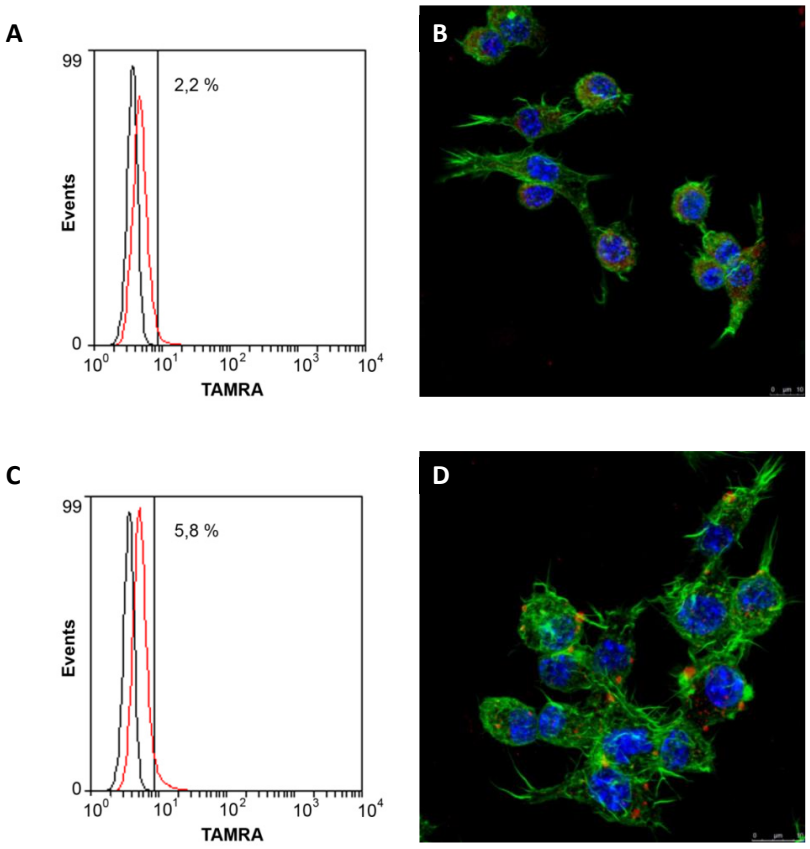

Figure 5: Cellular uptake profile of protamine:HA 1:4 and 4:1 nanoparticles. A and B show the flow cytometry analysis and a confocal image of Protamine:HA 1:4 nanoparticles, respectively. C and D show the same data obtained for protamine:HA 4:1 nanoparticles. In the cytometry analysis (A and C), the black curve corresponds to control cells and the red curve represents the cells treated with the internalized labeled nanoparticles. The percentages are related to the number of positive events. The systems were prepared with protamine-TAMRA conjugate (red channel). The cytoskeleton of RAW 264.7 cells was labeled with Alexa Fluor 488-phalloidin (green channel) and the nuclei with DAPI (blue channel).

Cytokines secretion studies

Cytokines have an important role linking innate and adaptive immune responses. Antigen presenting cells (APCs) recognize pathogens that induce their activation and maturation with the secretion of proinflammatory cytokines, among other processes [38]. These cytokines are multifunctional

proteins that are mostly involved in the regulation of immune responses, hematopoiesis and inflammation. In particular, IL-1α has an important role in T and B cell growth and differentiation, whereas TNF α, has an important tumoricidal activity and participates in the growth and differentiation of B cells. As a consequence, cytokines secretion capacity does normally relate to the adjuvant properties of specific compounds [39, 40].

To investigate the adjuvant capacity of protamine:HA nanoparticles, we analyzed their cytokine secretion profile by immunofluorescence in murine macrophages. The results showed that rHBsAg loaded nanoparticles stimulated greater production of TNFα, IL-1α and IL-6 than the free antigen **(Figure 6).** Apparently, negatively charged nanoparticles, despite their lower internalization, exhibited a greater IL-1α production activity ($p<0.05$). This could be explained by the differences in size and structural organization of the materials, and their possible internalization through different pathways. For example, while the internalization through scavenger receptors does not always induce an inflammatory response, the internalization through other routes (Fcγ, mannose or complement receptor-mediated) in most cases produces an inflammatory response [41].

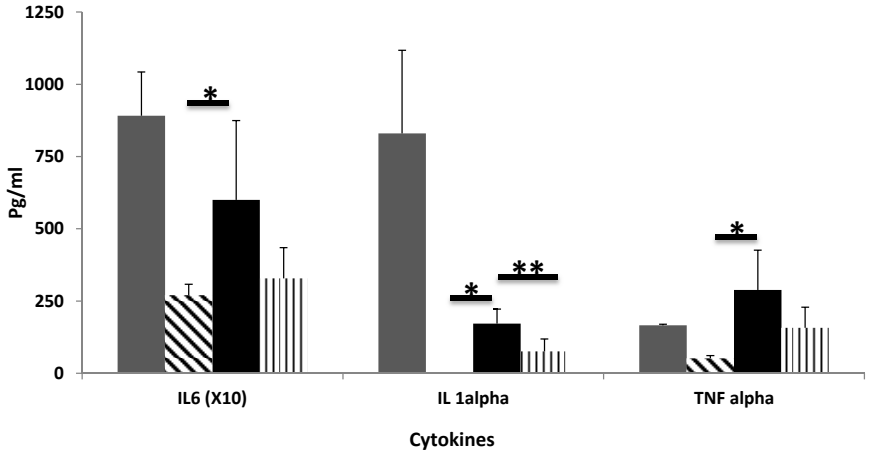

Figure 6: Cytokines released from macrophages after stimulation with rHBsAg alone (▨) or loaded into protamine:HA 1:4 (■) and protamine:HA 4:1 nanoparticles (▥). ■: Positive control: LPS. * Significant differences between groups ($p<0.05$).

Irrespective of this small difference in the cytokine release profile, the results suggest overall that both types of nanoparticles might exhibit potential adjuvant properties [42].

Immunization studies

In order to evaluate the potential of protamine-based nanoparticles as antigen delivery carriers, we chose hepatitis B as a model antigen and monitored the IgG responses following either intramuscular or nasal administration to mice. For intramuscular immunization we administered 10 µg of rHBsAg associated to the nanoparticles, at day 1 and day 28. As a positive control, we used alum-adsorbed rHBsAg administered according to the same protocol. The results in **Figure 7A** indicate that both protamine:HA nanoparticles 4:1 and protamine:HA 1:4 nanoparticles induce significant serum IgG antibody levels (> 100 mUI/mL). Although these antibody titers are slightly lower than those induced by the conventional adjuvant (alum), these values are all above those considered to be protective in humans (10 mUI/mL) [43]. Significant differences between the two nanoparticles formulations were only found at the first bleeding (42 days

post administration), being the formulation with positive zeta potential and higher internalization rate the one inducing higher specific IgG levels. This underlines the importance of the surface charge of the nanoparticles, thus confirming previous data in the literature where cationic liposomes [44] and cationic chitosan nanocapsules [45] produced stronger immune responses than the corresponding neutral nanostructures.

Overall, the adjuvant effect of protamine:HA nanoparticles is in agreement with their cytokine production profile showed previously and the protective levels achieved *in vivo*, although the response triggered by this antigen adsorbed in alum was higher. This may be related to the well-known hyper-responsiveness of mice to this antigen when inoculated with rHBsAg using alum as adjuvant.

We also investigated the potential of protamine:HA nanoparticles for nasal vaccination. In this study, we used 3 doses of 10 µg rHBsAg loaded in protamine:HA 4:1 or 1:4 (days 0, 28 and 56). The resulting antibody response is shown in **Figure 7B**. In contrast to what was found following i.m. administration, in this case we could see clear differences in the responses elicited by the different nanoparticle compositions. While the protamine:HA 1:4 formulation failed to elicit immune responses intranasally, the protamine:HA 4:1 , achieved relatively high specific IgG levels (with a peak of 80mUI/mL at week 18).

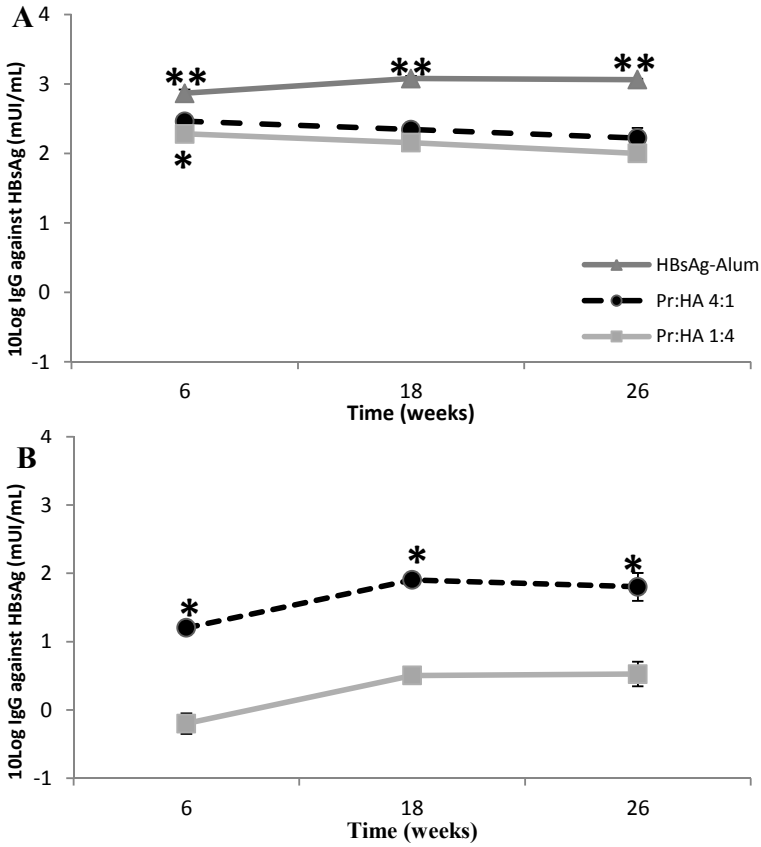

Figure 7: Serum antibody titers of BALB/c mice immunized with protamine:HA 1:4 and protamine:HA 4:1 nanoparticles. (A) After 2 immunizations (0 and 28 days) with rHBsAg-loaded nanoparticles or alum-adsorbed antigen through intramuscular (i.m.) administration. (B) After 3 immunizations (0, 28 and 56 days) with rHBsAg loaded into protamine:HA 1:4 or 4:1 nanoparticles by intranasal (i.n.) administration. In both cases the rHBsAg dose administered was 10 µg/mice. Results are presented as mean ± SD ($p<0.05$).

Similar levels of IgG have been reported with other cationic polymers such as chitosan in form of nanoparticles loaded with rHBsAg and administered by nasal route [18]. In this field, it is worth mentioning the work of Vicente *et al* [47], where chitosan nanocapsules loaded with the rHBsAg and imiquimod (TLR 7 agonist) trigger a higher immune response when compared with chitosan nanoparticles or the systems described in this work. Further studies loading the antigen together with other adjuvants (such as a TLR agonist) in protamine-

polysaccharide nanoparticles could allow knowing the potential of our systems for needle-free vaccination with hepatitis B antigen.

All these results show the versatility of the developed nanoparticle formulations based on protamine, their interaction with immunocompetent cells and their capacity to trigger a protective immune response against the antigen used as model.

4. CONCLUSIONS

Protamine:polysaccharide nanoparticles can be prepared using an easy and mild ionic cross-linking technique. These nanoparticles were able to associate significant amounts of rHBsAg without affecting its immunogenicity and they show high stability in physiological conditions and under long-term storage. Their adjuvant effect has been tested through the enhancement of different cytokines secretion and the protective levels achieved *in vivo*. These findings indicate the interest for the further development of this nanocarrier composition for intramuscular administration and/or a needle-free vaccine formulation.

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CAPÍTULO 4

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PROTAMINE NANOCAPSULES: A NEW PLATFORM FOR ANTIGEN DELIVERY

Este trabajo ha sido realizado en colaboración con Inmaculada Dalmau-Mena[1], Susana Martínez-Pulgarín[2], Covadonga Alonso[1,2] y José Ángel Escribano[1,2].

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ABSTRACT

Here we report for the first time a new antigen-drug nanocarrier consisting of an oily core of Miglyol or α tocopherol, and a shell made of protamine, stabilized with pegylated surfactants. This nanocarrier was rationally designed with the final goal of promoting the intracellular delivery of antigens to the immunocompetent cells and to trigger an efficient and long-lasting immune response.Protamine nanocapsules were prepared by the solvent displacement technique and characterized for their size, surface charge and the amount of polypeptide forming the shell around the oily core. They were also studied for their ability to associate influenza H1N1 antigen, which was chosen as a model antigen. The nanometric systems developed presented an average size of 250 nm, positive zeta potential and a great association capacity without loss of the antigen structure. The nanocapsules were stable upon storage at 4ºC in the form of an aqueous suspension for up to 3 months. They could also be freeze-dried and resuspendend without the need for the use of cryoprotectants. In addition, *in vitro* studies showed that protamine nanocapsules were efficiently internalized by macrophages without exhibiting any signs of toxicity below the dose of 76 μg/cm^2. Finally, the results of the *in vivo* studies indicated that protamine nanocapsules loaded with influenza antigen trigger an immune response that was comparable to the one achieved using alum as adjuvant. Their versatility for the loading of different antigens and oily immunomodulators together with their stability profile, and adjuvant properties render these nanocapsules as a novel platform for antigen or active molecules delivery.

1. INTRODUCTION

Different kinds of active molecules, either for solubility, degradation or uptake problems, need to be administered with delivery systems that can improve their properties and allow them to achieve their target for a successful therapy. The emerging of new nanosystems tries to solve the current deliver challenges of these complex active molecules.

This approach has been used in the immunization area for antigen delivery. In fact, the term nanovaccine has been recently coined to describe nanometric sized carriers studied and used in vaccine development [1]. Using different strategies, it is intended that this nanovaccines can be a new generation of vaccines that act as adjuvants, administered in a single dose, thermostable and/or administered via mucosa allowing a needle-free vaccination approach

Recently, our research group has published an innovative and interesting work about chitosan nanocapsules as nanovaccine. These nanocapsules consist of an oily core surrounded by a chitosan coating and allowed the efficient association of antigens in combination with immunostimulants. These chitosan nanocapsules have shown to be an adequate strategy for single-dose immunization against a hepatitis B and for improving vaccine stability through the production of a freeze-dried forme [2]. Based on these promising results and with the aim of further exploring the potential of polymeric nanocapsules for vaccine delivery, in the present work we have explored the feasibility to develop a new nanocapsules platform based on a natural cationic polypeptide, protamine.

The name protamine refers to a diverse family of aliphatic and strongly basic arginine-rich proteins (**Figure 1**), with average molecular mass of 4.5-5.5 kDa [3]. These proteins are synthesized in nature during the late stage of spermatid development in different animals or plants with the function of condensing

their genetic material [4]. In addition to this main physiological role, the repetitive sequence of arginine present in protamine (about 70%) also provides this polypeptide with an efficient translocation activity through biological membranes [5]. Protamine is an FDA approved drug for reverting the anticoagulant effects of heparin and is also used as an excipient in the formulation of NPH (Neutral Protamine Hagedorn) insulin to prolong its action. Additionally, it has been studied as a protein stabilizer [6], conjugated with enzymes to improve their internalization [7] and as an antibacterial agent [8].

1)Pro-**Arg-Arg-Arg-Arg-Arg-**Ser-Ser-Ser-**Arg-**Pro-Ile-**Arg-Arg-Arg-Arg-Arg-**Pro-**Arg-**Ala-Ser-**Arg-Arg-Arg-Arg-Arg-Arg-**Gly-Gly-**Arg-Arg-Arg-Arg.**
2) Pro-**Arg-Arg-Arg-Arg-**Ser-Ser-**Arg-Arg-**Pro-Val-**Arg-Arg-Arg-Arg-Arg-**Pro-**Arg-**Val-Ser-**Arg-Arg-Arg-Arg-Arg-Arg-Arg-**Gly-Gly-**Arg-Arg-Arg-Arg-Arg.**

Figure 1: Primary structure of the two principal polypeptides of protamine from chum salmon.

Protamine and its low molecular weight derivative are frequently used in gene therapy to improve intracellular the delivery of genetic material enhance gene expression [9-11]. In addition, protamine has also been investigated recently as biomaterial in drug delivery applications due to its non-toxic, biocompatible nature and its safety profile [12, 13]. For example, protamine has been used as composing polymer of innovative nanocarriers such as liposomes [14, 15], nanoparticles [16, 17] or layer-by-layer nanocapsules [18, 19].

In the immunization field, there are recent evidences that nanoparticles or microparticles associated or decorated with protamine show higher immune responses when compared with the systems without this component [20, 21]. Moreover, protamine complexes with mRNA, stimulating TNF-α and INF-α secretion in human blood cells and directly activating B cells [22] suggesting that Toll-like receptors could be recognizing this complex and can be acting as adjuvant.

With this previous information in mind, the main goal of this work has been to design and develop an original carrier consisting of an oily core stabilized with one or more surfactants and surrounded with protamine shell. The versatility of such nanocapsules allows the incorporation of different lipidic materials and oils in their core. Taking advantage of this, the use of oils with adjuvant properties such as α tocopherol (vitamin E) combined with a protamine shell emerges as an interesting strategy to combine the properties of both structural elements (shell and core) and maximize the adjuvant capacity of the developed nanovaccine.

These new carriers were characterized with respect to their physicochemical properties and their capacity to associate the model antigen (influenza haemagglutinin) was also evaluated. The optimized carriers were tested for their interaction with macrophages and an *in vivo* study was also performed as a proof-of-principle of their capacity to trigger immune responses against the associated antigen. In summary, this is the first report about protamine nanocapsules intended to describe the rational design of these carriers and demonstrate their potential use as a new platform for antigen or active molecules delivery.

2. MATERIALS AND METHODS

2.1 Chemicals

Protamine sulphate was kindly donated by LEO Pharma (Denmark). PEG-Stearate (Simulsol® M52) and Macrogol 15 hydroxystearate (Solutol® HS 15) were obtained of Seppic (France) and BASF (Germany) respectively. Miglyol® 812 was a kind gift from Sasol Germany GmbH (Germany) and α-tocopherol (TCPH) was supplied by Merck (Germany). 5-Carboxytetramethylrhodamine succinimidylester single isomer (TAMRA) and Alamar blue were obtained from Invitrogen (United Kingdom). Haemagglutinin from the H1N1 A/PR/8/34 influenza antigen (HI) was kindly offered by Algenex S.A (Spain). Antibodies for Western blot, mouse monoclonal antibodies against the terminal His tag domain of HI and goat anti-mouse IgG-HRP were supplied by Abcam pcl (United Kingdom). Dulbecco's Modified Eagle's Medium (DMEM), glutamine, penicillin and streptomycin were supplied by Gibco (USA). Tween® 20, Tween® 80, d-α tocopheryl polyethylene glycol 1000 succinate (TPGS), glucose, trehalose, Triton-X100, WGA (wheat germ agglutinin), PBS and sodium cholate were obtained from Sigma-Aldrich (Spain). All other products used were of high purity or reagent grade.

2.2 Preparation of protamine nanocapsules

Blank protamine nanocapsules were prepared by the solvent displacement technique following the procedure described and optimized by our group [23]. Different amounts of PEG-stearate (Simulsol® M 52) (0, 12, 24 and 48 mg) and sodium cholate (0, 5, 10 and 20 mg) were dissolved in 750 µl of ethanol, followed by the addition of Miglyol® (31.25, 62.5 or 125 µl) and 4.25 ml of acetone. This organic phase was immediately poured over 10 ml of an aqueous phase with 0.05% w/v protamine (one step procedure). The elimination of organic solvents was performed by evaporation under vacuum (Rotavapor Heidolph, Germany), to a constant volume of 5 ml. For the nanocapsule

preparation method in two steps, we used the same materials and amounts indicated but omitting protamine in the external aqueous phase. The resulting nanoemulsion (NE) was incubated in a subsequent step with a protamine solution (0.5 % w/v). The volume ratio used was 4:1 (NE:protamine solution).

Through the same solvent displacement technique, nanocapsules were also prepared using different oils (Miglyol vs tocopherol) in combination with others surfactants such as Solutol® HS 15, Tween® 20, Tween® 80 or TPGS with sodium cholate. The ratio between TCPH and the main surfactant used was 12:12, 60:12 and 60:60 (mass ratio).

Protamine nanocapsules were isolated by ultracentrifugation (Optima™ L-90K, Ultracentrifuge, Beckman Coulter, USA) at 30000 RPM for 1h (at 15ºC) and, then, resuspended in ultrapure water to a final theoretical protamine concentration of 1 mg/mL.

2.3 Physicochemical characterization of protamine nanocapsules

The hydrodynamic diameter and polydispersity index of the systems were determined by photon correlation spectroscopy (PCS) (Zetasizer®, NanoZS, Malvern Instruments, Malvern, UK), after sample dilution with ultrapure water. Zeta potential was measured by laser-Doppler anemometry after diluting the samples with 1 mM KCl. Morphological examination of nanocapsules was carried out by transmission electron microscopy (TEM, CM12 Philips, The Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid solution.

2.4 Quantification of protamine association to the nanocapsules

The amount of protamine associated to the nanocapsules was indirectly measured through the quantification of the polypeptide remaining in the aqueous medium after the isolation of the blank nanocapsules by

ultracentrifugation (described in 2.2). Protamine was quantified by Ultra Performance Liquid Chromatography (Acquity UPLC, Waters, Spain). The column Acquity UPLC® BEH C18 1.4 µm 2.1X50 mm was used with flow rate of 0.3 ml/min and the following gradient: 0-2 min, 100% A; 2 – 2.1 min 70% A; 2.1-6 min 100 % A. The column was maintained at 35ºC. Solvent A was trifluoroacetic acid 0.1% (v/v) and solvent B was acetonitrile with 0.1%TFA (v/v). The detector wavelength was set at 214 nm.

2.5 Association of influenza antigen to the nanocapsules and determination of its structural integrity

The association of influenza antigen (HI) as model antigen was performed using isolated protamine nanocapsules (section 2.2). These nanocapsules were incubated with different amounts of antigen, in 6:1, 4:1 and 2:1 mass ratios, in equal volumes. Incubation was performed at room temperature for 1 hour.

The ability of protamine nanocapsules to associate the antigen was measured by an indirect method by Western blot as described Dickinson *et al* [24]. Loaded nanocapsules were isolated by ultracentrifugation (section 2.2) and the aqueous solution was analyzed. For this purpose, samples were reduced with β-mercaptoethanol and loaded onto a 4% acrylamide stacking gel. Different concentrations of HI were treated in the same way and loaded onto the same gel. Samples were then subjected to electrophoresis on a 10 % separation gel at 200V (Bio-Rad, USA). Antigen and samples for the calibration curve were transferred to a PVDF (polyvinylidene fluoride) membrane at 90V for 90 min. Membranes were soaked subsequently for 1 hour with non-fat milk 5% (w/v) and incubated for another hour in the presence of the primary antibody. After the incubation process, the membranes were carefully washed and the secondary antibody was added, followed by incubation for an additional hour. Finally, the membranes were washed and the antibody–antigen complexes were visualized by chemiluminescence using the ECL Plus Western Blotting

Detection Reagents (Amersham Biosciences, UK) in UVP imaging system (EC3™ Imaging System, UVP, USA). The analysis of the bands was performance using ImageJ software (NIH; USA).

On the other hand, Western blot assay also allowed the determination of the antigen's structural integrity when associated to the nanocapsules. For this purpose, the isolated nanocapsules loaded with HI were treated in the same way as the remaining dissolution after their isolation and the Western blot technique was applied as explained above.

2.6 Stability of protamine nanocapsules upon storage

The stability of the nanocapsules in suspension was evaluated by monitoring the evolution of particle size during storage at 4ºC for up to 3 months and also upon incubation in PBS at 37ºC for up to 7 days. Particle size analysis was carried out as indicated in section 2.3.

2.7 Freeze-drying of protamine nanocapsules

Different concentrations of protamine nanocapsules (1, 0.75, 0.5 and 0.25 % w/v) were lyophilized (Labconco Corp, USA) in presence/absence of trehalose or glucose at 5 or 10% (w/v). Samples were frozen at -20 ºC and then subjected to an initial drying step at -35ºC followed by a secondary drying at 0ºC, both steps lasting for 24 hour at a high vacuum atmosphere. Finally the temperature was slowly increased up to room temperature till the end of the process.

The freeze-dried formulations were resuspended with ultrapure water by manual resuspension and their physicochemical characteristics were evaluated as mentioned in section 2.3.

2.8 Study of cytotoxicity of protamine nanocapsules

Raw 246.7 cells (ATCC, USA) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) of penicillin-streptomycin at 37ºC in

a humidified atmosphere containing 5% carbon dioxide. Previous to the experiment, $1x10^5$ cells per well were seeded and incubated for 24 hours. Different concentrations of antigen-loaded protamine nanocapsule (between 1212, - 9 µg/cm^2) were incubated with the cells. As positive control, cells were incubated with Triton 100X (1:10 dilution in DMEM) and as negative control, untreated cells were used. After 4 hours of incubation, the medium was removed together with the non-internalized NC and replaced with fresh DMEM. At 24 hours, the medium was eliminated and 100 µl of Alamar blue diluted in DMEM (1:10) were added. This was incubated for 3 hours and the fluorescence was quantified by (Ex: 530 nm and Em: 590 nm). The same procedure was repeated at 48 hours.

2.9 Study of internalization of protamine nanocapsules

For the uptake studies, fluorescent nanocapsules were prepared with TAMRA labeled protamine (Pr-TAMRA). For the fluorescent labeling, protamine was dissolved in 0.1 M sodium bicarbonate buffer (pH 9.0) and TAMRA was slowly added under stirring. After one hour of incubation at room temperature, the labeled protamine was dialyzed for 48 hours for to remove the free TAMRA (Slide-A Lyzer® dialysis cassette 2000 MWCO, Thermo). Following this, the nanocapsules were prepared according to the procedure described in 2.2.

For uptake studies we used two different cell lines. Vero cells (monkey kidney epithelial cells), were obtained from ATCC (CCL-81) and grown in a controlled manner at 37ºC, 5% CO_2 atmosphere in DMEM supplemented with 5% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. The second cell type was a primary culture of alveolar macrophages from pig lungs labeled with a surface lectine (WGA (wheat germ agglutinin) in a dilution of 1:200 in PBS.

Cells ($1x10^5$) were plated in 35 mm plates and left overnight. Fluorescent protamine nanocapsules were incubated at a concentration of 60 µg/ml (diluted in DMEM) for 30 minutes at 37ºC. Then the cells were washed and analyzed by TCS SP2 confocal microscopy (Leica, Germany).

2.10 Immunization studies using HI-loaded protamine nanocapsules

Female BALB/c mice were purchased from Harlan (Spain). The animals were provided with food and water ad libitum, and environment with constant temperature (22ºC) and 12h light and 12h dark cycles. The protocol was adapted to the guidelines of the Spanish regulations (Royal Decree 1201/2005) and approved by the ethical committee of the "Instituto Nacional de Investigación y Tecnologías Agrarias y Alimentarias" (INIA).

Mice were randomly distributed in groups of 5 animals and were immunized with three doses of protamine nanocapsules (weeks 0, 3 and 5) by the subcutaneous route. The doses were either 7.5 or 2 µg of HI per mice. As a control, the same doses adsorbed on alum were used (same schedule). Blood samples were collected before the second and third immunizations (weeks 3 and 5) and at weeks 7 and 28. Humoral immune response was evaluated by ELISA using serum dilution (from 1:100 to 1:12800) and anti-mouse IgG-HRP as secondary antibody. Plates were read at 405 nm (Multiskan EX, Thermo Electron Corp., Finland)

2.11 Statistics

The experimental design and Kruskal-Wallis analysis were performed with Statgraphic centurion XVI (Statpoint Technology). Differences were considered significant at a level of $p<0.05$.

3. RESULTS AND DISCUSSION

This work describes the development of protamine nanocapsules, a new nanocarrier designed as a generic platform for the delivery of bioactive molecules (peptides, proteins or DNA) and/or lipophilic molecules. The rational design is focused in the possibility of overcoming cellular or mucosal barriers, which makes them interesting and appropriate carriers for gene therapy or for a vaccination approach, allowing the delivery of antigens, DNA, ODN, siRNA or other active molecules to target sites.

The developed nanocapsules form a core-corona structure in which the active substances can either be confined into an inner reservoir or adsorbed onto the shell [25]. In addition, the oily core allows the incorporation of oils with potentially interesting properties. The components chosen for the external shell may also facilitate the interaction and internalization of the carrier with cells and may improve its stability in biological media [26].

For the proof of concept on the potential of these nanocarriers for antigen delivery, we chose haemagglutinin from the H1N1 A/PR/8/34 influenza (HI) as a model viral protein antigen. Despite its use as a model in this case, influenza is a disease that affects millions of people, with severe morbidity and mortality affecting mainly newborn, elderly and immunocompromised patients and therefore it is an actual research interest in terms of vaccination and treatment.

Development and characterization of protamine nanocapsules

In the first step of the rational design of protamine nanocapsules, we selected the solvent displacement technique as a mild and scalable preparation technique. As described in the literature, in this method, a phospholipidic surfactant, typically lecithin, is used for the stabilization of nanoemulsions and nanocapsules [27]. Unexpectedly, this type of surfactants was found to be incompatible with our approach, since phase separation and formation of

aggregates occurred when preparing the nanocapsules, independently of the oils, co-surfactants or solvents used in the formulation. These findings, together with fact that corresponding nanoemulsions (same composition except protamine), present nanometric size with low polydispersity index (**Table 1**) led us to the conclusion that a specific interaction of protamine with the phospholipids might be responsible for the aggregation.

Table 1: Physicochemical characteristics of protamine nanocapsules and the corresponding control prepared with different oils in the core using one-step or two-step methodologies. The characteristics of the nanocapsules labelled with TAMRA and those of nanocapsules loaded with influenza antigen H1N1 (HI) are also presented. TCPH: α tocopherol and TAMRA: 5-Carboxytetramethylrhodamine succinimidylester single isomer.

Formulation	Core composition	Size (nm)	PI	Potential (mv)	HI assoc. (%)
Nanoemulsion	Miglyol	270 ± 12	0.2	-27 ± 8	
Pr-NC (two steps)		265 ± 6	0.2	+19 ± 2	
Pr-NC (one step)		277 ± 5	0.2	+21 ± 5	
Nanoemulsion	TCPH	235 ± 9	0.2	-38 ± 1	
Pr-NC (one step)		241 ± 10	0.2	+34 ± 3	
Pr-TAMRA NC		226 ± 7	0.2	+39 ± 2	
Pr-NC:HI (4:1 mass ratio)		205 ± 10	0.1	+13 ± 2	71 ± 10

In fact, this destabilization of O/W emulsion by flocculation was described by Chargaff in the presence of protamine and phospholipids. Authors attributed this phenomenon to a high binding affinity between protamine and the negative domains of the phospholipid [28, 29].

As alternatives to phospholipids, the first selection criteria of possible surfactant was its HLB value, ideally higher than 8 for the stabilization of our primary O/W nanoemulsion. In addition, we preferably chose surfactants having polyethylene glycol (PEG) in their structure to take advantage of the characteristics of this polymer such as the improvement of the nanocarriers

stability in biological media and the avoidance of unspecific opsonisation [30, 31].

The development of protamine nanocapsules was based on an experimental design. As surfactants, we employed PEG-stearate (with 40 unit of PEG in their structure) alone or with sodium cholate. For PEG-stearate, we used 0, 12, 24 and 48 mg, combined with 0, 5, 10 and 20 mg of sodium cholate (2 levels with 4 factors). With respect to the oil component, we used Miglyol® in 3 different amounts: 31.25, 62.5 and 125 µl. The protamine concentration in the external aqueous phase was constant in all experiments (0.05 w/v). Using statistic-based design, we studied the influence of these parameters on the nanocapsules physicochemical characteristics such as particle size and surface charge.

Table 1 shows the physicochemical characteristics of one representative example of the nanocompositions. The corresponding nanoemulsion (NE, without protamine) has similar size and polydispersity index to the protamine nanocapsules prepared either in one or two steps. It is possible to see the inversion of the surface charge of the nanosystem in the presence of protamine, indicating the formation of the polymeric shell around the oily nanodroplets. .

In **Figure 2** we can see the influence of the three main components (PEG-stearate, sodium cholate and Miglyol®) on the protamine nanocapsules physicochemical characteristic (size and zeta potential). For the formulations studied, the incorporation of PEG-stearate was found to be essential and without this surfactant, aggregates could be observed in all cases. The formulations displayed nanometric size between 180-350 nm and positive zeta potential ranging from +40 mV to close to neutral values. However, the zeta potential values were always higher than those obtained for the respective nanoemulsions (between -25 and -15 mV), indicating that protamine was deposited around the oil droplet.

The analysis of particle size for these formulations showed that the amount of Miglyol® used in the formulation was the principal variable affecting this parameter (**Figure 2A**). It is possible to see a decrease in the size of the systems when the amount of oil is low, regardless of the oil:surfactans ratio. Previous studies using the same preparation method reported a decrease in particle size with the shell polymer amounts, which does not occur in protamine nanocapsules [32]. Nevertheless, these studies do not evaluate the influence of the amount of oil, which in our case is apparently the most important parameter to take in account.

Component	Function				
Miglyol® (µl)	Oil	31.25	62.5	125	
Peg-stearate (mg)	Surfactant	0	12	24	48
Sodium Cholate (mg)		0	5	10	20

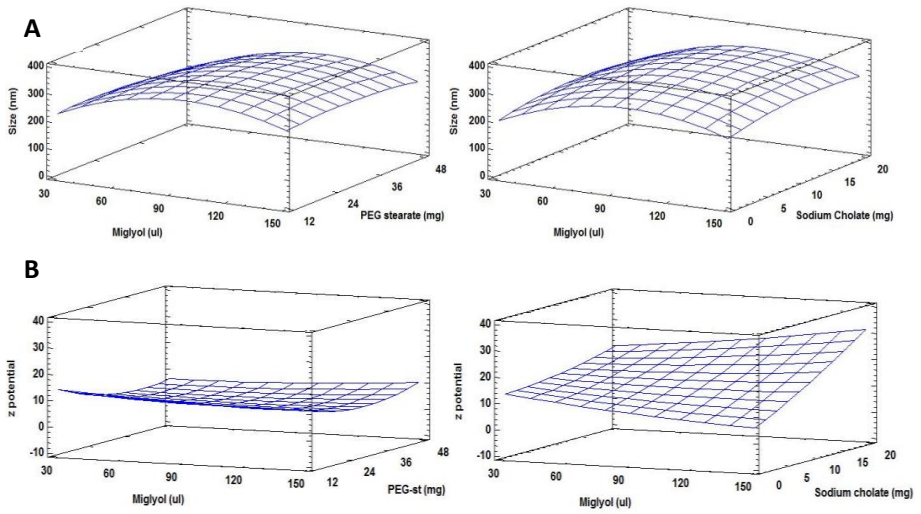

Figure 2: Influence of the amount of surfactants (PEG-stearate and sodium cholate) and Miglyol® included in protamine nanocapsules on the mean particle size and zeta potential of the formulations. (A) Size. (B) Zeta potential.

With respect to the surface charge, **Figure 2B** shows that this physicochemical property is clearly affected by the combination of surfactants studied.

Concretely, zeta potential values increase when sodium cholate concentration increases, leading to increasing negativity of the nucleus, which can therefore attract more of the positively charged protamine to form the outer shell and defines the overall positive zeta potential value. On the other hand, the formulations without sodium cholate have closely neutral surface charge, a typical characteristic of systems that include nonionic PEG-containing surfactants [33].

In order to further study the versatility of the developed nanosystems, we have also studied the feasibility of incorporating different oils as core components. Therefore we changed Miglyol® for α tocopherol (TCPH). This oil was chosen for its safety profile, its presence in the regular diet and its adjuvant and antioxidant properties [34, 35].

In **Table 1**, it is possible to see that the replacement of Miglyol for TCPH does not affect the nanometric size and the polydispersity index of the formulations. Also, the zeta potential remains positive, suggesting the presence of protamine in the shell.

Moreover, PEG-stearate (which has PEG in a lineal form) has shown to be necessary in the development of protamine nanocapsules. For this reason, we evaluated the feasibility of using other PEG-containing surfactants such as Macrogol (15)-hydroxystearate (Solutol® HS15), Tween® 20 and Tween® 80, which have less PEG monomers than PEG-stearate and in a ramified form. A special case is d-α tocopheryl polyethylene glycol succinate (TPGS), which is a conjugate between TCPH and PEG, in a linear configuration (similar to PEG-stearate) but with less PEG monomers [36].

In **Table 2** we can see the physicochemical characteristics of protamine nanocapsules depending on the different surfactants studied and with different mass ratios. It is possible to observe that all formulations have nanometric size

with low polydispersity index and zeta potential values between 0-+30 mV. As previously noted, the particle size depends on the oil:surfactant ratio, thereby when the nanocapsules had the same amount of oil and surfactant , the size decreases below 200 nm. This decrease in particle size could be very interesting for other applications where it has been shown that this parameter is an important feature, such as for accesing the lymphatic system [37].

Table 2:

Mean particle size, polydispersity index (PI), zeta potential and protamine association of nanocapsules with different surfactants and α tocopherol as oil core (n>3 ± SD).TPGS: D-α-Tocopherol polyethylene glycol succinate

Surfactant	Units of PEG (Form)	Ratio (mg) (TCPH: Surfac.)	Size (nm)	PDI	ζ Potential (mV)	Protamine assoc. (%)
PEG-stearate	40 (linear)	12:12	181 ± 11	0.1	+6 ± 1	10 ± 3
		60:12	234 ± 5	0.2	+1 ± 2	
		60:60	203 ± 3	0.1	-4 ± 0,5	
PEG-stearate / Sodium Cholate	40 (linear)	60:12	247 ± 9	0.2	+24 ± 3	57 ± 9
Macrogol (15)-hydroxystearate	23 (Ramified)	12:12	146 ± 1	0.1	-1 ± 0.4	19 ± 10
		60:12	250 ± 39	0.3	+23 ± 5	
		60:60	176 ± 23	0.2	-2 ± 0,5	
Tween® 20	20 (Ramified)	12:12	143 ± 15	0.4	+18 ± 3	
		60:12	232 ± 148	0.3	+11 ± 1	
Tween® 80	20 (Ramified)	12:12	105 ± 25	0.2	+19 ± 2	
		60:12	221 ± 26	0.2	+13 ± 4	20 ± 3
TPGS / Sodium Cholate	23 (linear)	60:12	189 ± 5	0.2	+21 ± 6	

When we compare the influence of PEG in the different formulations we can see that the size is higher when there are more linear PEG monomers present

since these are is oriented towards the aqueous phase, increasing the hydrodynamic diameter of the nanocapsules.

The protamine association was quantificated by UPLC, It is possible to see that surfactants with shorter PEG chains, such as Solutol® and Tween® 80 produced an increase in protamine association compared with PEG-stearate (20 % vs 10 % association respectively). In addition, the association of protamine was higher when we used a combination of surfactants that increase the negativity of the core, allowing a greater interaction between protamine and the nucleus. The best results were obtained when we used a combination of surfactants such as PEG-stearate and sodium cholate. Due to this result, we have chosen this formulation for further studies.

Association of influenza antigen to the nanocapsules

Hemagglutinin from the H1N1 A/PR/8/34 influenza antigen (HI) is a protein with a molecular weight of 63 KDa and an isoelectric point close to 7. The antigen was used as model in a solution at pH 9 which could guarantee negative charged and therefore facilitates its association to protamine nanocapsules by ionic interaction. This process was performed by incubation with isolated protamine nanocapsules at different nanocapsule:antigen ratios (section 2.5).,

The capacity of protamine nanocapsules to associate HI was quantified by Western blot. As it can be seen in **Table 1**, the association of HI to isolated blank nanocapsules in a mass ratio 4:1 (protamine:HI) does not affect the nanometric size of the formulation. The surface charge decreases when HI is associated, suggesting that the antigen was effectively associated to the nanocapsules shell. This surface presentation of the antigen is expected to allow rapid recognition by immunocompetent cells and triggers an efficient immune response.

TEM images (**Figure 3**) show that protamine nanocapsules loaded with HI maintain their spherical shape and distribution within a homogenous population. Western blot images show that the antigen structure is not affected by the association to the nanocarriers. This is expected since the association method (by incubation) it is a mild procedure, without use of organic solvent or other components that could damage the associated molecule. The association efficacy was near to 70%, demonstrating the high capacity of the nanocapsules to associate the antigen (HI). These results are similar to those reported recently by our group, where polysaccharidic nanocapsules (composed by highly deacetylated chitosan and a squalene core) achieved 69% association efficiency with the same antigen [38].

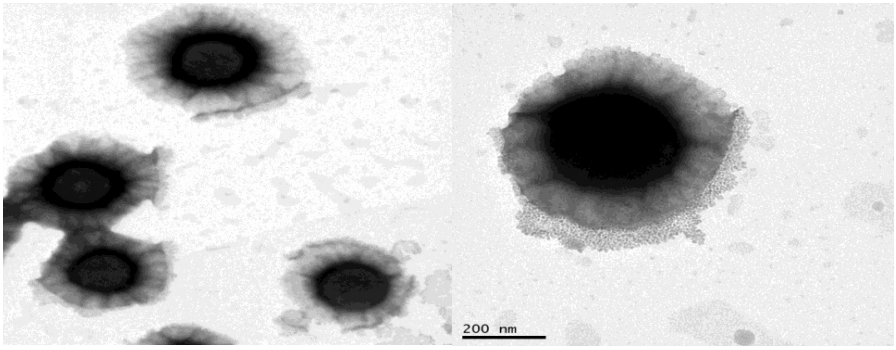

Figure 3: Transmission electron microscopy (TEM) images of protamine nanocapsules loaded with influenza antigen.

Stability of the nanocapsules and freeze-drying studies

Antigen-loaded protamine nanocapsule suspensions maintained their nanometric size for at least 3 months under storage (4ºC) and for 7 days in physiological conditions (PBS at 37ºC). This profile can be seen in other nanocapsular systems and it may be due to the polymer or polypeptide corona enclosing the oily nanodroplets and therefore giving stability to the formulation [32]. To have one stable formulation at 4°C is a positive result but the challenge is more ambitious: achieving room temperature stability. Therefore, freeze-drying can be and interesting approach for the accomplishment of this purpose.

Lyophilization is a strategy to increase the stability of colloidal systems and labile molecules, easing their shipment and increasing their storage time in adequate conditions [39]. Different concentrations of blank protamine nanocapsules were freeze-dried in presence or absence of trehalose or glucose at 5 or 10%. As it is shown in **Figure 4**, the reconstituted powder maintains the nanometric size even without the use of cryoprotectants. This is an excellent property which decreases the cost of the formulation for a possible scaling up.

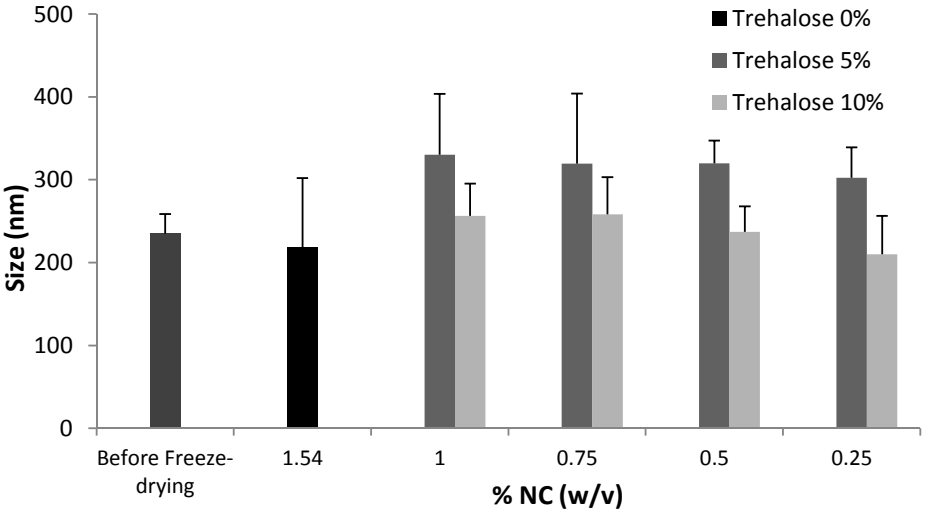

Figure 4: Particle size of protamine nanocapsules (oily core: α tocopherol) upon freeze-drying and further reconstitution in water. The nanocapsules were freeze-dried at different concentrations in the presence of trehalose at 5 or 10 % or in the absence of this cryoprotectant.

These results are different from those obtained for other nanocapsules rich in arginine where cryoprotectants were found to be crucial for the recovery of the initial particle size of the formulation [26]. This leads us to speculate that the protamine could play a important role in the freeze-drying process, stabilizing the system and possibly acting as a cryoprotectant.

Nor the concentration of nanocapsules neither the type of cryoprotectants showed influence on the physicochemical characteristics of the freeze dried nanocapsules. **Figure 4** shows the results obtained with trehalose as a

representative example from the freeze drying study. Due to its less hygroscopic character, this sugar is considered to have advantages over other cryoprotectants for the freeze drying of nanocarriers [40].

Cell viability and uptake studies

Different concentrations of protamine nanocapsules loaded with influenza antigen were incubated *in vitro* with the macrophage cell line RAW 264.7 and Alamar blue was used for the evaluation of cell viability by measuring mitochondrial activity [41]. As shown in **Figure 5**, cells viability remained unaltered in a wide range of nanocapsule concentrations (9-76 μg/cm^2).

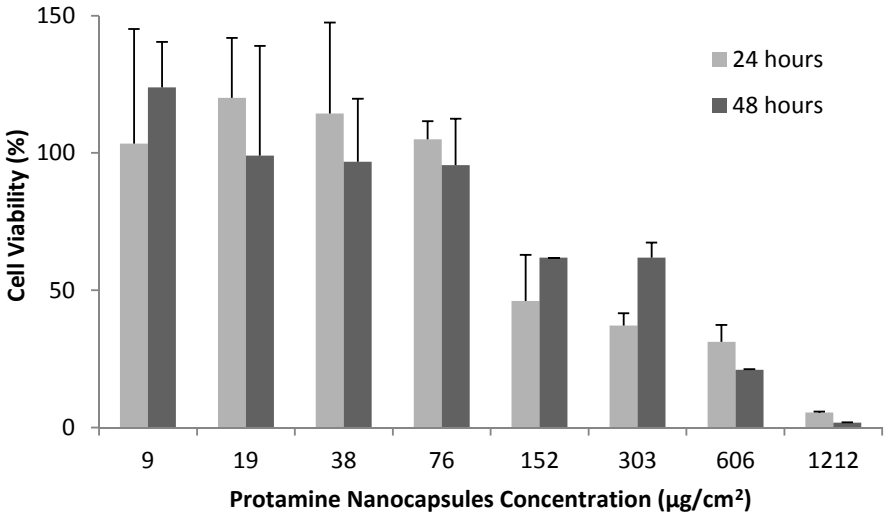

Figure 5: Cell viability of Raw 264.7 cells upon contact with different concentrations protamine nanocapsules loaded with influenza antigen (NC:HI 4:1) for 24 and 48 hours.

Over this range, viability decreased gradually with increasing concentrations, showing minor differences between 24 and 48 hours. This decrease in cell viability may be due to the fact that polycations, such as protamine, present higher cytotoxicity than neutral or negative macromolecules at high concentration. It is nevertheless possible to control this effect by adjusting the concentration used and the exposure time [42]. Within this regard, the highest

concentration of protamine nanocapsules without toxic effect (76 μg/cm^2) is considered to be sufficient for further *in vitro* and *in vivo* studies. In addition to cellular toxicity, internalization studies were also performed since the recognition and uptake of the nanocarriers by the immune system cells is considered to be crucial step to trigger an immune response. Protamine was labeled with TAMRA and with this modified polypeptide it was possible to prepare nanocapsules without changing their physicochemical characteristics or the association of influenza antigen (**Table 1**). Labeled nanocapsules were incubated for 30 min with a primary culture of alveolar macrophages and a line of monkey kidney epithelial cells (Vero cells). As it is shown in **Figure 6**, both cell types of cells have efficiently internalized the protamine nanocapsules. This result confirms that the positive charge given by the protamine at the systems is an attractive feature for phagocytic cells such as macrophages [43]. Moreover, the ability of protamine to enhance membrane translocation has been reported and this effect has already been described as the basis of the high internalization levels observed for other protamine nanosystems [44]. Different mechanisms have been postulated for this property, although it was known that the basic guanidine group of the arginine forms stable hydrogen bonds with sulfates or phosphates present in biological membranes. This interaction is considered essential for membrane translocation activity and can consequently improve the cellular uptake of protamine-based nanosystems [45].

In vivo studies

The immunization studies were performed in BALB/c mice, measuring the systemic humoral immune response elicited after subcutaneous immunization. Animals were immunized with protamine nanocapsules loaded with influenza antigen in two doses (2 and 7.5 μg/ mice) and with 7.5 μg of antigen adsorbed in alum as a control (commercially available as Fluval P). The first immunization

was followed by two boost-doses (at weeks 3 and 7) and the blood samples were monitored for 28 weeks.

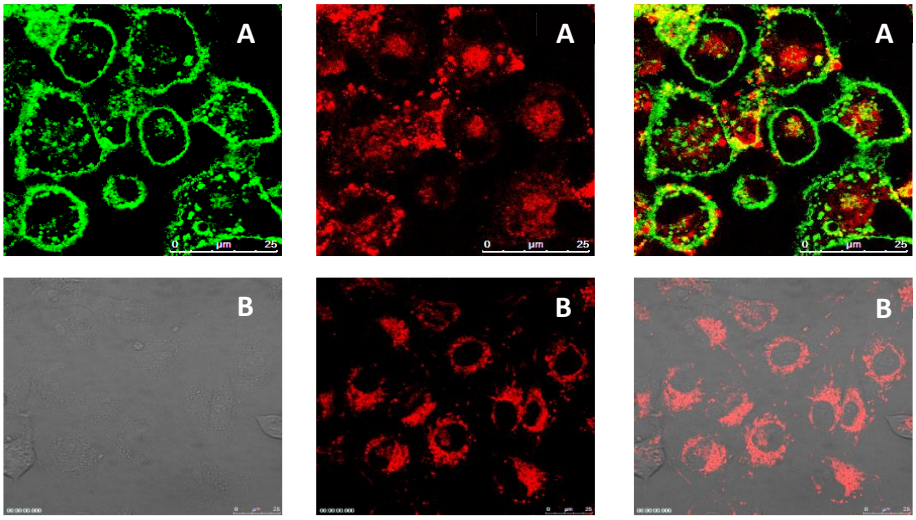

Figure 6: Visualization of the internalization of TAMRA-labeled protamine nanocapsules loaded with influenza antigen (HI) by confocal microscopy. A: image of primary culture of alveolar macrophages from pig lungs, where the green channel (A1) was used to visualize the plasma membrane (labelled with fluorescent-WGA), the red channel (A2) illustrates the internalized TAMRA-labeled protamine nanocapsules loaded with HI and the overlapped image is shown in (A3). B: Vero cells (B1) transmitted image (B2) internalized TAMRA-labeled protamine nanocapsules loaded with HI and (B3) overlay.

In **Figure 7** shows the profile of immune response generated by the different antigen formulations and doses. Protamine nanocapsules presented an elevated peak of immune response in the 3rd week, higher than that achieved for the antigen adsorbed in alum. This immune response is still comparable with the commercial formulation in the following two points and becomes slightly lower at the last time point.

On the other hand, when we used three times less antigen dose (2 µg) with the nanocapsule formulation, the immune response only showed a slight decrease, maintaining its effectiveness and protection features. In addition, the capacity of inhibition of hemagglutination induced by the protamine nanocapsules is

much higher than the dilution considered protective against influenza diseases (1:12800 *vs* 1:64 dilution) [46]. These results open an option to administer low doses of antigen loaded in protamine nanocapsules and to obtain an efficient and long-lasting immune response. Further studies will allow corroboration of this hypothesis. These results are similar to the ones obtained with chitosan nanocapsules, where the immune response generated with 2 µg of the same antigen was similar to the one produced with 7.5 µg of antigen, leading the authors to conclude about the immunopotentiating effect of the carrier used [38].

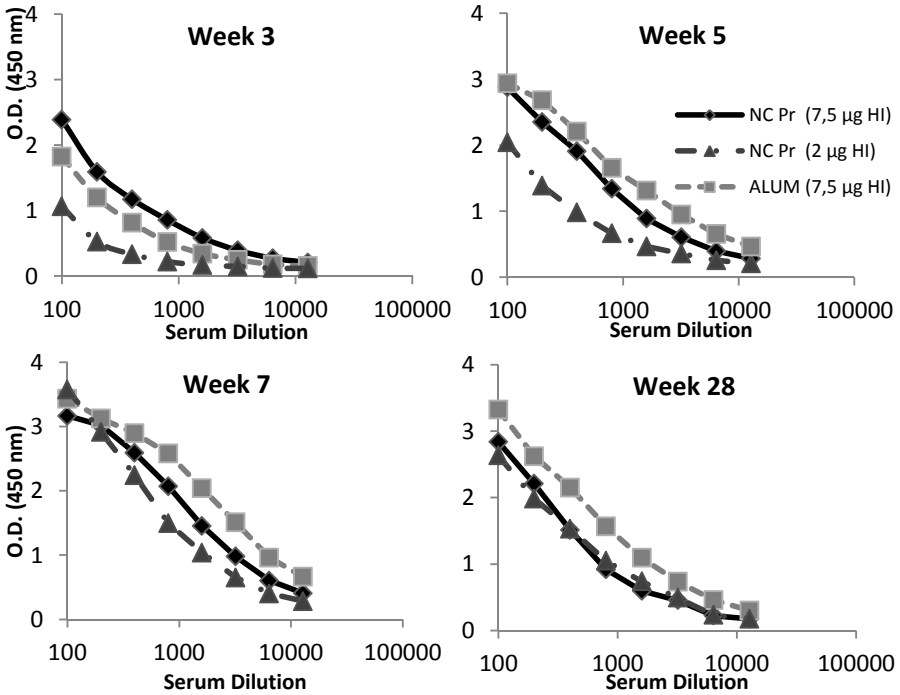

Figure 7: Serum neutralizing antibody levels against influenza antigen (HI) in mice (5 for each group) elicited upon three subcutaneous injection (0,3 and 5 weeks) of HI-loaded protamine nanocapsules containing two different doses of antigen: 7.5 µg (◆) and 2 µg (▲).Alum-adsorbed HI at the dose of 7.5 µg was used as a control (■).

These *in vivo* results show the potential of the protamine nanocapsules as a platform for antigen delivery. Protamine as a polycation, due to its repetitive

sequences of arginine, presents immunostimulating effects and is able to quickly transport the antigen to the draining nodes, where they can induce a fast and efficient immune response [47].

4. CONCLUSION

Here we present the first report on protamine nanocapsules as a new platform for drug and antigen delivery. This original prototype consists in an oily core surrounded by a protamine shell and stabilized by PEGylated surfactants. Protamine nanocapsules are very versatile systems regarding their composition, which allows the easy modulation of their physico-chemical properties such as particle size and zeta potential. *In vitro* results confirm the high biological and physical stability of the system. In an immune cell line, the system presented non-toxic effects in wide range of concentrations together with high levels of cell internalization. Finally, *in vivo* studies have shown that this system can carry the antigen and trigger an adequate immune response, similar to the one obtained with the conventional adjuvant and even using a lower antigen dose.

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CAPÍTULO 5

CAPÍTULO 5

NOVEL THERMOSTABLE NANOVACCINES ANTIGEN-ASSOCIATED PROTAMINE NANOCAPSULES

Este trabajo ha sido realizado en colaboración con Mercedes Peleteiro[1] y África González-Fernandez[1].

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ABSTRACT

One of the main challenges in the development of vaccines is their stability at room temperature, which could allow avoiding the cold chain, administering more effective and safer vaccines. In this work we describe the development and optimization of thermostable nanocarriers consisting of an oily core with immunostimulating activity containing squalene or α tocopherol, surrounded by a protamine shell. These formulations can efficiently associate the recombinant hepatitis B surface antigen (rHBsAg) without losing the antigen epitope. Freeze-dried protamine nanocapsules maintain their physicochemical characteristics for at least 12 months at room temperature and they preserve the integrity and bioactivity of the associated antigen. Nanocapsules show high internalization in immunocompetent cells and efficiently stimulate cytokine secretion and complement activation. Formulations loaded with rHBsAg achieve protective levels upon intramuscular and/or intranasal administration to mice. Overall, our data put, protamine nanocapsules forward as an innovative thermostable nanovaccine platform for improved antigen delivery.

1. INTRODUCTION

Nanoparticles are raising expectations in the antigen delivery field because they combine adjuvant properties with special abilities for overcoming biological barriers. In fact, this specific behavior has rendered nanoparticles promising strategies towards achieving single dose and needle-free vaccines [1]. Among the materials used in the development of antigen delivery nanoparticles polylactic acid (PLA) and related polymers, as well as chitosan, are probably those receiving the greatest deal of attention [2, 3]. This is mainly due to their adequate safety profile and their promising behavior in preclinical research as well as in clinical trials. More recently, the interest has also been focused on the use of biomaterials with inherent adjuvant properties; materials that are able to activate the pattern-recognition receptors (PRRs) of immunocompetent cells and modulate the cytokines secretion profile [4].

Our group has designed a new antigen delivery vehicle, named as chitosan nanocapsules and explored their potential for immunization [5]. These nanocapsules, composed of an oily core and a chitosan shell, are very versatile and may offer advantages as compared to the matrix-type nanoparticles. For example, they can associate different antigens in their shell and lipophilic immunostimulants in their core. Furthermore, the chitosan shell itself and the specific antigen localization may also contribute to their potential role as immunoactive carriers. In fact, these nanocarriers were found to enhance the immunogenic response of antigens, i.e. recombinant hepatitis B surface antigen (rHBsAg) following either, intramuscular or nasal administration [5, 6].

As an alternative, in the current work we have chosen protamine as material for the elaboration of these nanocapsules. Protamine is a rich-arginine polypeptide that has been is approved by the FDA as an excipient in insulin formulation and also as a drug used to neutralize the effect of heparin in cardiac surgery [7]. In addition to this interesting safety record, protamine

exhibits cell penetrating properties [8] and the ability to enhance the production of cytokines and the proliferation of antigen-specific T cells [9]. There is also a preliminary report on the use of protamine to enhance the adjuvant properties of liposomes upon nasal vaccination [10].

On the other hand, in our attempt to rationally design these nanocapsules, we have selected as a core material, two different types of oils: squalene and α tocopherol (vitamin E), both of them with proven adjuvant activity [11]. In fact, squalene and α tocopherol have been included as adjuvants in some marketed influenza vaccine formulations such as MF59^{TM} and ASO3^{TM} [12].

Finally, the model antigen chosen to assess the potential of protamine nanocapsules for immunization was recombinant Hepatitis B surface antigen (rHBsAg). Although this antigen gives very high responses upon injection in association to alum [13], it has important limitations that remain to be solved. For example, the alum-adsorbed vaccine needs storage in a narrow range of temperature (2-8 ºC), a fact that represents an important limitation for the worldwide distribution of this vaccine [14]. On the other hand, this vaccine needs to be administered according to a multiple injection protocol, and the possibility to be administered in a needle-free format is seen as a major milestone towards improving immunization coverage, especially in developing countries.

Based on these premises, in this work we report the development and the comprehensive analysis of protamine nanocapsules as antigen delivery vehicles. Namely, the resulting nanovaccines were characterized with respect to their physic-chemical properties, their capacity to load and release rHBsAg, there stability upon storage as a freeze-dried powder, their ability to stimulate cytokine production in blood mononuclear cells and their *in vivo* performance, following either intramuscular or intranasal administration. With this work we expect to provide a view of the potential of protamine nanocapsules in the

development of advanced and safe vaccines that could potentially help increasing the immunization coverage worldwide.

2. MATERIALS AND METHODS

2.1 Chemicals

Protamine sulfate was produced by Yuki Gosei Kogyo, Ltd. (Japan) and offered by LEO Pharma (Denmark). Recombinant hepatitis B surface antigen (rHBsAg) antigen was kindly donated by Shantha Biotechnics Limited (Hyderabad, India). Poliethylenglicol (PEG)-Stearate (Simulsol M52) was from Seppic (France). Squalene (SQL) and α tocopherol (TCPH) were supplied by Merck (Germany). Enzyme linked immunosorbent assay (ELISA) kit (Murex rHBsAg Version 3) was from Diasorin (United Kingdom). Antibodies for Western-blot detection, chicken polyclonal antibody to hepatitis B virus surface antigen and rabbit polyclonal to chicken conjugated with horseradish peroxidase were purchased from Abcam pcl (United Kingdom). For complement analysis by Western-Blot, mouse monoclonal antibody (mAb) against human complement factor 3 (C3) was from Abcam (Cambridge, UK). Secondary polyclonal goat anti-mouse IgG conjugated to alkaline phosphatase were from Dako (Glostrup, Denmark). For ELISA, mouse and rabbit mAbs against HBsAg were purchased from Biokit (Barcelona, Spain) and Acris Antibodies GmbH (Hiddenhausen, Germany), respectively. Secondary Abs (both polyclonal goat anti-rabbit and anti-mouse IgG, IgG1, IgG2a conjugated to horseradish peroxidase) were from Southern Biotech (Birmingham, AL). 5-Carboxytetramethylrhodamine succinimidyl ester (TAMRA) was from Invitrogen (United Kingdom). Fetal bovine serum (FBS), glutamine and penicillin/streptomycin were purchased by PAA (Austria). Glucose, trehalose, PBS, sodium cholate and aluminum hydroxide gel were obtained from Sigma-Aldrich (Spain). All other chemicals used were of reagent grade or higher purity.

2.2 Preparation of protamine nanocapsules

Blank protamine nanocapsules were prepared by a solvent displacement technique [15]. Briefly, PEG-stearate (12 mg) and sodium cholate (5 mg) were dissolved in ethanol (750 µl) and mixed with a solution of the oil, either TCPH (60 mg) or SQL (62.5 µl) in acetone (4.25 ml). The organic phase was immediately poured into 10 ml of aqueous phase with protamine (0.05 % w/v). The formation of the systems was evident due to the milky appearance of the mixture. The elimination of organic solvents was performed by rota-evaporation under vacuum (Heidolph, Germany) until a constant volume of 5 ml.

The isolation of the protamine nanocapsules either with SQL or with TCPH was carried out by ultracentrifugation (OptimaTM L-90K, Ultracentrifuge, Beckman Coulter; USA) at 61740 x g for 1h, at 15 ºC. The isolated nanocapsules were resuspended in ultra-pure water to a final theoretical protamine concentration of 1 mg/mL.

2.3 Characterization of protamine nanocapsules

The hydrodynamic diameter and polydispersity index of the systems were determined by photon correlation spectroscopy (PCS) (Zetasizer®, NanoZS, Malvern Instruments, Malvern, UK), after sample dilution with ultrapure water. The zeta potential was measured by laser-Doppler anemometry diluting the samples with 1 mM KCl. Morphological examination was carried out by transmission electron microscopy (TEM, CM12 Philips, Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid solution.

2.4 Association of rHBsAg to protamine nanocapsules and determination of its structural integrity

The preparation of protamine nanocapsules loaded with rHBsAg was performed after incubation for 1h at room temperature of the isolated

protamine nanocapsules with the model antigen, at a mass ratio of 4:1 (protamine:rHBsAg). The volume of the protamine nanocapsules suspension and the antigen solution used was the same.

The ability of protamine nanocapsules to associate the rHBsAg antigen was determined indirectly, calculating the difference between the total amount of antigen used in the incubation process and the amount of free rHBsAg in the remaining aqueous medium after the isolation process. This amount of non-associated antigen was quantified by ELISA following the manufacturer's instructions.

Western blot assay (Wb) was used as a qualitative tool for the determination of the antigen's structural integrity. For this purpose, we followed the same procedure described by Prego *et al* [16] with minor modification. In our case, the primary antibody was incubated for 1 hour at room temperature and the antibody–antigen complexes were visualized using the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, UK) in UVP imaging system (EC3™ Imaging System, UVP, USA).

2.5 In vitro release studies of rHBsAg from protamine nanocapsules

In vitro release studies of the rHBsAg from protamine nanocapsules were performed to assess the ability of this system to release the associated antigen in its active form. Thus, 50 µl of isolated rHBsAg-loaded protamine nanocapsules (TCPH core) were incubated with 1.95 ml of PBS (pH 7.4) with Tween 80® (0.02 % v/v) and placed in an incubator at 37ºC, under moderate shaking. Previously, we ensured that the physicochemical characteristics of protamine nanocapsules were not modified in the above conditions. At different time points (1, 4, 8 and 24 hours), the suspension was ultracentrifuged, (as described in section 2.2) and the remaining aqueous medium was analyzed for released rHBsAg by ELISA. These samples were also

submitted to Western blot analysis in order to check the structural integrity of the antigen.

2.6 Stability of rHBAg-loaded freeze-dried nanocapsules

Protamine nanocapsules (1% w/v) with TCPH in their core and loaded with rHBsAg in a mass ratio 4:1 (protamine:rHBsAg) were lyophilized (Labconco Corp, USA) in presence of 5% (w/v) trehalose. For this purpose, the samples were frozen at -20 ºC and then subjected to an initial drying step at -35 ºC followed by a secondary drying at 0 ºC, both for 24 hours at a high vacuum atmosphere. Finally, the temperature was slowly increased up to 25 ºC till the end of the process.

The lyophilized product was stored at 25 ºC (Climatic chamber, Binder; Germany) for up to different times (1, 3, 6, 9 and 12 months). The formulations were resuspended, manually, in ultrapure water and their physicochemical characteristics were checked (section 2.3). The integrity of the antigen was evaluated by Western blot (section 2.4).

2.7 Cytotoxicity of protamine nanocapsules in macrophages

Cell toxicity was measured by xCELLigence® system in a macrophage cell line (RAW 264.7), following manufacturer's instructions (Roche Diagnostics, Germany). This system can analyze the condition of the cells, including number, viability and morphology at real time through the measurement of the s electrical impedance.

Briefly, 1.5 X 10^4 cells/well were cultured in special 16 well plates carrying gold electrodes and incubated on the equipment (3 plates can be analyzed simultaneously) inside the incubator at 37 ºC in the presence of 5% CO_2 for 18 hours until reaching the exponential phase. Then, protamine nanocapsules with TCPH or SQL were added at three different concentrations (25, 50 and 100

µg/ml, referred to protamine amount), in duplicate and cells were incubated at the same conditions for 48 hours. Cells without nanocapsules, nanocapsules alone, and medium alone were used as controls. The impedance obtained is correlated with the amount of attached cells per each well. The monitoring was performed every 15 minutes until the end of the experiment.

2.8 Internalization of protamine nanocapsules by macrophages

In order to visualize the uptake of these systems by phagocytic cells; a selected prototype of nanocapsules containing TCPH was prepared using fluorescently labeled protamine. For this, 100 µl of 5- carboxytetramethylrodamine succinimidyl ester (TAMRA) solution in DMSO (10 mg/ml) was slowly added to 1-ml of a protamine solution (10 mg/ml) in 0.1 M sodium bicarbonate buffer (pH 9.0) under stirring. The resulting solution was incubated for 1 hour at room temperature and finally dialyzed (Slide- A Lyzer® dialysis cassette 2000 MWCO, Thermo) for 48 h, to remove the free TAMRA. The protamine nanocapsules were then prepared with this labeled protamine (Pr-TAMRA) following the procedure described in section 2.2.

The phagocytic assay was performed using Raw 264.7 cells. Briefly, 5 x 10^5 cells were plated in a 24-wells plate (Falcon 3047, BD Biosciences, USA) with 1 ml of RPMI 10% FBS in the presence or not of labeled nanocapsules at a concentration of 50 µg/ml respect to the theoretical amount of Pr-TAMRA associated at the NC, for 30 minutes.. After three washes with PBS to remove non-internalized nanocapsules, cells were observed under an inverted fluorescent microscope (IX50, Olympus Optical Co GmbH, Germany).

Cells were also analyzed in a flow cytometer. After incubation with the labeled nanocapsules, cells were washed once with PBS and then incubated with 200 µL of Accutase® (PAA, Austria) for 10 minutes at 37 ºC in the presence of 5% CO_2. Finally, cells were transferred to a tube, washed with complete medium

and centrifuged. The resulting suspension of cells was analyzed using a flow cytometer (FC500, Beckman-Coulter, USA). The final confirmation of internalization of nanocapsules was performed using a confocal microscope (Leica SP5, Germany). Cells were seeded on a glass cover-slip (Menzel-Gläser, Germany) in a 24-well plate and incubated with nanocapsules as described above. After several washes, cells were fixed with 4% formaldehyde during 10 minutes and then incubated with Alexa Fluor 488-phalloidin for 20 minutes in order to stain the cellular cytoskeleton. Finally, the cover-slips containing the attached cells were mounted over slides in the presence of ProLong® Gold Antifade mounting medium (Invitrogen, USA) containing DAPI to stain the nucleus.

2.9 Cytokine Secretion by peripheral blood mononuclear cells (PBMCs)

This study was performed with PBMCs of three independent healthy human donors (the project was *approved* by the *Clinical* Research *Ethical Committee* of *Galicia, and w*ritten *informed consent* for participation in the study was *obtained* from the donors). Heparinized blood was diluted with equal volume of PBS and centrifuged through a Ficoll-Hypaque (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient in a relation 7:3 (Blood diluted:ficoll) at 260 x g for 30 minutes at 20 ºC, in order to separate mononuclear cells from the granulocyte and erythrocyte populations. Mononuclear cells in the interface between Ficoll and plasma were collected with a Pasteur pipette and washed twice with complete medium by centrifugation (145 x g, 5 min, 20 ºC).

For the assessment of cytokine production, 2×10^5 peripheral blood mononuclear cells (PBMCs) were incubated during 24 hours in 96-well plates (37 ºC, 5% CO2) in the presence of protamine nanocapsules with SQL or TCPH nucleus at two concentrations (10 and 100 µg/ml). As negative and positive control, cells were incubated with complete medium or with 1 µg/mL of lipopolysaccharide (LPS) plus 10 µg/mL phytohaemagglutinin (PHA),

respectively. After 24 hours, the plate was centrifuged (100 x g, 5 min, 4 ºC) and supernatants were collected and stored at -20 ºC until the analysis was performed.

The secretion levels of different cytokines were determined using the human Th1/Th2 FlowCytomixTM assay (eBioscience, Austria), following manufacture`s intructions. Briefly, 25 µL of Ab-coated microspheres were incubated with 25 µL of culture supernatants and 50 µL of biotin-conjugated secondary Abs for 2 hours at room temperature (RT) on a microplate shaker. After several washes, 50 µL of streptavidin conjugated to phycoerythrin and 100 µL of PBS-T were added to the preparation and incubated for 1 hour at RT on a microplate shaker. Finally, phycoerythrin-bound beads were studied by flow cytometry.The analysis was done using Flow Cytomix Pro 3.0 Software (eBioscience, Austria)

2.10 Study of complement cascade activation by protamine nanocapsules

The study of the complement cascade activation induced *in vitro* by protamine nanocapsules was performed analyzing the C3 cleavage products by Western blot. For this purpose, human plasma was incubated with different concentrations of protamine nanocapsules (25 - 500 µg/ml range). The different dilutions were prepared in PBS (pH 7.4). Equal volumes of plasma, veronal buffer (pH 7.4) and protamine nanocapsules were mixed and incubated at 37 ºC for 1 hour. The positive (cobra venom factor) and negative (PBS) controls were prepared in the same way. The mixture was centrifuged (16000 x g, 0.5 hr) and the supernatants were loaded onto a 10 % SDS- PAGE gel and then transferred to a PVDF membrane (Immun-Blot, Biorad; Hercules, CA) using a transblot semidry transfer equipment (Bio-Rad; Hercules, CA). PVDF membranes were blocked overnight with non-fat milk 5% (w/v) at 4 ºC. The membranes were incubated for 1 hour in the presence of mouse mAb against human C3, carefully washed and secondary anti-mouse antibodies conjugated with alkaline phosphatase were added, followed by an additional hour of

incubation. Finally, the membranes were revealed with 5-Bromo-4-chloro-3-indolyl-phosphate p-toluidine salt (BCIP).

2.11 Immunization studies upon intramuscular and intranasal administration

Groups of 7 BALB/c female mice with an average weight of 20 g and 4 weeks of age were randomly assigned and immunized with vaccine formulations. The animals were kept conscious during the immunization and subsequent sample collection.

Two groups of mice received two intramuscular (im) administrations of rHBsAg loaded protamine nanocapsules (at day 0 and 28) carrying either SQL or TCPH. Control mice received the conventional alum-adsorbed antigen, at a dose of 10 µg of rHBsAg,

The alum-adsorbed antigen was prepared in situ just before immunization. For this, the aluminum hydroxide and rHBsAg solutions were incubated in a volumetric ratio 3:1 (rHBsAg:alum) for 30 min at 4 ºC under moderate agitation [16].

On the other hand, two group of mice (n=7) received three doses at days 0, 28 and 56 of 10 µg rHBsAg associated to protamine nanocapsules (SQL or TCPH) by intranasal administration (in) and another group received a combined (im-in) schedule of protamine nanocapsules (TCPH nucleus). This group received the first dose was by im route and boost doses intranasally at days 28 and 56.

Serum samples were collected from the mouse maxillary vein in the weeks 6, 18 and 26. A pool of sera for each group was prepared and IgG endpoint titers for rHBsAg were determined by standard ELISA.

2.12 ELISA for the quantification of specific total IgG subtypes

rHBsAg diluted in Carbonate Buffer (pH 9.6) at 5µg/mL was incubated overnight at 4ºC in maxisorp 96-wells plates. Then plates were blocked with PBS-BSA 1% for 1 hour at 37ºC in order to reduce non-specific interactions. To quantify anti rHBsAg IgG titers in µg/mL and in mIU/mL, a mouse monoclonal IgG anti rHBsAg and rabbit IgG anti rHBsAg of known concentrations in µg/mL and mIU/mL, respectively, were serially diluted and used to transform data first from absorbance to µg/mL and then to mUI/mL. For this purpose, both controls and serum samples were incubated for 2 hours at 37ºC. Then, secondary Abs (goat anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase) were added to each well and incubated for 1h at 37ºC. Finally, bound antibodies were revealed with ABTS and the titers were expressed in concentration (mIU/ml).

For the study of the IgG subtypes, the pool of sera of the different groups of mice immunized with rHBsAg associated to protamine nanocapsules (TCPH nucleus) were analyzed by the same ELISA protocol as described above but using goat anti-mouse IgG1 and IgG2a Abs (conjugated to horseradish peroxidase) as secondary Abs. Finally the IgG1/IgG2a ratio was calculated.

2.13 Statistics

The Kruskal-Wallis test was performed using Statgraphic centurion XVI (Statpoint Technology). Differences were considered significant at a level of $p<0.05$.

3. RESULTS AND DISCUSSION

Recently, we reported the utility of protamine nanocapsules as an antigen delivery carrier, using influenza antigen as model antigen [17]. In the present work, our objective was to further improve these nanocarriers including oils with demonstrated adjuvant capacity and evaluate their potential using recombinant hepatitis B antigen (rHBsAg) as a model antigen. More precisely, the new nanocarriers were rigorously evaluated *in vitro* for their antigen loading and release properties, stability profile and interaction with immunocompetent cells. Moreover, they were studied *in vivo* for their ability to trigger and enhance a specific immune response in mice against rHBsAg antigen.

Development and characterization of protamine nanocapsules

Protamine nanocapsules were developed based on our previous experience with polyaminoacid and polysaccharide nanocapsules [18, 19]. Thus, different variables such as surfactant type (PEG- stearate and sodium cholate) and a specific oil:surfactant ratio (60:12 w:w) were selected according to our previous experience [17]. As an improvement step in this technology, we selected squalene and α tocopherol to form the oily core of the nanocapsules. These oils have been tested in different pharmaceutical forms and their adjuvant capacity has been demonstrated in different clinical trials. Most importantly, both of them can be found as components in EMA-approved adjuvants [12]

In **Table 1**, we can see that the two blank formulations containing different oils have a nanometric size with low polydispersity index and positive surface charge, being the latter due to the presence of protamine forming a shell around the nanocapsules. As shown in **Figure 1**, the nanocapsules are in the form of individual and homogeneous structures with spherical shape regardless their core composition.

Table 1: Mean particle size, polydispersity index (PdI), zeta potential and percentage of association of hepatitis B antigen to protamine nanocapsules with different core compositions (n>3 ± SD).

Oil component		Size (nm)	PdI	ζ potential (mV)	rHBsAg (%)
Squalene	Blank	215 ± 5	0.1	+18 ± 1	---
	4:1 (NC:rHBsAg)	226 ± 19	0.2	+8 ± 3	82 ± 1
α tocopherol	Blank	250 ± 23	0.2	+36 ± 3	---
	4:1 (NC:rHBsAg)	210 ± 38	0.2	+20 ± 5	78 ± 13

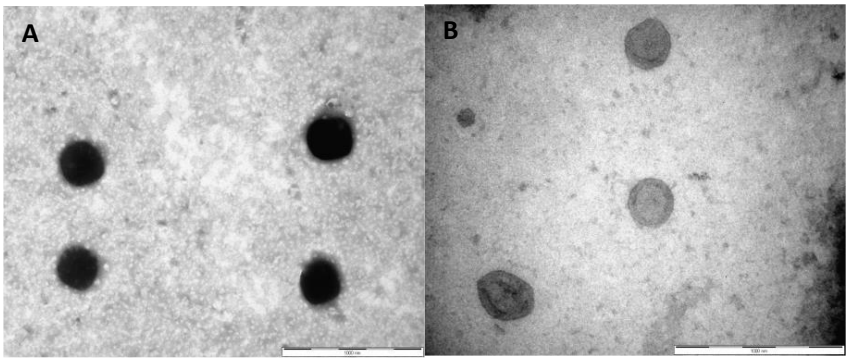

Figure 1: Transmission Electron microscopy images of protamine nanocapsules with squalene (A) or α tocopherol (B) as oil in their nucleus.

Antigen association and release from protamine nanocapsules

The antigen, rHBsAg, was associated to the nanocapsules by incubation at room temperature for up to 1 hour. rHBsAg is a a virus-like protein nanoparticle with a size of 22 nm and a negative surface charge [20, 21]. These tiny nanoparticles can be easily attached to the nanocapsule's surface by simple interaction with the positively charged guanidine groups of arginine, the main aminoacid present in protamine. This interaction is supposed to be mediated by ionic forces, although other types of interactions such as hydrophobic and hydrogen bonds may also be involved in this process. An evidence of this interaction is

illustrated by the significant decrease of the zeta potential of the nanocapsules (**Table 1**).

The association or rHBsAg onto the shell of the nanocarriers is very suitable as it allows the surface exposition of the antigen, thus making the antigen more accessible to antigen presenting cells (APC) and increasing its likeliness to eventually trigger an immune response.

On the other hand, the results in **Table 1** also show that the percentage of antigen associated to the nanocapsules, as determined by ELISA, is very high, irrespective of the nature of the oily core. In addition, Western blot analysis indicated that the antigen size and epitope are both preserved during the process (data not shown). These findings are in agreement with those found for chitosan nanocapsules where the antigen association was near 70% and a reduction of the positive zeta potential of the nanocapsules was observed [22].

In order to evaluate the rHBsAg release, we incubated protamine nanocapsules in PBS (pH 7.4) at 37 ºC for 24 hours. Prior to the experiments we confirmed that the physicochemical properties of protamine nanocapsules were unaffected throughout the duration of the experiment (data not shown). **Figure 2** shows the profile of antigen release from the nanocapsules. It is possible to observe that near 50 % of rHBsAg is gradually released within the first 24 hours, showing that the antigen-protamine interaction in the formulation is reversible. Moreover, the results of the ELISA and Western blot analysis confirmed that the antigen maintains its integrity during the release process.

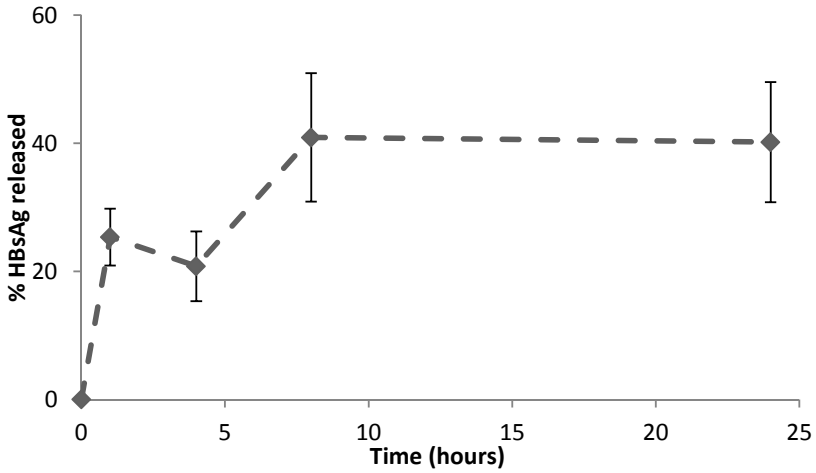

Figure 2: *In vitro* rHBsAg release from protamine nanocapsules (TCPH nucleus) in PBS (pH: 7.4) with Tween ® 80 (0.02 %) buffer after incubation at 37 ºC. Data represents the mean ± SD, n>9.

Stability of freeze-dried nanocapsules

Most vaccines are only stable at temperatures between 2 and 8 ºC. This is a relevant problem, especially in developing countries where the cold chain is often broken with the consequent loss of effectiveness [23]. As a consequence, to improve vaccine stability at room temperature is, nowadays, one of the greatest challenges in vaccine formulation.

In order to assess the stability of rHBsAg-loaded protamine nanocapsules during storage, we first converted them into a freeze-dried powder. As seen in **Figure 3.A**, protamine nanocapsules with a TCPH core maintained their nanometric size and surface charge upon storage at room temperature (25 ºC) for up to 12 months. These results suggest that the formulation was effectively preserved.

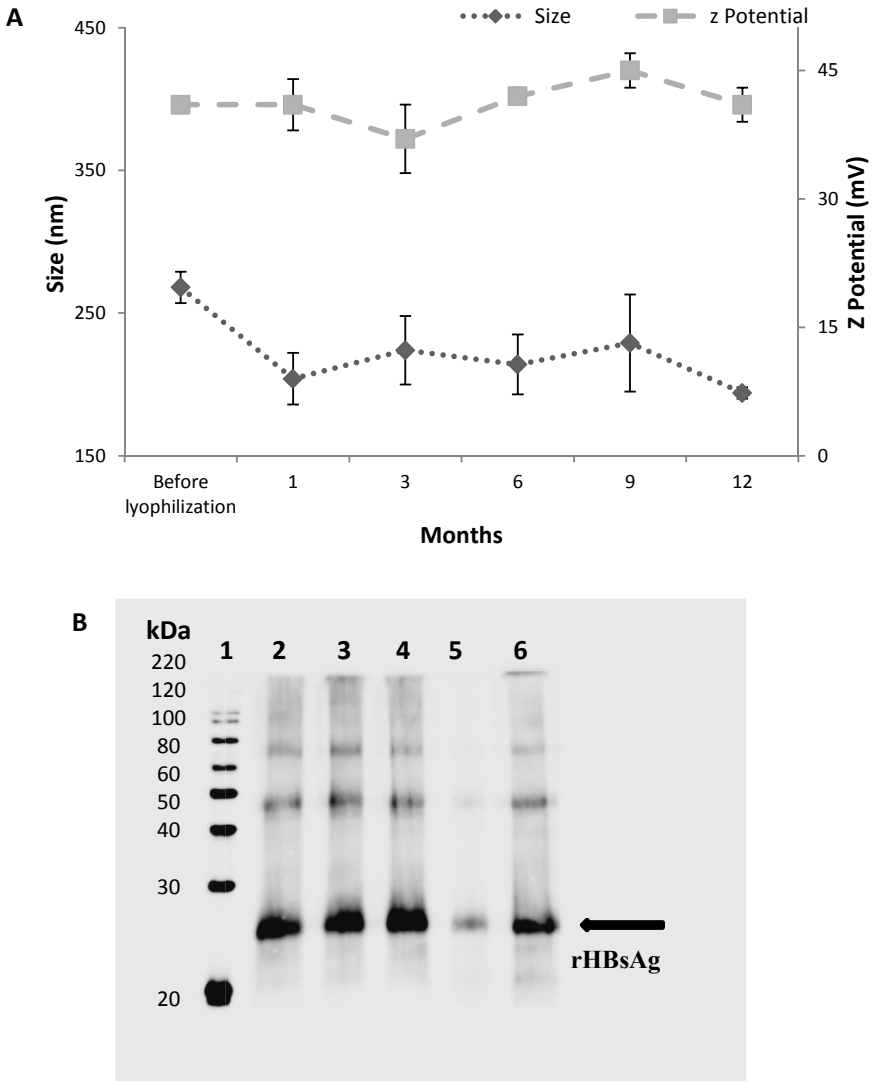

Figure 3: (A) Particle size and zeta potential of freeze-dried and reconstituted rHBsAg-loaded protamine nanocapsules (TCPH nucleus) conserved at room temperature for different time periods (25 ºC) (n=3 ± SD). (B) Western-blot analysis of rHBsAg of samples at 1 year.

Regarding the stability of the associated antigen, a Western blot image (**Figure 3.B**) of a sample at 12 months time point shows that the antigen loaded onto protamine nanocapsules (lanes 2, 3 and 4) did not lose its structure or antigenicity neither in the freeze-drying process nor over time, compared with a control of non-lyophilized antigen (lane 6). Lane 5 shows that the freeze-dried

free antigen, maintained its structure upon freeze-drying. It is possible to observe a relative loss of the amount of antigen in this case, in comparison with the antigen loaded in the NC (lanes 2, 3 and 4), confirming that the antigen preserves better its properties when it is associated to protamine nanocapsules.

Overall these results show that rHBsAg-loaded protamine nanocapsules are thermostable for at least 12 months.

Interaction of protamine nanocapsules with macrophages: internalization and toxicity profile

In order to provide some insights on the mechanistic behavior of the potential nanovaccine, we studied the cell viability and internalization of the protamine nanocapsules in a macrophages cell line (RAW 264.7). In this sense, it is important to keep in mind that the recognition and internalization of the nanosystems by APC is an essential step for triggering the immune response [24].

Figure 4 shows the exponential cell growth profile and the changes observed upon contact with protamine nanocapsules containing ∝-tocoferol at different concentrations (25, 50 and 100 µg/ml) for up to 48 hrs. The results show that doses of 25 and 50 µg/ml did not interfere with the cell growth in the first 24 hours of contact. However, the exposure of the cells to a higher concentration (100 µg/ml) of nanocapsules interferes in the cell growth showing a decrease in their viability. These results are slightly different from others obtained with chitosan nanocapsules using the same method. Blank chitosan nanocapsules doses of 25 and 50 µg/ml in contact with RAW 264.7 cells affect their viability is in the first 24 hours. However, an abrupt decrease in cell viability at 48 hours is not observed [6]. Similar results were obtained with high doses of blank

nanocapsules (100 µg/ml), where both prototypes interfere in the cells viability at early times.

The results obtained for nanocapsules containing SQL were very similar to those presented above, thus indicating that the oily core did not influence the cytotoxicity profile. These results were confirmed by the Quick Cell Proliferation Assay, a colorimetric method that measures the metabolic activity of living cells through the reduction of the added reagent (data not shown).

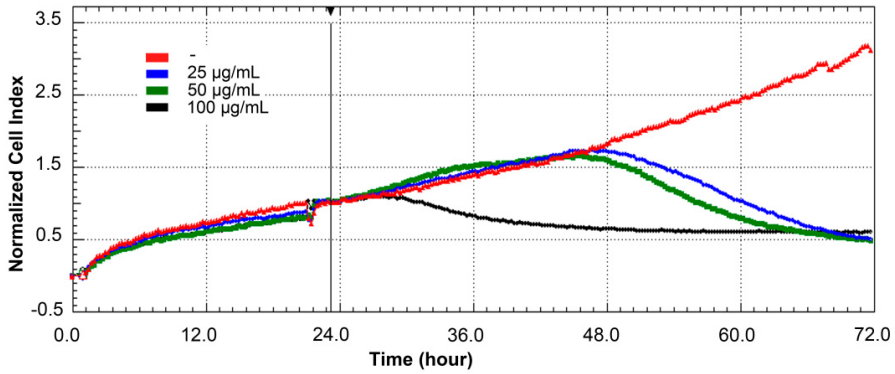

Figure 4: Effect of protamine nanocapsules (TCPH) on the viability of RAW 264.7 cells at 24 and 48 hours, determined by xCELLingence system (n=4± SD). Different concentrations of nanocapsules (25 in blue, 50 in green and 100 in black µg/ml) were incubated. Red line was the negative control (cells incubated only with medium). Vertical black line indicates when nanocapsules were added to the cells (after 18 hours of growing)

To study the cellular uptake of the nanocapsules, we incubated, fluorescent nanocapsules (labeled with TAMRA) with RAW 264.7 cells for up to 30 min. Before incubation, we verified that the physicochemical characteristics of the protamine nanocapsules were not affected by the use of TAMRA-labeled protamine (date not shown).

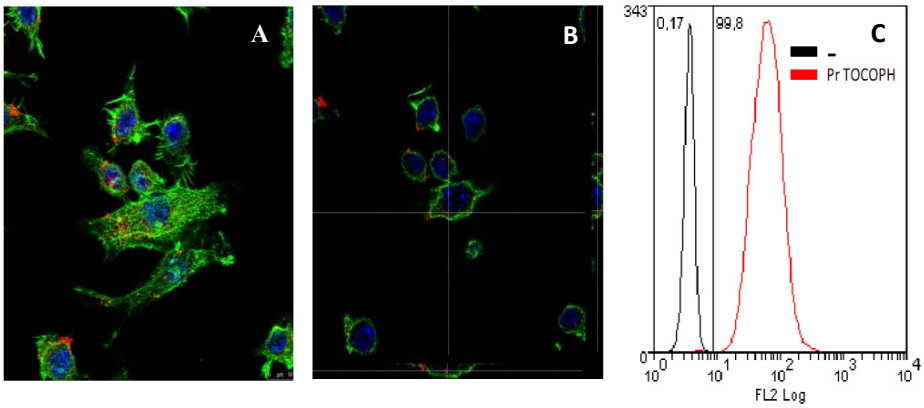

Figure 5: Internalization of TAMRA-labeled protamine nanocapsules (TCPH nucleus), at theoretical concentration TAMRA-Pr of 50 µg/ml in RAW 264.7 cells measured using two different techniques: Confocal microscopy (green channel: Alexa fluor 488-phalloidin, blue channel: DAPI and red channel: TAMRA-Pr NC): image of the maximum projection of a z-stack (A) and orthogonal section of the same z-stack (B) and flow cytometry analysis (C).

The confocal microscopy images presented in **Figure 5A-B** show that the fluorescent nanocapsules are located inside the cells and also attached to the cell membrane. In **Figure 5B**, which shows confocal images using orthogonal sections on the z stack it is possible to observe the presence of the nanocapsules inside the cells. Finally, using flow cytometry studies (**Figure 5C**) we could observe that almost 100 % of the cells were fluorescent after incubation with the nanocapsules. These results are in agreement with those previously reported for protamine-oligonucleotides complexes incubated with U937 cells [25]. The well-known membrane-translocating properties of protamine shared with other polysaccharides [26] or polypeptides such as Tat peptide [8], described in the literature, could be responsible for this high internalization of the nanocapsules. In this sense, the positive surface charge of protamine is expected to play an important role on the important cellular uptake [27].

Cytokine secretion mediated by protamine nanocapsules in blood mononuclear cells

Cytokines are small peptides, proteins or glycoproteins that have an important role in T-cell mediated immunity, inflammatory response, cancer, autoimmunity or allergy [28]. In the design of vaccines and antigen delivery systems the study of these endogenous molecules is very important due to their role, not only in antibody response, but also in cell-mediated immunity. In fact, cytokines are the main messengers between T cells, macrophages, dendritic cells and other immune cells involved in the immune response.

A way of obtaining preliminary information in this regard is to analyze the cytokines secretion profile of blood mononuclear cells exposed to the nanocapsules. More precisely, the cytokine profile provides estimative information about the implication of T helper (Th) cells in the immune responses. For example, interleukin 12 (IL-12), IL-2, interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β) are produced by Th1 cells. In contrast, IL-4, IL-5, IL-6 and IL-10, have a Th2 profile and, thus, are involved in the development of humoral responses.

Additionally there are other cytokines that have an important role in the development of the immune response, such as the proinflammatory cytokines IL-1β, IL-8 and TNF-α, all of them mainly produced by macrophages. IL-1β is involved in inflammatory responses and in cellular activities like apoptosis [29], IL-8 is a potent chemotactic for lymphocytes and neutrophils [30], and tumour necrosis factor alfa (TNF-α) is involved in the inflammation processes, destroys tumour cells, regulates immune responses and can induce cell proliferation or apoptosis [31].

The different kind of cytokines induced by protamine nanocapsules was evaluated in human peripheral blood mononuclear cells at two different doses

(10 and 100 µg/ml). The results summarized in **Table 2** indicate that, regardless of the nature of the oily core, both types of nanocapsules induce the production of a mixture pattern of cytokines, mostly proinflammatory (IL-8, IL-1 β and TNFα), but also IL-2 and TNFα (Th1 profile) and IL-4 and IL-6 (Th2 profile). It can also be noted that the secretion of some cytokines was stimulated in a concentration dependent manner, whereas others (IL-5 and IL-10) were not secreted at any concentration tested.

This mixed pattern of cytokine secretion is in agreement with those previously reported using protamine-condensed mRNA [32]. The protamine, but not mRNA alone, was able to strongly activate blood mononuclear cells and promote cytokine secretion, although the type of cytokines induced there were IL-2 and IL-4. The same authors have also shown that protamine-mRNA complexes can activate dendritic cells [33].

Overall, these results allowed us to speculate about the potential immunostimulating properties of protamine nanocapsules.

Complement activation capacity of protamine nanocapsules

The complement system is a family of several proteins and cell surface receptors that act in a cascade manner, participating in the activation of both innate and adaptative immunity. Although it can be activate by three pathways: classical, lectine and alternative [34], there are some common elements to all routes, such as the cleavage of complement factor 3 (C3). Thus, the analysis of the C3 cleavage by Western blot is indicative of the activation of the complement cascade by any of the three routes.

Table 2: Cytokines production by human peripheral blood mononuclear cells incubated with protamine nanocapsules with TCPH in the core at two doses (10 and 100 µg/ml). The positive control was LPS (1 µg/ml) plus PHA. N/tested: number of responsive persons / three donors tested**.** (10 µg/ml+: 1-10. ++: 10-100. +++: 100-1000. ++++: > 1000 fold higher than negative control (cells incubated in culture medium).

		Concentration (µg/ml)	Pr-NC		LPS + PHA	
			Response	N/tested	Response	N/tested
Th1 Profile	**IL 12**	10	+	1/3	++	3/3
		100	-			
	IFN γ	10	-	-	+++	3/3
		100	-	-		
	IL 2	10	++	1/3	++	3/3
		100	++	1/3		
	TNF β	10	+	1/3	++	2/3
		100	++	1/3		
Th2 Profile	**IL 10**	10	-	-	++	1/3
		100	-	-		
	IL 6	10	++	2/3	++++	3/3
		100	++	2/3		
	IL4	10	++	1/3	++	2/3
		100	+++	1/3		
	IL5	10	-	-	+	1/3
		100	-	-		
Other proinflammatoy Cytokines	**IL 8**	10	+++	3/3	++++	3/3
		100	++++	3/3		
	IL 1 β	10	+++	1/3	+++	3/3
		100	+++	3/3		
	TNF α	10	++	1/3	++++	3/3
		100	++	1/3		

Figure 6 shows that both types of protamine nanocapsules, containing either SQL or TCPH, induce C3 cleavage at a concentration range of 50-500 µg/ml. This result may be due to the interaction of the guanidine groups of the protamine present in the nanocapsules shell with the C3b α-chain [35]. However, the intensity of this interaction was also affected by the nature of the oily core.

Namely, TCPH-containing nanocapsules induced complement activation at a concentration of 25 µg/ml, whereas those containing SQL were only active at higher concentration (100 µg/ml). Consequently, the complement activation capacity of protamine nanocapsules does not only depend on the protamine shell, but also on the oil present in the core.

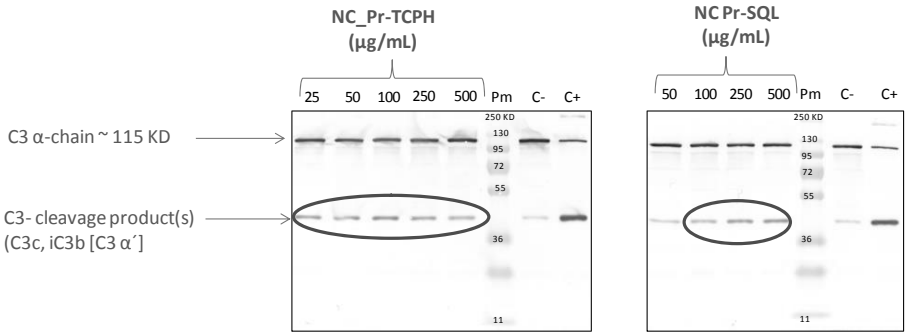

Figure 6: Complement activation induced by protamine nanocapsules analyzed by Western blot. Different concentrations of protamine nanocapsules with TCPH (left) or SQL (right) were studied. Negative control was PBS (C-) and positive control was cobra venom factor (C+).

In the case of vaccine design, the complement activation activity caused by the delivery carrier, rather than being a negative feature, could be considered an indication of its capacity to induce dendritic cell maturation and, thus, to initiate adaptive immunity responses. In fact, the activity of alum (the most used adjuvant in vaccines) is highly related to its ability to activate the complement cascade [36].

Moreover, there is information in the literature showing the effect of the particle size on the complement activation capacity. For example, Pedersen *et al* found that dextran-coated iron oxide core particles of a size around 250 nm induced greater complement activation than larger particles (600 nm) [37]. Therefore, the size of protamine nanocapsules might additionally contribute to the inherent activation properties of its constituents. Overall, because of their size, structure and composition and their synergistic effect on the complement

activation, protamine nanocapsules could be considered as promising adjuvants

Immune response of rHBsAg-loaded protamine nanocapsules

On the basis of the positive features of protamine nanocapsules containing both, an antigen and two immunostimulating biomaterials (protamine and oil), our next goal was to assess their immune response upon in vivo administration and to analyze the potential contribution of the oil core to the intensity of the response generated. More precisely, we analyzed the humoral specific immune response (anti rHBsAg serum IgG) elicited by these nanovaccines following either intramuscular (i.m.) or intranasal (i.n.) administration to BALB/c mice. Alum-adsorbed rHBsAg administered i.m. in two doses was used as a positive control.

Immunization by the intramuscular route

The formulations were administered at the dose of 10 μg at time cero and after four weeks**.** The results in **Figure 7** indicate that both nanocapsule formulations elicited steady antibody levels in the range of 100–1000 mIU/ml.

In contrast, the alum vaccine led to a very high initial response that decreased over the time, with antibody levels at the end of the study, which were close to those obtained by our nanocapsules. Although the decrease of the antibodies occurs in all formulations, it was much more acute in the case of the alum vaccine (**Figure 7**). It is worthwhile to indicate that the levels elicited by the nanovaccines are far above to those considered to be protective in humans (10 mIU/ml) [13] and are in the same order of magnitude of those achieved by other nanovaccines containing the same antigen [22, 38].

On the other hand, the statistical analysis of the response elicited by the two nanocapsular formulations, led us to conclude that protamine nanocapsules

containing TCPH elicited a greater response than those containing SQL. This is in agreement with the capacity of these two formulations to activate the complement. This result is in accordance with the ones obtained by Morel *et al*, who studied the ASO3 (adjuvant that has α tocopherol and squalene) showing that the presence of α tocopherol increased the secretion of different cytokines and that this component it is necessary for an optimal antibody response [39].

Overall, these results suggest the potential utility of protamine nanocapsules as a new generation of thermostable adjuvants. It should be, however, accepted that the antigen model selected in this study, rHBsAg, might not be the one benefitting the most from this nanotechnology, this being due to the great adjuvant effect of alum for this specific vaccine

We could, thus, speculate that the benefit of this novel adjuvant carrier might be of greater value for other antigens for which alum does not give the adequate response.

Immunization by the intranasal route

Because of the positive surface charge of protamine nanocapsules and, thus, their potential interaction with negatively charged mucosal surfaces, we decided to explore their potential as antigen carriers for nasal immunization. The humoral IgG responses elicited upon nasal immunization with 3 doses of rHBsAg-loaded protamine nanocapsules are shown in **Figure 7B**. The results show significant and steady IgG levels all along the study. Even though these levels were slightly lower than those achieved upon i.m. injection of the alum-vaccines, they were above the threshold reported for a protective response in humans (10 mlU/ml) [13]. This protective response is in line with the results reported by our group for chitosan-based nanoparticles and nanocapsules with tetanus toxoid [40] and rHBsAg [16, 22]. The positive superficial charge is a common parameter in these formulations. In fact, the positive charge has been

demonstrated to enhance the immune response [41], inducing the maturation and improving the function of antigen presenting cells.

On the other hand, in agreement with the results obtained upon i.m. immunization, the formulation containing TCPH exhibited a higher response than the one containing SQL. Consequently, these results confirm the role of the oily core plays in the adjuvant activity of these novel nanocapsules.

Finally, we also evaluated a combined i.m. - i.n. administration schedule consisting of one intramuscular dose followed by two intranasal doses. Interestingly, this protocol of administration led to a response that was comparable to the one obtained by the same formulation (TCPH nucleus) administered in two doses through the i.m. route. Therefore this schedule could be of interest in cases where the response achieved by simple nasal administration does not reach the levels required for an adequate protection.

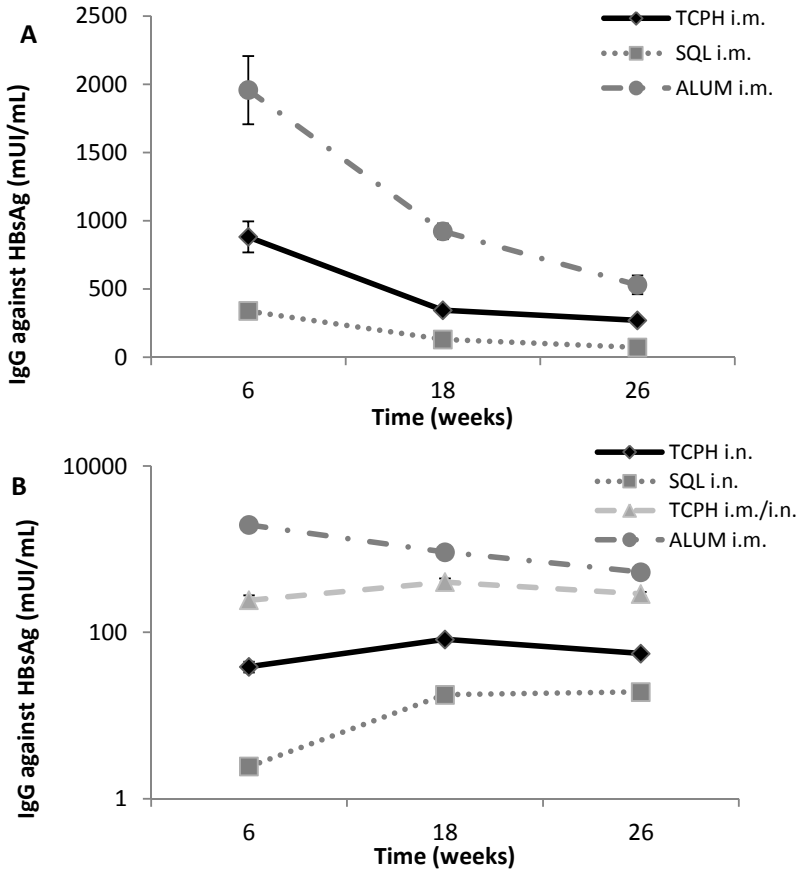

Figure 7: Serum antibody titers of BALB/c mice immunized with protamine nanocapsules containing squalene (SQL) or α tocopherol (TCPH). (A) Serum IgG titers after 2 immunizations (0 and 28 days) with rHBsAg associated to nanocapsules or adsorbed in alum by intramuscular (i.m.) route. (B) Serum IgG titers after 3 immunizations (0, 28 and 56 days) with rHBsAg associated to nanocapsules by intra nasal route (i.n.), or a combined schedule where the first dose was i.m. and the boosters were by i.n. compared with rHBsAg adsorbed in alum i.m. in two doses (0 and 28 days); for all cases the dose was 10 µg rHBsAg/mice. In both graphics the values at the each time point are significantly different from each other ($p<0.05$).

Modulation of immune response

In order to study the effect of protamine nanocapsules in the modulation of the immune response, IgG1 and IgG2 levels were quantified in sera of mice immunized with the carriers including TCPH in their core. The ratio of serum IgG1/IgG2 subtypes indicates the predominant type of immune response

generated, either humoral or cellular response, mediated by Th2 or Th1 lymphocytes, respectively. From our results the conclusion is that the type of immune response elicited by protamine nanocapsules containing TCPH is dependent of the route of administration. Namely, following i.m. immunization the nanocapsules produce a strong and long-lasting Th2-type response, this being the one typically observed for alum-based vaccines [42]. On the other hand, following intranasal administration, we observed a predominant Th2 response in early time points, which evolved towards a balanced Th1/Th2 response (**Figure 8**). Finally, the combination schedule of i.m. and i.n. administration gave a response comparable to the one observed after i.m. administration alone.

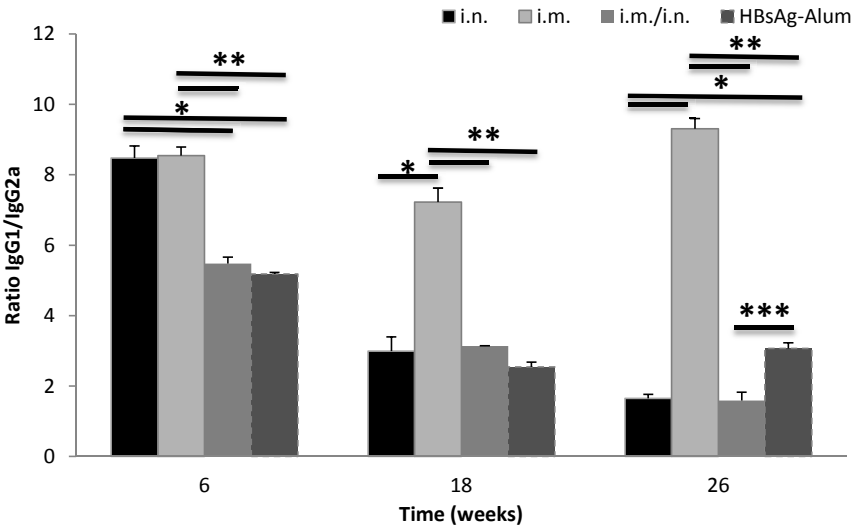

Figure 8: Comparison of the IgG subtype ratio (IgG1/IgG2) elicited by protamine nanocapsules with α tocopherol in the core by intramuscular, nasal or combined schedule of administration. The ratio of alum-adsorbed rHBsAg is shown as control. (* $p<0.05$).

The predominant humoral *vs.* cellular response elicited by the antigen-loaded nanocarriers might be attributed to different patterns of interaction of the nanocapsules with the antigen presenting cells and naïve Th cells [43]. These results confirm what was found in the cytokine profile after the *in vitro*

activation of blood monocytic cells (**Table 2**) and are also in accordance with those reported for liposomes and trimethylchitosan nanoparticles encapsulating OVA [44].

Overall, these results suggest the potential of protamine nanocapsules as adjuvants for parenteral administration and nasal administration. Moreover, it is also possible to combine both modalities of administration in order to achieve both, humoral and cellular responses.

4. CONCLUSIONS

Here we present a new thermostable vaccine delivery vehicle, protamine nanocapsules, which may represent a promising alternative to the currently available adjuvants. This is based in a number of relevant features, such as: (i) it may load simultaneously antigens and immunomodulators such as oils and oily soluble compounds; (ii) it preserves the stability of the associated antigen for at least one year at room temperature; (iii) it is able to stimulate human immune cells and promote the secretion of cytokines that are relevant for immunostimulation (iv) it is able to elicit humoral and cellular responses directed against the associated antigen following i.m. and nasal administration, respectively. In conclusion, these nanocapsules offer interesting opportunities for the formulation of challenging antigens for which a mixed Th1/Th2 response might be required. Furthermore, the represent a promising option for needle-free vaccination strategies and for the preservation of the stability of antigens at room temperature. Current studies are aimed at assessing the value of this novel technology for other antigens.

5. ACKNOWLEDGEMENTS

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DISCUSIÓN GENERAL

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La protamina pertenece a una familia de polipéptidos ricos en arginina con capacidad para atravesar membranas biológicas, ampliamente estudiada en vehiculización de material genético. Recientes estudios han demostrado que puede estimular el sistema inmune [1]. Esta conjunto de moléculas está aprobado por la FDA y la EMA como fármaco y también como excipiente, siendo su principal uso el unirse a la insulina para realizar una formulación de acción retardada [2]. Esta particularidad, que podría ser considerada trivial, es una gran característica si se pretende llevar un producto al mercado en el menor tiempo posible, ya que su perfil de seguridad ha sido testado durante varios años. Con este antecedente se ha escogido este material de partida y se ha tratado de desarrollar diferentes tipos de formulaciones de tamaño nanométrico, utilizando tecnología sencilla para la vehiculización y liberación de antígenos.

Como se puede apreciar en la **Figura 1**, dos son los tipos de nanosistemas que se han desarrollado y optimizado en el presente trabajo. En una primera etapa, se desarrollaron nanopartículas de protamina junto con un polisacárido aniónico. En una fase posterior, se desarrollaron nanocápsulas de protamina, las cuales han sido optimizadas con diferentes aceites en su núcleo con capacidad adyuvante y con el fin de poder asociar el antígeno a su superficie catiónica. Esta acción multifuncional pretende presentar el antígeno asociado y en conjunto poder liberar un aceite con capacidad de co-estimular al sistema inmune.

Desarrollo y caracterización fisicoquímica de los nanosistemas

Las nanopartículas de protamina fueron preparadas a través de la técnica de reticulación iónica. Este sencillo procedimiento nos permite encapsular moléculas delicadas como antígenos o material genético, ya que no necesita

fuerzas como sonicación o extrusión, que podrían dañarlo [3]. Para el desarrollo de estas formulaciones, fue necesaria la adición de un polisacárido que pueda gelificar en presencia de la protamina. Para ello se escogieron como contra iónes el ácido hialurónico y el alginato, ya que presentan ambos estudios de biocompatibilidad y un reconocido perfil de seguridad [4, 5]. La adición de cualquiera de estos dos polisacáridos sobre la protamina permite obtener partículas de tamaño nanométrico, con una baja polidispersión y diferentes cargas superficiales dependiendo del componente mayoritario en la formulación. En forma general cuando es protamina, el potencial zeta es positivo, y cuando el componente mayoritario es el polisacárido se puede apreciar una inversión de carga. Como se puede ver en la **Tabla 1** las formulaciones que tienen alginato son levemente más pequeñas que las que poseen ácido hialurónico, en ambos casos se obtienen formulaciones con una adecuada estabilidad, por lo que se cree que la protamina está realizando el entrecruzamiento necesario para poder obtener las partículas mencionadas. Otras aminas endógenas con carga positiva han sido utilizadas como reticulantes de polímeros cargados negativamente para la elaboración de nanopartículas a través de esta misma técnica de preparación [6].

Respecto al diseño racional de las nanocápsulas de protamina, se realizó a través de la técnica de desplazamiento del solvente, la cual ha sido adaptado previamente por nuestro grupo de investigación para otro tipo de nanocápsulas polipéptidicas como de poliarginina [7], ácido poliglutámico [8] o poliasparagina [9]. El desarrollo de este tipo de sistema, que presenta una estructura tipo núcleo-corona, se ha centrado en la posibilidad de poder superar barreras biológicas (ej: mucosas, membrana celular, etc), por lo que puede ser una plataforma de vehiculización de antígenos y de otro tipos de moléculas (ya sean principios activos, material genético, etc).

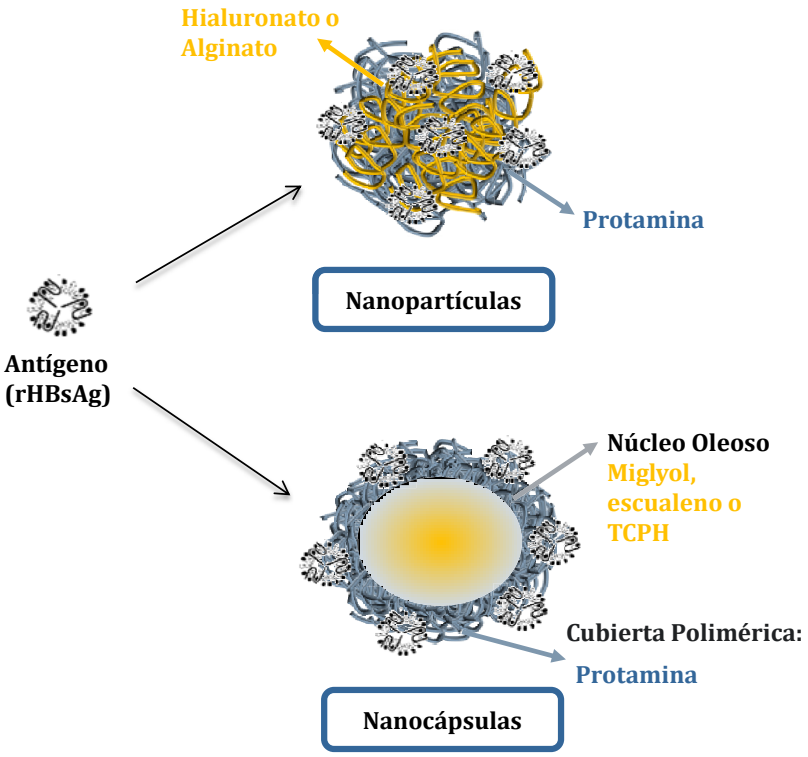

Figura 1: Diagrama de los nanosistemas a base de protamina desarrollados y la estrategia utilizada para la encapsulación de antígenos.

En una primera etapa, el desarrollo y optimización de este sistema nanocapsular se basó en la búsqueda de nuevos surfactantes ya que la lecitina, componente que se emplea regularmente en su preparación, interacciona con la protamina ocurriendo una desestabilización del sistema por medio de floculación [10]. Para ello se buscaron surfactantes con un valor de Balance Hidrófilo Lipófilo (HLB) mayor a 8, para poder estabilizar la emulsión aceite en agua (o/w), que no fueran fosfolípidos y preferiblemente que tuvieran en su estructura polietilenglicol (PEG) para aprovechar las características propias de este polímero como aumentar la estabilización de las nanocápsulas y evitar su opsonización por el sistema fagocítico mononuclear (MPS) [11].

La versatilidad propia de este sistema permite introducir en su núcleo oleoso aceites que presenten actividad inmuno-adyuvante como lo son el escualeno

(SQL) [12] y el α tocoferol (TCPH) [13], ambos presentes en formulaciones de vacunas aprobadas para su uso en humanos [14]. Por otra parte, es posible modular las características fisicoquímicas en terminos de tamaño y carga superficial dependiendo de la proporción utilizada respecto al ratio aceite/surfactante y fase acuosa [15].

En la **Tabla 1** se puede observar las características fisicoquímicas de las formulaciones de nanocápsulas con Miglyol®, SQL y TCPH como núcleo oleoso. Las nanoestructuras desarrolladas presentan tamaño nanométrico, una baja polidispersión y un potencial zeta positivo, indicativo que la nanoemulsion de aceites ha sido recubierta por la protamina. Se utilizaron combinaciones de PEG-estearato y colato de sodio como surfactantes observandose una mayor adsorción de la cubierta del polipéptido catiónico (cercano al 60% cuantificado por UPLC), respecto a otros como Tween® 80 y 20. Macrogol 15, etc.

Comparando las nanopartículas con las nanocápsulas desarrolladas, nos encontramos con nanopartículas protamina:HA y protamina:ALG que poseen potencial zeta modulable y tamaños más pequeños que las nanocápsulas de protamina, las cuales presentan siempre potencial zeta positivo, que indica la presencia del polipéptido en superficie.

Tabla 1: Caracterización fisicoquímica de las formulaciones desarrolladas. NP: nanopartículas. Pr: Protamina. HA: Hialuronato. ALG: alginato. NC: nanocápsulas. TCPH: α tocoferol.

Formulación	Tamaño (nm)	PDI	Potencial ζ (mV)
NP Pr:HA 1:4	126 ± 13	0.2	-36 ± 4
NP Pr: ALG 1:4	94 ± 4	0.2	-31 ± 7
NP Pr:HA 1:4	265 ± 1	0.1	+21 ± 3
NP Pr: ALG 1:4	100 ± 6	0.1	+19 ± 2
Pr-NC (Miglyol)	277 ± 5	0.2	+21 ± 5
Pr-NC (TCPH)	241 ± 10	0.2	+34 ± 3

Asociación de antígeno a los nanosistemas desarrollados

El principal antígeno utilizado en nuestros estudios fue el antígeno recombinante de la hepatitis B (rHBsAg). El incluir esta molécula en sistemas nanométricos ya se considera un reto, ya que este antígeno es per se una partícula de 22 nm con carga superficial negativa [16].

Como se puede ver en la **Figura 1** la estrategia utilizada en el desarrollo de las nanopartículas se basa en la incorporación del antígeno en su matriz polimérica, hecho que tiene lugar en un paso en el momento de su preparación. Esto permite que el antígeno quede protegido. En el caso de las nanocápsulas la estrategia es asociar el antígeno a las mismas a través de diferentes tipos de fuerzas, principalmente interacciones electrostáticas. En este caso la incorporación del antígeno tiene lugar en un paso posterior a la elaboración de las nanocápsulas, permitiendo que el antígeno quede expuesto y pueda ser fácilmente reconocido por el sistema inmune. Asimismo se evita que el antígeno esté en contacto con los solventes orgánicos durante proceso de preparación. Esta novedosa estrategia ha sido ya testada en otras plataformas nanométricas, como SVP^{TM} (del inglés: Synthetic Vaccine Particles), donde el antígeno se encuentra en la corona de la nanoestructura. Se trata de una tecnología segura, escalable y producida bajo condiciones GMP, la cual está siendo testado para diferentes enfermedades infecciosas, cáncer y enfermedades respiratorias [17].

Los nanosistemas fueron visualizados mediante microscopia electrónica de transmisión (TEM). Las imágenes obtenidas tanto para nanopartículas como para nanocápsulas muestran una población homogénea de partículas y de forma esférica **(Figura 2).**

En la **Tabla 2** se puede observar que las nanopartículas de protamina encapsulan eficazmente el rHBsAg en su entramado polimérico, con un leve

incremento del tamaño y sin variar las propiedades de carga superficial. Asimismo, las nanocápsulas de protamina asocian también este mismo antígeno, si bien en una menor proporción respecto a las nanopartículas, pero sin modificar considerablemente el tamaño de las nanoestructuras. El potencial zeta se ve disminuido, haciendo evidente la interacción del antígeno con la cubierta de protamina. Este resultado es similar para todos los prototipos desarrollados con núcleo de SQL o TCPH, demostrando que la asociación de este antígeno depende principalmente de la cubierta polipeptídica y no del aceite presente en el núcleo.

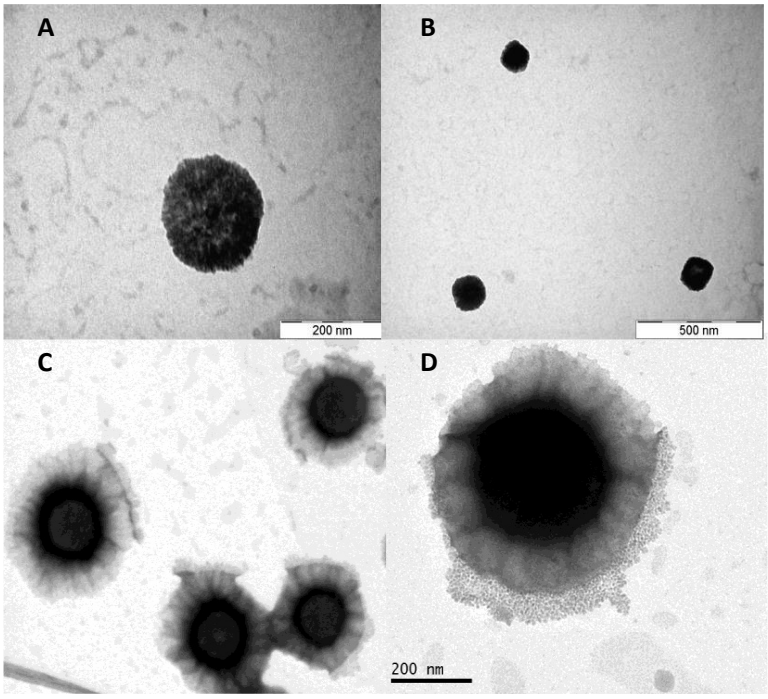

Figura 2: Imágenes de microscopia electrónica de transmisión. A-B: Nanopartículas de Protamina:HA 4:1. C-D: Nanocápsulas de protamina. HA: ácido hialurónico.

Para estudiar la versatilidad de las nanocápsulas de protamina, también se asoció a su estructura el antígeno de influenza H1N1, una proteína modelo de 63 kDa y punto isoeléctrico cercano a 7. Como se observa en la **Tabla 2**, su asociación tampoco varia respecto al rHBsAg, asociando eficazmente este tipo

de antígeno. De este modo queda demostrado que la formulación desarrollada puede transportar antígenos de distintas estructuras químicas y tamaño (tipo partícula viral *vs* proteica).

Tabla 2: Caracterización fisicoquímica de las formulaciones desarrolladas asociando el antígeno recombinante de la hepatitis B (rHBsAg) y el antígeno de influenza (HI). NP: nanopartículas. Pr: Protamina. HA: Hialuronato. NC-Pr: nanocápsulas de protamina. SQL: escualeno. TCPH: α tocoferol.

Formulaciones	Loading	Tamaño (nm)	PDI	Potencial ζ (mV)	Eficacia de encapsulación (%)
Pr: HA 1:4	-	126 ± 13	0.1	-37 ± 4	-
	5 % rHBsAg	197 ± 10	0.1	-31 ± 3	92 ± 1
P: HA 4:1	-	265 ± 10	0.2	+21 ± 3	-
	5 % rHBsAg	563 ± 38	0.2	+23 ± 2	90 ± 2
NC- Pr (SQL)	-	215 ± 5	0.1	+18 ± 1	-
	4:1 (NC:rHBsAg)	226 ± 19	0.2	+8 ± 3	82 ± 1
NC- Pr (TCPH)	-	250 ± 2	0.2	+36 ± 3	-
	4:1 (NC:rHBsAg)	210 ± 38	0.2	+20 ± 5	78 ± 13
	4:1 (NC:HI)	205 ± 10	0.1	+13 ± 2	71 ± 10

Para ambos sistemas se estudió la integridad del antígeno una vez asociado mediante la técnica de Western blot y como se esperaba, tanto la reticulación iónica, para las nanopartículas, como el desplazamiento del solvente, para las nanocápsulas, no afectan la estructura y por ende la antigenicidad de la molécula asociada.

Estabilidad de los sistemas y estudios de liofilización

El estudio de estabilidad de ambos sistemas se realizó analizando la evolución del tamaño a través del tiempo en distintas condiciones. De forma general se puede concluir que tanto nanopartículas como nanocápsulas han mantenido un tamaño constante por lo menos 3 meses en condiciones de almacenamiento (4ºC), además de presentar un buen perfil de estabilidad en PBS a 37ºC (para simular condiciones biológicas).

A pesar de que los resultados obtenidos en condiciones de almacenamiento fueron buenos, una vacuna termoestable que fuera capaz de evitar la cadena de frío tendría un gran impacto, sobre todo en los países en vías de desarrollo. Para ello nos planteamos el uso de la liofilización como una estrategia para poder aumentar la estabilidad de las diversas formulaciones y poder probar su termo-estabilidad.

Los resultados tanto para las nanopartículas como para las nanocápsulas desarrolladas demostraron que el tamaño de partícula no sufre cambios significativos tras el proceso de liofilización y reconstitución. Es más, para ambos sistemas se ha comprobado que no es necesario el uso de otro excipiente como crioprotector, lo cual puede deberse a la arginina presente en la protamina, ya que este aminoácido ha sido utilizado para prevenir daños en la composición de vacunas en el proceso de liofilización [18].

Con esta información, se quiso avanzar un paso más y se liofilizaron muestras de nanocápsulas de protamina cargadas con rHBsAg, en presencia de trehalosa al 5%. El uso de trehalosa responde a que consideramos que sea necesaria para la estabilidad del antígeno. Estas muestras fueron almacenadas a 25 ºC y resuspendidas a diferentes tiempos a lo largo de un año. Como se puede observar en la **Figura 3**, las nanocápsulas con TCPH en su núcleo y cargadas con rHBsAg mantienen su tamaño nanométrico y su carga superficial al menos durante 12 meses. Más aún, las imágenes de Western blot demuestran que el antígeno asociado a las nanocápsulas liofilizadas conserva de mejor manera su integridad estructural que el control de antígeno liofilizado y conservado a la misma temperatura después de haber sido resuspendido.

Con estos resultados, podemos concluir que las nanocápsulas de protamina cargadas con el antígeno rHBsAg son termoestables durante un período de al menos 12 meses.

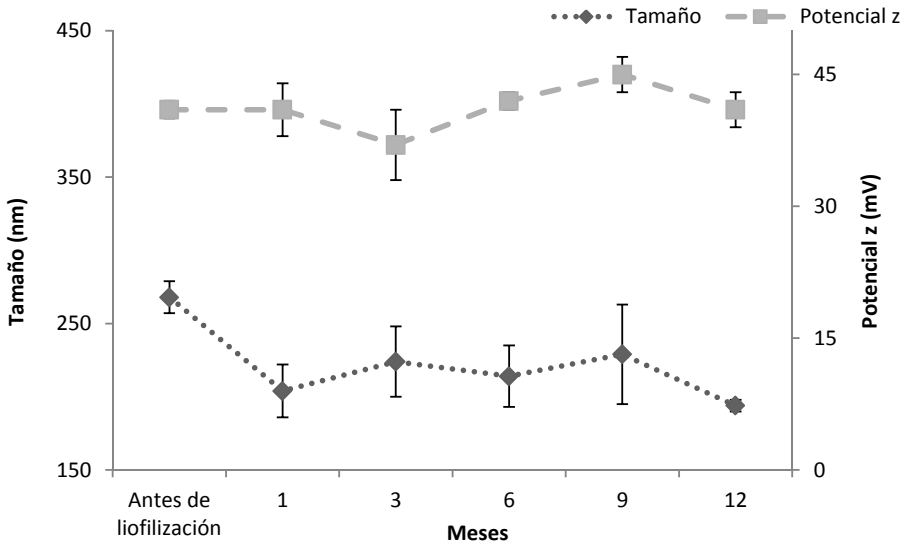

Figura 3: Caracterización fisicoquímica de la formulación de nanocápsulas de protamina (núcleo de TCPH) cargadas con el antígeno recombinante de la hepatitis B (rHBsAg), conservada a temperatura ambiente (25ºC) y reconstituida a diferentes meses. (n=3 ±DS). (TCPH: α tocoferol)

Estudios *in vitro*

Se realizaron diversos estudios en células inmunocompetentes, para poder observar la interacción de los nanosistemas desarrollados con el sistema inmune.

La influencia de los diferentes sistemas sobre la viabilidad celular fue testada en macrófagos RAW 264.7, a través de 3 métodos diferentes. La elección de uno u otro sólo responde a poder encontrar diferencias entre ellos, lo cual no fue posible. Para las diferentes formulaciones de nanopartículas testadas (protamina:HA 4:1 y 1;4), así como dos de las formulaciones de nanocápsulas de protamina (con SCL o TCPH en el núcleo) se pudo apreciar una baja toxicidad en un amplio rango de concentraciones.

El estudio de la interacción e internalización de las partículas desarrolladas se llevó a cabo en la misma línea celular (RAW 264.7) mediante microscopía confocal y citometría de flujo. Para ello fue necesario preparar los sistemas con

un marcaje fluorescente. Esto se logró a través de la unión de TAMRA (un derivado de la rodamina) con la protamina. El marcaje fluorescente no cambió considerablemente las propiedades fisicoquímicas de los sistemas obtenidos.

Tanto nanopartículas como nanocápsulas fueron reconocidas e internalizadas eficientemente por los macrófagos RAW 264.7. De manera particular, en la **Figura 4** se puede observar como tras 30 minutos de incubación las nanocápsulas de protamina son reconocidas y captadas por estas células (aproximadamente de 100%). Este hecho se puede atribuir a diversos factores; en primer lugar, la cubierta de protamina le confiere al nanosistema sus propiedades de translocación de membranas y ésto se refleja en un incremento de la internalización de los diferentes vehículos [19]. Por otra parte, es bien sabido que los sistemas nanométricos de carga positiva son eficientemente internalizados, incluyendo las células inmunocompetentes [20], propiedad que le aportan los grupos guanidinos de la protamina presente en la cubierta del sistema desarrollado.

Realizar una comparación entre los estudios de internalización de nanopartículas y los de nanocápsulas sería errónea, ya que las concentraciones utilizadas para ambos sistemas son diferentes y este parámetro es fundamental en la internalización de los sistemas nanométricos [21]. Sin embargo, la comparación entre ambos sistemas nanoparticulares demuestra un mayor reconocimiento e internalización de las formulaciones con carga superficial positiva, lo que confirma lo anteriormente señalado.

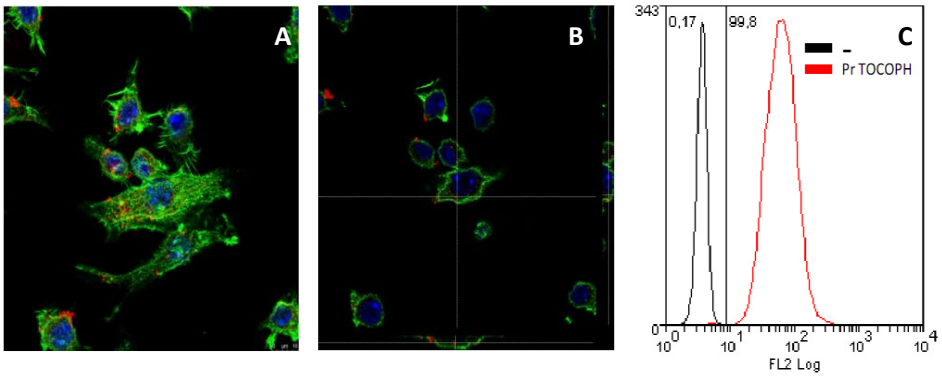

Figura 4: Internalización de nanocápsulas de protamina marcadas con TAMRA (Pr-TAMRA NC) (α tocoferol en el núcleo), a una concentración teórica de 50 µg/ml de Pr-TAMRA en una línea celular de macrófagos (RAW 264.7). A y B: imágenes de microscopía confocal (canal verde: Alexa fluor 488-faloidina, canal azul: DAPI y canal rojo: Pr-TAMRA NC). C: análisis de citometría de flujo.

De manera complementaria, se llevaron a cabo estudios de secreción de citocinas para los diferentes sistemas. En el caso de las nanopartículas, los ensayos fueron realizados en macrófagos de ratón (BALB/c) y se estudió la influencia de las partículas cargadas con el rHBsAg *versus* este antígeno solo. Como se puede ver en la **Figura 5**, la secreción de las citocinas estudiadas es siempre mayor cuando el antígeno está encapsulado en las diversas estructuras nanométricas. Entre los dos sistemas, las partículas con mayor cantidad de ácido hialurónico (Protamina:HA 1:4) estimulan mayor secreción de citocinas que el sistema protamina:HA 4:1, aunque solo en la secreción de IL1α se observan diferencias estadísticamente significativas. El perfil de ambos sistemas muestra su potencial como adyuvante en el proceso de inmunización, ya que la presentación del antígeno en forma particulada es capaz de modular y estimular el sistema inmune a través de la secreción de diversas citocinas.

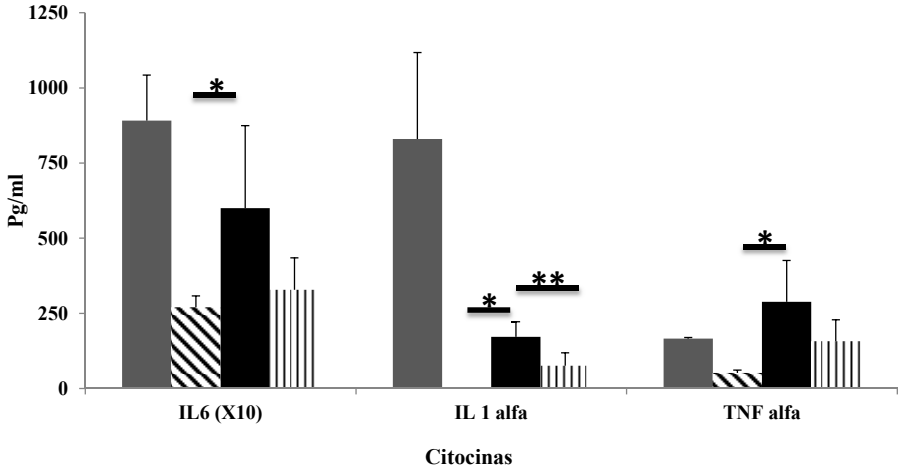

Figura 5: Citoquinas liberadas desde macrofagos murinos despúes de la estimulación de HBsAg solo (▨) o encapsulado en NP Pr:HA 1:4 (■) y NP Pr:HA 4:1 (▥). El control positivo fue LPS(▩). Diferencias significativas entre los diferentes grupos ($p<0,05$).

Para el estudio de citocinas en nanocápsulas se utilizaron células mononucleares procedentes de sangre periférica de 3 voluntarios sanos y se analizó el perfil de liberación de citocinas de los dos prototipos de nanocápsulas de protamina desarrollados (con SQL o TCPH en su núcleo). Las formulaciones blancas (sin antígeno) se evaluaron a dos concentraciones diferentes. Los resultados hallados señalan una importante secreción de citocinas pro-inflamatorias, encontrándose un balance entre las citoquinas con un perfil Th1 y Th2 sin hallar diferencias significativas entre ambas composiciones de nanocápsulas.

El último estudio *in vitro* realizado fue la evaluación de la capacidad de las nanocápsulas de protamina para activar el sistema del complemento. Dicho sistema es una familia de más de 25 proteínas que actúan en forma de cascada y participan, entre otras funciones, en la activación de la inmunidad innata y adaptiva [22]. Para estudiar su activación se analizó el factor de complemento C3 a través de Western blot. Se seleccionó el C3 por ser un punto común de las 3 vías de activación del complemento (i.e. clásica, alternativa y lectinas). Las

nanocápsulas de protamina fueron capaces de inducir la activación del complemento en un amplio rango de concentraciones, lo cual puede deberse a la interacción de los grupos guanidinos con la cadena α C3b [23]. Cabe destacar sin embargo que la formulación con TCPH activa el complemento a una dosis menor que aquella que posee SQL, lo que indicaría que no solo es la protamina la que interfiere en la activación del complemento sino también el núcleo oleoso, teniendo el TCPH un mayor perfil de activación.

Estudios *in vivo*

Las formulaciones de nanopartículas basadas en protamina y las nanocápsulas de este mismo polipéptido han sido testadas para estudiar su respuesta *in vivo*. Para el estudio de las nanopartículas se establecieron grupos de 10 ratones BALB/c a los que se les administró por vía intramuscular 10 µg de rHBsAg cargado en las nanopartículas de Protamina: HA 1:4 y 4:1, los días 1 y 28. El control fue la misma dosis de antígeno adsorbido en álum, como adyuvante convencional. Como se puede ver en la **Figura 6**, ambas formulaciones de nanopartículas inducen niveles elevados de anticuerpos contra la hepatitis; aunque si bien estos niveles son menores a los obtenidos por el antígeno adsorbido en el álum, son mayores a 10 mUI/mL, valor aceptado como protector contra esta enfermedad [24]. Existe diferencia entre ambas formulaciones, protamina:HA 4:1 presenta una mayor respuesta inmune, la cual puede deberse tal como se demostró en los estudios *in vitro,* a una mayor internalización por los macrófagos. Esta información es confirmada en la literatura, donde nanopartículas positivas inducen una mayor respuesta inmune que a las correspondientes estructuras neutras [25, 26].

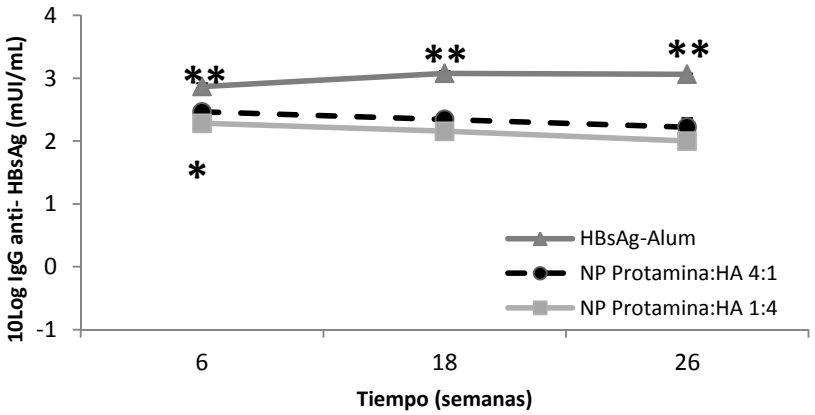

Figura 6: Respuesta inmune en ratones BALB/c inmunizados con nanopartículas de Protamina:HA 4:1 y 1:4 cargadas con 10 µg de antígeno después de 2 inmunizaciones (día 1 y 28) por vía intramuscular. Como control se utilizó la misma dosis adsorbida en alum. Resultado representan el promedio ± D.S. (n=10), ($p<0,05$).

En el caso de las nanocápsulas se les administró el rHBsAg las nanocápsulas de protamina con núcleo de TCPH o SQL a grupos de 7 ratones BALB/c, siguiendo el mismo régimen anteriormente mencionado para las nanopartículas. Como muestra la **Figura 7**, para ambas formulaciones se obtuvieron valores protectores y del mismo orden de magnitud que otros sistemas nanométricos conteniendo el mismo antígeno [27]. Al comparar ambas formulaciones, se puede observar que la formulación con TCPH en su núcleo desencadena una respuesta mayor que la producida por el SQL. Esto estaría en concordancia con la capacidad que posee esta formulación en activar el complemento. Más aún, en la literatura correspondiente al AS03, adyuvante que posee TCPH y SQL, demuestran que la presencia de TCPH es fundamental para una mayor respuesta inmune [28].

Es posible observar tanto para las nanocápsulas como para las nanopartículas que la respuesta generada por el adyuvante comercial es superior en ambos estudios. Esto puede deberse al reconocido efecto que tiene el álum con el rHBsAg, lo que abre la posibilidad de que esta tecnología probada con otros

antígenos pueda dar una respuesta similar o mayor que la formulación con álum.

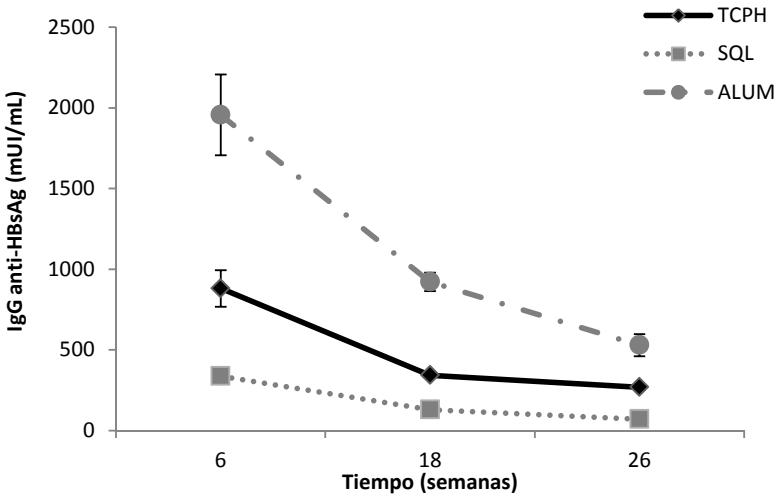

Figura 7: Respuesta inmune en ratones BALB/c inmunizados con nanocápsulas de protamina con escualeno (SQL) o α tocoferol (TCPH) en su núcleo y cargadas con 10 µg de antígeno después de 2 inmunizaciones (día 1 y 28) por vía intramuscular. Como control se utilizó la misma dosis adsorbida en alum. Resultado representan el promedio ± D.S. (n=7). Todos los puntos para un mismo tiempo presentan diferencias estadísticamente significativoas ($p<0,05$).

Uno de los desafíos de la inmunización actual es poder generar vacunas que sean libres de agujas, por ello se quiso explorar la posibilidad de estos vehículos en una formulación por vía nasal. Para ello, se cuantificó la respuesta humoral (IgG) producida tras 3 inmunizaciones de 10 µg de rHBsAg cargado en las diferentes formulaciones.

Para el estudio realizado con las nanopartículas, como se puede apreciar en la **Figura 8A**, solo la formulación de protamina:HA 4:1 logró proporcionar niveles protectores (con un pico de 80 mUI/mL a la semana 18). Esto puede deberse a su carga positiva, la cual permite una mayor interacción con el mucus que recubre el epitelio aumentando el tiempo de residencia en la cavidad nasal y por tanto teniendo una mayor probabilidad de cruzar esta barrera y llegar a las

células inmunocompetentes para desencadenar una respuesta inmune adecuada.

Respecto al mismo estudio con las nanocápsulas, en la **Figura 8B** se puede apreciar que con ambas formulaciones se obtienen valores protectores pero, al igual que en el estudio por vía intramuscular, se puede ver una diferencia mayor para la composición que posee núcleo de TCPH, resultados que confirman que el núcleo oleoso tiene un importante rol en el efecto adyuvante de estos sistemas.

Las formulaciones desarrolladas basadas en protamina puedan atravesar la barrera nasal y desencadenar una respuesta inmune. Más estudios acerca de su interacción con el mucus y un posible efecto mucoadhesivo serán necesarios para avalar esta hipótesis. Sin embargo, esto estaría en concordancia con lo descrito en la literatura donde se reporta que la protamina podría incrementar la permeabilidad celular en diversos epitelios [29], como por ejemplo el nasal.

Finalmente se evaluó un calendario combinado de administración intramuscular (primera dosis) con un refuerzo de dos dosis por vía intranasal. Para ello se utilizó la formulación de nanocápsulas de protamina con núcleo de TCPH, con la misma dosis utilizada en los estudios previos. Como se puede observar en la **Figura 8B**, la respuesta generada con este calendario es similar a la obtenida para la administración i.m., lo cual convierte a este calendario en una interesante estrategia de inmunización, ya que se puede obtener una adecuada protección y disminuir el uso de agujas así como los costes relacionados a ello.

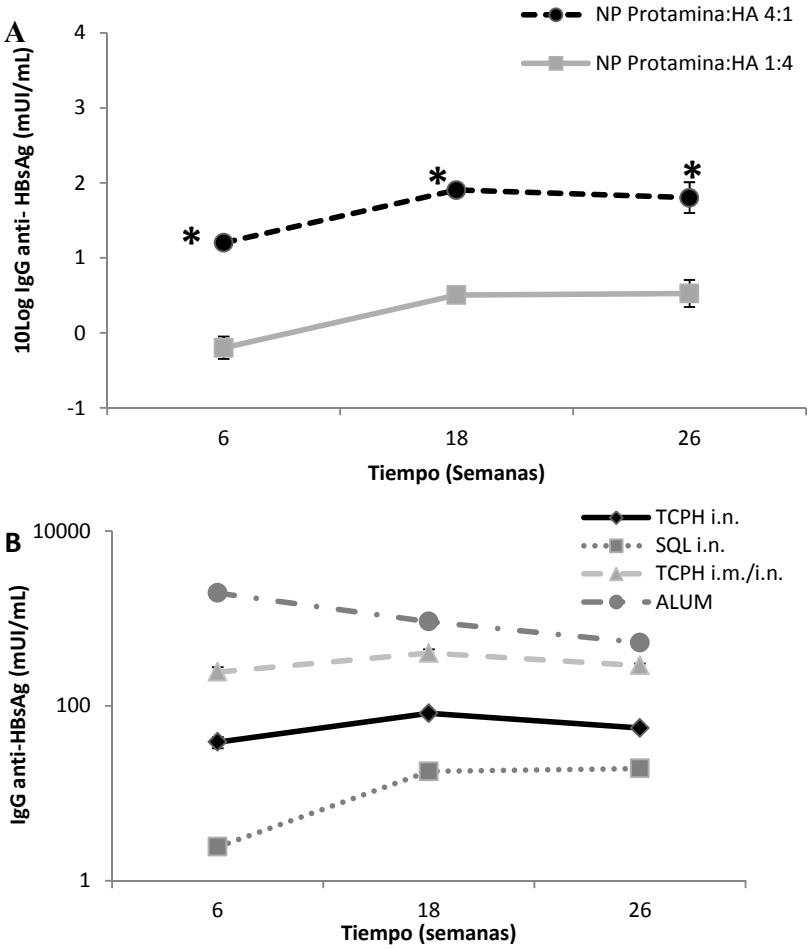

Figura 8: Respuesta inmune en ratones BALB/c inmunizados con los diversos nanosistemas basados en protamina por vía intranasal. **A**: nanopartículas de Protamina:HA 4:1 y 1:4 cargadas con 10 µg de antígeno después de 3 inmunizaciones (día 0, 28 y 56) (n=10). **B**: Nanocápsulas de protamina con escualeno (SQL) o α tocoferol (TCPH) en su núcleo y cargadas con 10 µg de antígeno después de 3 inmunizaciones por vía nasal (día 0, 28 y 56) y TCPH con una primera dosis intramuscular (i.m.) y dos refuerzos intranasal (día 28 y 56) (n=7). Resultado representan el promedio ± D.S. ($p<0,05$).

Con el fin de estudiar el efecto que poseen las nanocápsulas de protamina en la modulación de la respuesta inmune, se cuantificaron los niveles de IgG1 e IgG2 en los ratones que habían sido inmunizados con los sistemas de nanocápsulas de protamina con núcleo de TCPH y rHBsAg como antígeno. Como se puede observar en la **Figura 9**, la respuesta inmune generada es dependiente de la vía

de administración. Por vía intramuscular se obtiene una fuerte respuesta de tipo Th2 (humoral), respuesta típicamente observada en las formulaciones que utilizan álum como adyuvante [30]. Mientras que por vía nasal, aunque al inicio proporciona una respuesta de tipo Th2, en los siguientes sangrados se puede observar un balance de tipo Th1/Th2 (celular/humoral). El tipo de respuesta inmune generada por las nanocápsulas de protamina puede deberse a las diferentes formas de interacción del sistema con las células presentadoras de antígenos y con las células Th inmaduras y puede ser una información necesaria para el desarrollo de otros tipos de vacunas.

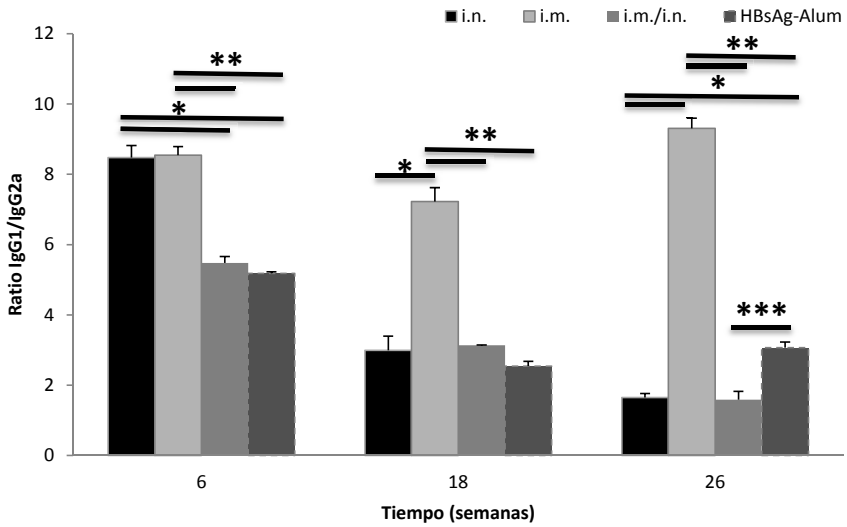

Figura 9: Comparación de los subtipos de IgG (ratio IgG1/IgG2) producido por la inmunización de nanocápsulas de protamina (con α tocoferol en el núcleo), cargadas con rHBsAg como antígeno, administrada por vía intranasal (i.n.), Intramuscular y con un calendario combinado (i.m./i.n.). El ratio del antigeno adsorbido en alum es mostrado como control. ($p < 0,05$).

Para observar la versatilidad que poseen las nanocápsulas de protamina con diferentes antígenos, se estudió su comportamiento *in vivo* con el antígeno de la influenza H1N1. Con este fin, grupos de 5 ratones BALB/c fueron randomizados e inmunizados con 2 y 7,5 µg de antígeno vehiculizado en las

nanocápsulas de protamina con TCPH en el núcleo. Como control se utilizó una dosis de 7,5 µg de antígeno adsorbido en álum y se cuantificó la respuesta antes de cada inmunización (semana 3 y 7) y en lasemana 28. En la **Figura 10** es posible observar el perfil de respuesta inmune generado. Las nanocápsulas de protamina presentan un primer pico elevado en la primera semana de monitorización comparado con el control, seguido de dos puntos levemente menores que el álum. Por otra parte, se puede observar que al utilizar 3 veces menos de dosis (2 µg) con la formulación de nanocápsulas, la respuesta inmune generada es levemente menor a la de 7,5 µg, sin existir diferencias significativas entre ambas, lo que abre una opción de poder administrar menor dosis demostrando así el efecto inmuno-potenciador de la formulación desarrollada.

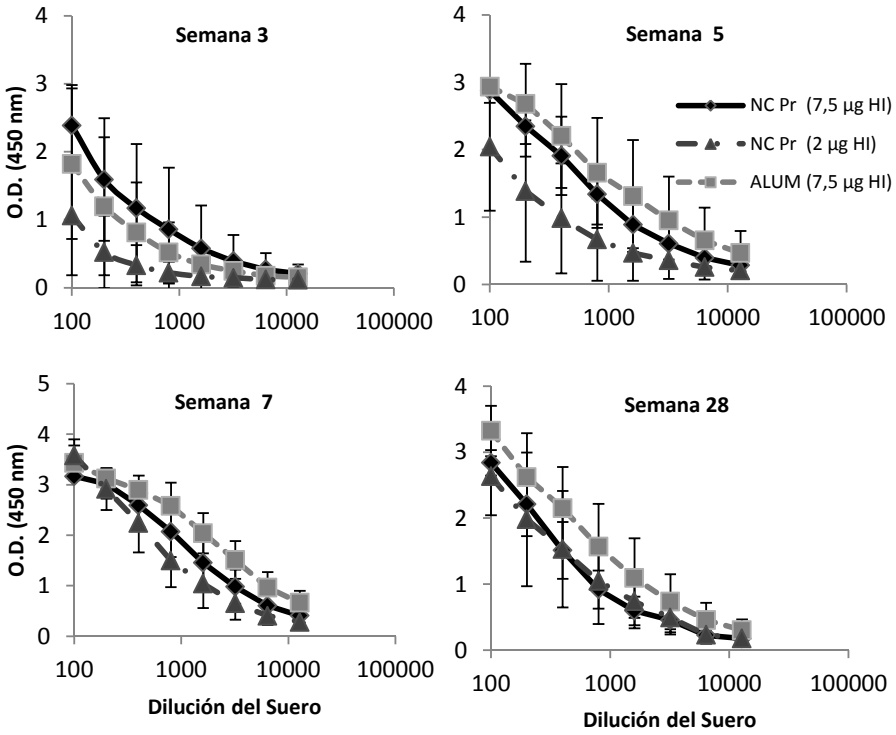

Figura 10: Niveles de anticuerpo neutralizante frente al antígeno de la influenza tras tres inmunizaciones a ratones BALB/c (0,3 y 5 semanas) por vía subcutánea con nanocápsulas de protamina (nucleo de α tocoferol) con dos diferentes dosis de antigeno: 7,5 µg (◆) and 2 µg (▲). El antígeno adsorbido en alum en una dosis de 7,5 µg fue usado como control (■).

Finalmente, como conclusión general se puede decir que se han desarrollado y optimizado nanopartículas y nanocápsulas a base de protamina, las cuales se presentan como sistemas versátiles, capaces de ajustar sus propiedades fisicoquímicas y que permiten la asociación eficiente de diversos antígenos formando parte de una misma estructura, conservando su tamaño nanométrico y su carga superficial. Estudios *in vitro* han podido determinar la capacidad adyuvante de los sistemas desarrollados y estudios *in vivo* han servido para demostrar la posibilidad de utilizar estos vehículos para potenciar y/o modular la respuesta inmune generada frente a un antígeno específico.

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CONCLUSIONES/CONCLUSIONS

CONCLUSIONES

El trabajo experimental recogido en la presente memoria se ha centrado en el diseño y desarrollo de nuevos sistemas transportadores de antígeno de tamaño nanométrico constituidos por protamina, que fueran capaces de vehiculizar el antígeno asociado y desencadenar una respuesta inmune específica frente a éste, cuando se administran por vía parenteral o por una vía no invasiva como la nasal. Los resultados obtenidos nos han permitido formular las siguientes conclusiones, de acuerdo a las etapas programadas:

1.1 Se han desarrollado nuevos sistemas de tamaño nanométrico a base de protamina con ácido hialurónico o alginato a través de la técnica de reticulación iónica, con la posibilidad de ser convertidas en polvo seco tras el proceso de liofilización, sin la necesidad de otro excipiente.

1.2 Estas nanopartículas asocian eficazmente el antígeno recombinante de superficie de la hepatitis B (rHBsAg) sin perder la antigenicidad de la molécula asociada.

1.3 Los sistemas desarrollados son reconocidos e internalizados eficientemente por el sistema inmune, y son capaces de generar una respuesta protectora tras su administración por vía parenteral.

1.4 El potencial zeta positivo de los sistemas desarrollados es esencial para alcanzar niveles protectores frente a rHBsAg al ser administrada por vía nasal como posible estrategia de vacunación libre de agujas.

2.1 A través de la técnica de desplazamiento del solvente se han desarrollado nanocápsulas de protamina capaces de modular su tamaño y carga superficial dependiendo de los componentes presentes en el núcleo oleoso.

2.2 Estas formulaciones optimizadas asocian eficazmente el antígeno modelo (sea rHBsAg o de influenza H1N1 (HI)) en la cubierta polipeptídica.

2.3 Es posible obtener nanocápsulas de protamina en polvo seco tras el proceso de liofilización y este producto es estable a temperatura ambiente conservando la integridad del antígeno asociado al menos durante 12 meses.

2.4 Las nanocápsulas de protamina optimizadas son altamente internalizadas por células inmunes generando la activación del complemento y la secreción de diversas citocinas.

2.5 Estudios *in vivo* muestran la capacidad de estas formulaciones para generar una respuesta inmune sistémica protectora por vía parenteral tanto para rHBsAg y HI, así como por vía nasal para rHBsAg. Dichas respuestas se mantienen a lo largo del tiempo.

2.6

CONCLUSIONS

The experimental work enclosed in this thesis has been oriented towards the design and development of new protamine-based nanocarriers that they could be able to deliver antigens and trigger specific immune responses against them, when administered by parenteral or non-invasive routes such as the nasal one. The results obtained allow us to formulate the following conclusions, according to the programmed work steps:

1.1 New protamine-based systems with nanometric size have been developed using hialuronic acid or alginate through the ionic cross-linking technique, with the possibility of freeze-drying the formulations without cryoprotectants.

1.2 These nanoparticles associate efficiently the recombinant hepatitis B surface antigen (rHBsAg) without affecting its structure.

1.3 The developed systems are recognized and internalized by immune cells and are able to trigger a protective immune response following administration by parenteral route.

1.4 The positive zeta potential of the developed systems is essential to achieve a protective immune response against rHBsAg when administered nasally, opening room to a possible needle-free vaccination approach.

2.1 Protamine nanocapsules have been developed through the solvent displacement technique. It is possible to modulate the size of this system depending on the components present in the oily core.

2.2 The optimized formulations associate the model antigen (H1N1 or rHBsAg) effectively in the polypeptidic shell.

2.3 It is possible to obtain a freeze-dried form of the developed protamine nanocapsules and this product is stable at room temperature, preserving the integrity associated antigen, for at least 12 months.

2.4 Optimized protamine nanocapsules are highly internalized in immune cells, generating complement activation and secretion of diverse cytokines.

2.5 *In vivo* studies show the ability of these formulations to generate a protective systemic immune response against both HI and rHBsAg (parenteral administration and rHBsAg (nasal administration). These immune responses are maintained over time.

ANEXOS

ANEXOS

POLYPEPTIDES AND POLYAMINOACIDS IN DRUG DELIVERY

González-Aramundiz JV, Lozano MV, Sousa-Herves A, Fernandez-Megia E, Csaba N, *Expert Opin. Drug Deliv*. 2012. 9 (2): 183-201.

Expert Opinion

Polypeptides and polyaminoacids in drug delivery

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Introduction: Advances achieved over the last few years in drug delivery have provided novel and versatile possibilities for the treatment of various diseases. Among the biomaterials applied in this field, it is worth highlighting the increasing importance of polyaminoacids and polypeptides. The appealing properties of these polymers are very promising for the design of novel compositions in a variety of drug delivery applications.
Areas covered: This review provides an overview on the general characteristics of polyaminoacids and polypeptides and briefly discusses different synthetic pathways for their production. This is followed by a detailed description of different drug delivery applications of these polymers, emphasizing those examples that already reached advanced preclinical development or have entered clinical trials.
Expert opinion: Polyaminoacids and polypeptides are gaining much attention in drug delivery due to their exceptional properties. Their application as polymers for drug delivery purposes has been sped up by the significant achievements related to their synthesis. Certainly, cancer therapy has benefited the most from these advances, although other fields such as vaccine delivery and alternative administration routes are also being successfully explored. The design of new entities based on polyaminoacids and polypeptides and the improved insight gained in drug delivery guarantee exciting findings in the near future.

Keywords: cancer therapy, drug delivery systems, gene delivery, nanomedicine, polyaminoacid, polypeptide

Expert Opin. Drug Deliv. [Early Online]

1. An introduction to polypeptides and polyaminoacids: definition, properties and synthesis

Polyaminoacids (PAA) and polypeptides are polydisperse structures formed by condensation of amino acid monomers through amide bonds that, contrary to proteins, cannot fold into globular structures. They are regarded as important building blocks for the design of functional materials thanks to their ability to form well-defined secondary structures (i.e., α-helix and β-sheets). These secondary structures contribute significantly to the self-assembling character of polypeptide chains, leading to novel supramolecular structures with potential biomedical applications [1]. PAA and polypeptides are generally biocompatible and nontoxic. In addition, they can carry versatile reactive functional groups at their side chains (carboxylic acids, hydroxyl, amino and thiol groups) that allow for a variety of chemical modifications. All these features render PAA and polypeptides excellent polymers for drug delivery (DD) applications.

In this Expert Review, the term 'PAA' will be used when referring to synthetic materials. PAA usually incorporate a single amino acid monomer in their backbone. The cationic polylysine (PLL), polyarginine (PArg) and the anionic polyasparagine (PAsp) and polyglutamic acid (PGA) constitute typical examples of PAA. Conversely,

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Article highlights.

- Polyaminoacids and polypeptides are attractive biomaterials for drug delivery purposes due to their versatility, nontoxicity and biocompatibility.
- Synthesis improvements have allowed the achievement of more precise molecular weight polymers with lower polydispersity, which undoubtedly have a positive effect on their performance.
- The application of polyaminoacids and polypeptides to drug delivery has yielded the construction of new entities with interesting properties such as multidrug encapsulation, enhanced uptake or stimuli-responsiveness.
- Polyaminoacids and polypeptides have contributed to significant advances in biomedicine in the fields of cancer therapy and gene delivery. They also hold great promise for future developments in other areas such as vaccine delivery and tissue engineering.

This box summarizes key points contained in the article.

the term 'polypeptide' will be devoted to naturally occurring biopolymers of composite amino acid sequence such as protamine.

Interestingly, copolymers composed of PAA/polypeptides and synthetic polymers have recently gained much interest in different scientific areas, such as polymer and materials science, nanotechnology and biomedicine. These biomimetic hybrid polymers or 'molecular chimeras' combine the favorable properties of both polymer blocks and can form supramolecular structures with very interesting properties and applications otherwise unlikely with conventional materials. For instance, amphiphilic diblock copolymers of this type can form PAA-based micelles and vesicles in water, with potential applications in drug and gene delivery. In order to predict and direct the self-assembly behavior of these amphiphilic macromolecules, it is necessary to control a variety of chemical and physical parameters such as molecular weight, polydispersity, macromolecular architecture and chemical nature. The synthesis of such well-defined structures has been a major challenge for polymer chemists until recently.

1.1 Chemical synthesis of PAA

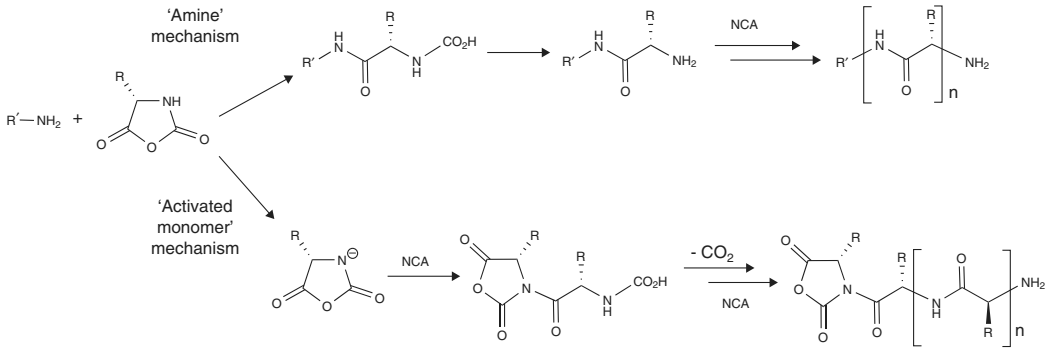
Traditionally, amino acids have been polymerized by conventional solid-phase peptide synthesis. A large number of oligomers and short peptides can be easily synthesized by this well-established method. However, the preparation of peptides larger than 100 residues usually results in undesired byproducts due to incomplete deprotection and coupling steps. To date, various alternative approaches to produce PAA and their block copolymers have been developed. Among them, the ring-opening polymerization (ROP) of α-amino acid-*N*-carboxyanhydrides (NCAs) is the most commonly applied technique (Scheme 1) [2,3]. This method is economic and usually leads to polymers in good yields and large quantities. In addition, NCAs are straightforwardly prepared [4-6].

Commonly, NCA polymerizations are initiated by different nucleophiles and bases, such as primary amines or alkoxide anions [2,3]. Unfortunately, no universal initiators and polymerization conditions have been identified and optimal results often require fine-tuning. Aspartic acid, glutamic acid and lysine appear to be the most suitable amino acids in terms of NCA preparation and subsequent polymerization [7]. Generally speaking, the main limitation of NCA polymerization has been the presence of side reactions that restrict control over molecular weight and usually lead to broad molecular weight distributions [8].

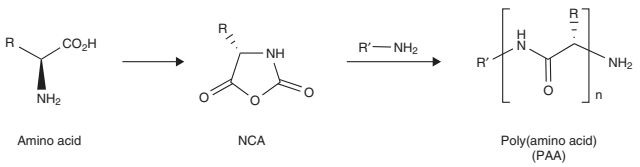
Two likely pathways for NCA polymerizations have been proposed, the 'amine' and the 'activated monomer' (AM) mechanisms (Scheme 2) [2,3]. The amine mechanism consists on a nucleophilic ROP initiated by species with stronger nucleophilic than basic character, typically primary amines. In the AM mechanism, the NCA is first deprotonated to become the nucleophile that initiates chain growth. It is generally attributed to strong bases such as metal alkoxides or tertiary amines.

Interestingly, a given system can switch back and forth between the amine and AM mechanisms during polymerization, which results in side reactions. Thus, the propagation step for one mechanism is a side reaction for the other one. As a result, block copolymers prepared from NCAs using, for instance, amine initiators reveal structures different from predicted and a considerable homopolymer contamination. These side reactions also prevent control of chain-end functionality, which is of crucial importance in the preparation of hybrid copolymers.

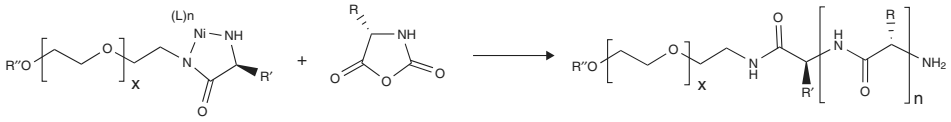
These potential side reactions in the polymerization of NCAs have made the synthesis of PAA with controlled molecular weight and low polydispersity unsuccessful until 1997, when Deming discovered novel NCA initiators based on transition metal complexes [9]. Since then, the efficiency and fidelity in the synthesis of PAA materials have greatly improved. In another seminal contribution, Hadjichristidis and coworkers reported in 2004 the preparation of PAA with control over chain length and length distribution under high-vacuum conditions [10]. Also in 2004, Olivia Giani and coworkers lowered the temperature of the polymerization process to 0°C as a strategy to obtain well-defined PAA [11]. Another innovative strategy to control amine-initiated NCA polymerizations has been described by Schlaad and coworkers, who employed primary amine hydrochloride salts as initiators to obtain well-defined PAA segments of narrow chain length distribution ($M_w/M_n < 1.03$) [12]. More recently, Lu and coworkers have reported on the controlled living polymerization of NCAs mediated by hexamethyldisilazane (HMDS) [13]. These authors have identified trimethylsilyl carbamate (TMS-CBM) as an efficient chain-propagating group and demonstrated that alternative TMS-protected amines were also efficient initiators, allowing the introduction of different functionalities for further chemical transformations [14], including 'click reactions' [15].



Scheme 1. Ring-opening polymerization of α-amino acid-*N*-carboxyanhydrides (NCAs).



Scheme 2. 'Amine' and 'activated monomer' mechanisms.



Scheme 3. Nickelacycle macroinitiators in a controlled α-amino acid-*N*-carboxyanhydride (NCA) polymerization.

1.2 Synthesis of PAA copolymers

Since the first reports on the synthesis of PAA hybrid block copolymers in the mid-1970s, the preparation of numerous PAA–synthetic polymer (AB type) and PAA–synthetic polymer–PAA (ABA type) block copolymers has been described [16]. Usually, these copolymers are prepared in two steps because of the chemical incompatibility of the two polymerization processes. This approach benefits from the fact that most synthetic polymers can be prepared with controlled chain length, low polydispersity and a high degree of amine functionalization at the chain ends. In most of these examples, the PAA block is composed of lysine or glutamate derivatives, since they form α-helixes with good solubility properties.

As in the case of PAA homopolymers, several improvements have been described for the controlled polymerization of hybrid PAA copolymers. For instance, the amine hydrochloride initiators developed by Schlaad and coworkers have been efficiently employed in the preparation of well-defined protected polystyrene-PLL (PS-PLL) block copolymers [12], and for the copolymerization with γ-benzyl-L-glutamate and β-benzyl-L-aspartate [17]. Deming and coworkers have also demonstrated that amido-amine nickelacycle end groups can be incorporated into synthetic polymers and subsequently used as macroinitiators in a controlled NCA polymerization (Scheme 3) [18]. The application of this technology renders copolymers where the length of the PAA segment can be tuned with good control and no unreacted homopolymers are detected. This methodology for the preparation of block copolymers seems to be of general scope and has been used with a wide range of amino-terminated polymers.

An alternative approach to obtain PAA block copolymers is the convergent coupling of pre-synthesized polymer segments. Among the various reactions used for this goal, the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) [19] has revealed

as an extremely powerful coupling technology [20]. This orthogonal high-yielding reaction is compatible with a broad range of functional groups and hence is particularly attractive for the synthesis of biopolymer conjugates.

2. DD applications of PAA and polypeptides

Drug delivery systems (DDS) have provided great benefits to current therapies by i) increasing stability and bioavailability of therapeutic molecules, ii) decreasing their side effects and/or iii) enabling alternative and better administration routes. This field offers a whole range of structures with different properties that can be tuned according to the therapeutic needs. For example, multi-encapsulation of drugs, bioadhesion, enhanced uptake properties, biocompatibility, drug sustained release or surface modification with target moieties to achieve a localized delivery of the drug transported are some of the appealing properties that DDS can offer [21,22]. In this sense, composing polymers play a crucial role and, therefore, need to be thoughtfully selected in the design process of new entities. Polypeptides and PAA have significantly contributed to the formulation of novel DDS, and their applications are going to be disclosed in the following lines, not only focusing mainly on major achievements of recent years, but also including some of the pioneering early developments where appropriate. The authors are aware of the advances related to the use of proteins as biomaterials in this field; however, these are out of the scope of the present work.

2.1 Polymer therapeutics

Synthetic polymers have been explored as therapeutic agents over more than 50 years. The term 'polymer therapeutic' is used to describe polymer-based constructs that are considered as new chemical entities by regulatory agencies [23]. In the mid-1970s, Ringsdorf [24] proposed that polymer–drug conjugates could enhance the delivery of anticancer drugs to tumors. He envisioned that when an anticancer drug is conjugated to a polymeric carrier, its pharmacological properties could be manipulated by changing the physicochemical properties of the polymer. In addition, it was later recognized that polymer–drug conjugates tend to accumulate in solid tumors thanks to the so-called enhanced permeation and retention (EPR) effect [25]. To date, many polymers have been investigated as candidates for the delivery of different drugs, and a growing number of polymer therapeutics have been transferred to the market. In general, the ideal polymer for DD should be water-soluble, biodegradable (or have adequate molecular weight to allow elimination from the body), have a low polydispersity and be non-immunogenic. In this context, biodegradable PAA such as PLL and PGA have revealed as promising candidates with several formulations in clinical trials. In addition, PGA and PLL have a high drug-loading capacity derived from the presence of reactive side groups (carboxylic acid and amine), which have been employed for the attachment of different chemotherapeutic agents such as doxorubicin (DOX), paclitaxel (PTX) and camptothecin (CPT). To date, one of the most advanced PAA formulations is Opaxio® (formerly known as Xyotax®), currently in Phase III for the treatment of ovarian and non-small cell lung cancer (NSCLC) (**Figure 1**). This polymer therapeutic results from the attachment of PTX to PGA through ester linkages. This system has an impressive drug loading (~ 37 wt%) and is able to circulate for prolonged times in the bloodstream and subsequently accumulate in tumors, where the drug is released [26]. This long circulation half-life and low toxicity seem to be key factors for the clinical success not only of Opaxio™ but also of CT-2106, a poly(glutamate)-glycine CPT polymer therapeutic currently in Phase I/II [27].

In addition, PGA is well-known to be biodegraded by cysteine proteases (cathepsin B) at the lysosomes, an essential property to avoid accumulation in the organism after repeated administration. Interestingly, clinical results suggest that this degradation pathway is behind the increased activity of Opaxio in women with NSCLC [28]. These studies sustain that the presence of estrogen may influence the metabolism of Opaxio, due to the enhanced activity of cathepsin B and subsequent drug release.

Another type of polymer therapeutics is constituted by well-defined multivalent dendrimers and dendritic polymers, which may act as nanocarriers or as a drug themselves. VivaGel® is an L-lysine-based dendritic microbicide decorated with anionic groups, in which the dendrimer is not a carrier but an active ingredient. It has been evaluated in Phase II as a vaginal gel for preventing/reducing transmission of HIV and genital herpes (**Table 1**) [29,30]. As another example of dendritic polymer therapeutics, it is worth mentioning those based on PGA, recently evaluated for the delivery of DOX. This dendritic delivery system can be further modified with biotin for targeting purposes and deliver the anticancer drug by the cleavage of the pH-sensitive bonds that attach the drug to the structure [31].

Polymer therapeutics with stimuli responsive functions have also been described in the literature. These should undergo physical or chemical modifications in response to small changes in the environmental conditions. The benefits of stimuli-responsive systems are especially important when these stimuli are unique for disease pathology, allowing a specific response to pathological 'triggers.' Typical examples of biological stimuli are pH, temperature, ionic strength and redox microenvironments.

In a recent approach, Shen and coworkers combined both pH-sensitive groups and disulfide bonds that were reduced in the intracellular reductive environment for the nuclear delivery of CPT by a PLL-based conjugate [32]. In order to avoid the toxicity and problems associated to *in vivo* applications of cationic PLL, they converted PLL's primary amines into acid-labile carboxylic amides (PLL/amide). CPT was introduced into the polymer by means of an intracellular cleavable disulfide bond, and folic acid was attached to the PLL skeleton in order to obtain a cancer-cell-targeted

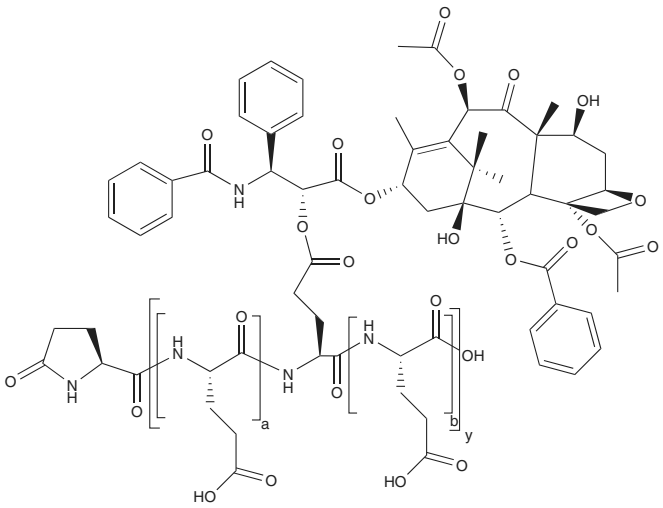


Figure 1. Chemical structure of Opaxio.
a, b, y denominate different numbers of monomer units.

nuclear-localization polymer–drug conjugate. The conjugate efficiently entered folate-receptor overexpressing cancer cells and moved to their nuclei with improved cytotoxicity.

2.2 Self-assembled nanostructures

A fascinating area in materials science and nanochemistry is concerned with the creation of supramolecular architectures with well-defined shape and function. Self-assembled nanostructures are held together through non-covalent forces, such as van der Waals forces, electrostatic interactions, hydrogen bonding and metal complexation [33]. Various self-assembled morphologies with spherical, rod-like and lamellar structures have been described and offer numerous possibilities to tailor their physical, chemical and biological properties by variation of their chemical structure or by conjugation to biomolecules. In this section, we will focus on self-assembled nanostructures most commonly employed in drug and gene delivery, which will be classified according to their supramolecular structure.

2.2.1 Polymeric micelles

Amphiphilic block copolymers with a large solubility difference between the hydrophilic and the hydrophobic segments can spontaneously form polymeric micelles in aqueous media. These micelles are characterized by a sub-100 nm core–shell structure, which provides a reservoir for hydrophobic drugs enveloped by a hydrophilic shell that improves drug solubility and suppresses protein adsorption (stealth effect) [34]. Furthermore, polymeric micelles can be functionalized with targeting ligands and biomarkers to provide control over biodistribution and site-specific cellular uptake (active targeting).

Among the various amphiphilic copolymers leading to micellar structures, block copolymers of PAA and poly(ethylene glycol) (PEG-PAA) obtained *via* ROP of NCA are especially appealing as they allow chemical modification at the amino acid side chains [35]. Within this context, Kataoka and coworkers have pioneered the use of PEG–PAA block copolymers for DD [36-38]. For instance, they prepared polymeric micelles from PEG–poly(aspartate) (PEG–PAsp) carrying DOX covalently conjugated at the side chains through amide bonds. This enhances the hydrophobicity of the PAsp block and facilitates the spontaneous formation of micelles in aqueous media [37].

Additionally, unbound DOX molecules could be physically entrapped in the micellar core by hydrophobic interactions. Physically entrapped DOX displays the major cytotoxic function, while conjugated DOX molecules work mainly by increasing the micelle stability [39]. This optimized PEG–PAsp (DOX) micelle has been studied in Phase I clinical trials as NK911 in Japan (**Table 1**) [40] and its structure is shown in **Figure 2**. Similarly, PTX has been physically entrapped in the core of hydrophobically modified PEG–PAsp polymeric micelles [41]. The resulting micellar system, known as NK105, is under Phase II clinical studies in Japan (**Table 1**) [42]. PEG–PGA micelles have also demonstrated to be useful for the delivery of the water-insoluble anticancer drug 7-ethyl-10-hydroxycamptothecin (SN-38), an analog of CPT. With this aim, Matsumura and coworkers covalently modified PEG–PGA with SN-38 at the PGA block, which self-assembled into micelles [43]. These SN-38-loaded micelles showed enhanced antitumor activity compared with the free drug and are currently under Phase I clinical trials as NK012 (**Table 1**) [44].

Table 1. Polyaminoacids (PAA)- and polypeptide-based drug delivery systems currently in clinical trials.

Polymer composition	Carrier type	Loaded drug	Clinical development	Identifier*/ref.
Polyglutamate	Polymer conjugate	Paclitaxel	Phase I (completed January 2009)	NCT00060359
			Phase II (completed September 2006)	NCT00069901
			Phase III (recruiting participants)	NCT00108745
		Methotrexate	Phase IV (completed December 2010)	NCT00695188
		Camptothecin	Phase I (completed July 2008)	NCT00059917
Polyglutamate–PEG	Micelles	SN-38	Phase II (recruiting participants)	NCT00951054
		Cisplatin	Phase I/II (recruiting participants)	NCT00910741
		Oxaliplatin	Phase I (Japan)	[144]
Polyaspartate–PEG	Micelles	Doxorubicin	Phase I (Japan)	
		Paclitaxel	Phase II (Japan)	
Polyglutamate–vitamin E	Nanoparticles	Insulin	Phase I	[145]
		Interferon β-1a	Phase I	[145]
		Interferon α	Phase II (recruiting participants)	NCT01010646
Polylysine	Dendrimers	SPL7013 Gel (VivaGel)	Phase II (completed June 2011)	NCT00740584
	Physical mixture/PEC	MUC-1 peptide vaccine	Phase 0 (recruiting participants)	NCT00986609
	Physical mixture /PEC	Poly-ICLC	Phase I (completed April 2011)	NCT00880867
			Phase II (recruiting participants)	NCT01245673
Polyarginine	Physical mixture/PEC	Vaccine for chronic hepatitis C virus	Phase II (completed July 2008)	NCT00601770

*According to clinicaltrials.gov [146].

Kataoka and coworkers have also developed PAA-based micelles containing cisplatin (cis-dichlorodiammineplatinum [II], CDDP). In this case, CDDP was introduced into PEG–PAsp block copolymer by metal complexation between platinum and carboxyl groups of the PAsp block. This complex spontaneously formed polymeric micelles that exhibited a higher accumulation in tumor sites than free CDDP [45]. The micelle composition was further modified to regulate the CDDP release and to extend the blood circulation time by using the more hydrophobic PEG–PGA copolymer instead of PEG–PAsp. The PEG–PGA(CDDP) micelle is currently undergoing a Phase I clinical trial as NC-6004 in the UK (**Table 1**). Alternative platinum-drug-loaded polymer micelles based on dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPt) and PEG–PGA have been obtained and shown longer circulation times and more than 20-fold higher accumulation in tumors than the free drug [46].

Further studies aimed to improve the biodistribution profile of PEG–PAA polymeric micelles have shown a crucial effect of the PAA length on biological performance. Micelles composed of a copolymer PEG–PGA with 20 glutamic acid units have resulted in reduced accumulations in the liver compared with longer PGA blocks. It has been proposed that shorter PGA segments may decrease the micellar core size and achieve a more efficient PEG shielding of the core, which helps to decrease hepatic toxicity and improve antitumor activity by increasing accumulation in tumor tissue [47].

In addition, stimuli-responsive polymeric micelles sensitive to different pH of specific cell compartments or tissues, as well as the differences in the concentrations of reductive agents (e.g., glutathione) outside and inside the cell, have been reported in the literature. For instance, pH-sensitive micelles that exploit acidic environment at tumor tissue to unload its contents have been prepared by different approaches, such as the employment protonatable groups (amines or carboxylic acids) or of pH-sensitive linkers (hydrazone, *cis*-aconityl or acetal groups, etc.) In this context, Kataoka's group has also prepared stimuli-responsive micelles containing DOX by conjugating the drug to the PAsp segment of a PEG–PAsp block copolymer through a hydrazone bond, which is stable under physiological conditions but cleavable under acidic intracellular environments [48] In addition, the micelles were modified with a folate moiety at the distal end of PEG, allowing for an active targeting to cancer cells and

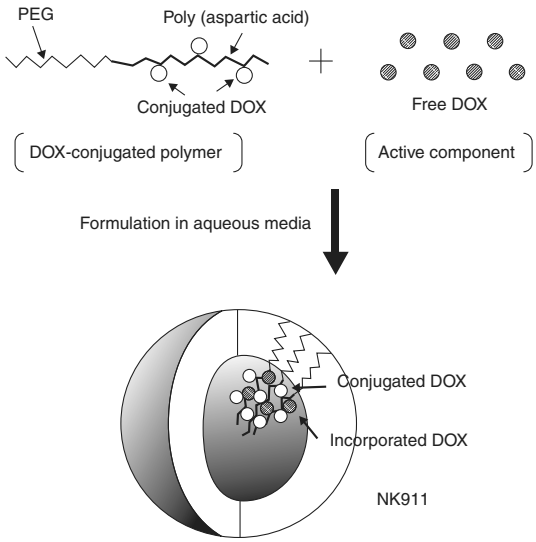


Figure 2. Schematic structure of NK911, a polymeric micelle consisting of a block copolymer of PEG and polyaspartic acid.
Reproduced with permission from Nature Publishing Group [40].
*= statistical differences of CP-Dox vs Dox at p=0.03 and vs PBS at p=0.00002
‡= statistical differences of CP-Dox vs Dox at p=0.0001 and vs PBS at p=0.00004

showing a significantly improved therapeutic effect compared with free DOX [49].

Another example to pH-triggered drug release was reported by Bae and coworkers [50]. They employed a pH-sensitive polymeric micelle composed of a mixture of PEG–poly(L-histidine), a pH-sensitive PAA with pKa values around physiological pH and biodegradable PEG–poly(L-lactic acid) block copolymers intended to encapsulate DOX. The resulting drug-loaded mixed micelles were stable under physiological pH but destabilized at the acidic pH of the target tumor site. When these mixed micelles were conjugated to folic acid, they resulted more effective in killing tumor cells due to accelerated drug release in the tumor region and folate receptor-mediated tumor uptake. Furthermore, the fusogenic activity of poly(L-histidine) in the endosomes facilitated the cytosolic delivery of DOX to achieve improved cytotoxicity.

Recently, as a result of the in-depth knowledge gained on protein structure and function, a new family of polymers called 'engineered peptide-based biopolymers' has been established [51]. Among them, those based on elastin-like polymers (ELP) have found application in DD. ELP self-assemble into polymeric micelles in aqueous solution in a reversible temperature-dependent way. Above a critical solution temperature (T_t) that is sequence specific, ELP assemble from a soluble expanded state to a controlled micellar collapsed state [52]. This behavior can be tuned depending on the polymer sequence to obtain polymeric carriers with customized properties [53]. Moreover, ELP have a typical phase-transition behavior, which can be also used for self-assembly purposes into nanostructures by selective desolvation of specific blocks of the recombinant ELP block copolymers [54].

The application of ELP to DD has yielded promising results in cancer therapy. Chilkoti and coworkers have recently published the formation of monodisperse nanostructures from a chimeric polypeptide–DOX conjugate that self-assembles in aqueous media. The chemical attachment of DOX to ELP through hydrazine linkages enables its encapsulation and its selective delivery at pH 5 by the cleavage of the acid-labile hydrazone bond. This pH-sensitive behavior assures that the drug remains within the structure while in the blood circulation, but it is released after internalization and exposure to the acidic endolysosomal environment. This phenomenon is shown by the improved pharmacokinetics of the system, showing long-circulating times for the micelles and a clear 3.5-fold enhanced drug concentration in tumor compared with free DOX at the same dose. Moreover, the accumulation at non-tumor sites is also reduced, being especially remarkable the decrease in drug concentration in the heart, which allows to achieve higher maximum-tolerated dose for the micelles compared with the free drug. In addition, an impressive remission of tumor burden was observed after a single injection of these DOX-loaded micelles (**Figure 3**) [55].

2.2.2 Polyion complex micelles

Another interesting type of micelles that can be prepared from PAA block copolymers is constituted by polyion complex

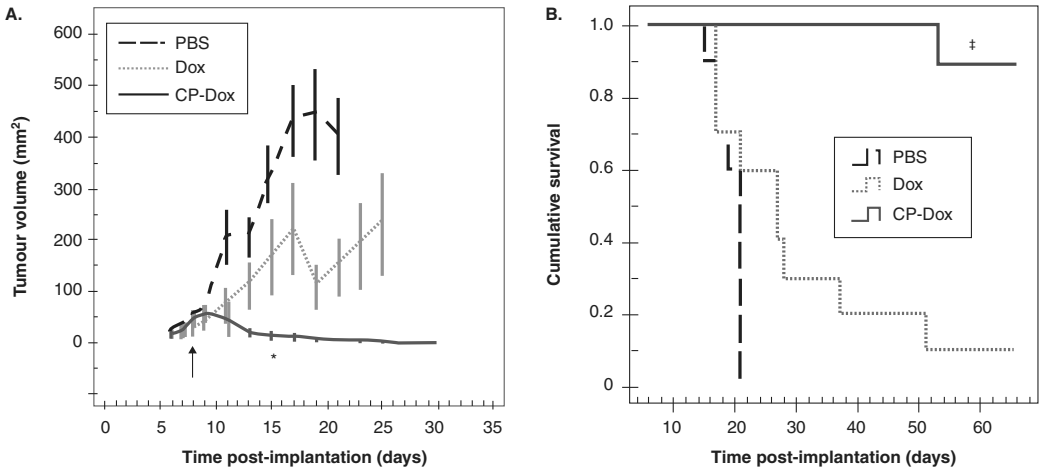


Figure 3. Antitumor activity of self-assembled polypeptide–doxorubicin nanoparticles. A. The decrease in tumor volume with camptothecin–doxorubicin (CP–Dox) at day 15. **B**. The high cumulative survival of mice with CP–Dox vs Dox and PBS.
Reproduced with permission from Nature Publishing Group [55].
*= statistical differences of CP-Dox vs Dox at p=0.03 and vs PBS at p=0.00002.
‡= statistical differences of CP-Dox vs Dox at p=0.0001 and vs PBS at p=0.00004.

(PIC) micelles [56,57]. Originally described by the groups of Kataoka and Kabanov, PIC micelles are formed by electrostatic interaction between oppositely charged polyion copolymers, usually in a stoichiometric charge ratio [58]. Similar to classical polymeric micelles, PIC micelles have a core-shell structure with a core of ionic blocks surrounded by a neutral hydrophilic corona, typically of PEG (**Figure 4**). It is well known that, at charge neutrality ratios, electrostatic interactions between oppositely charged polyelectrolytes result in phase separation and precipitation. By contrast, if a neutral segment such as PEG is linked to one of the interacting polyelectrolytes, soluble colloidal particles (PIC micelles) are formed instead. Indeed, the vast majority of examples of PIC micelles reported in the literature comprise PEG–PAA block copolymers.

Although research on PIC micelles is still in its infancy compared with more classical polymeric micelles, their therapeutic applications are rapidly increasing. Advantage has been taken from the charged nature of various types of biopharmaceuticals. Cationic PAA block copolymers have been used in the preparation of PIC micelles with DNA, siRNA, proteins and even viruses [58]. In the case of nucleic acids for gene delivery applications [59-62], the PEG segments surrounding the core have proven to prevent the complex from precipitation and to render the system with high resistance against DNase I [63,64] (also see Section 2.2.3.). Similarly, block copolymers of anionic PAsp have been used in the preparation of PIC micelles with proteins [65,66]. PIC micelles containing dendritic photosensitizers have been also reported for photodynamic therapy and as light-harvesting sensitizers [67-70]. *In vivo* results indicate that these dendritic micelles may constitute innovative formulations for the treatment of ophthalmologic diseases. More recently, remarkably stable PIC micelles prepared from PEG-dendritic block copolymers and PAA have been described by the group of Fernandez-Megia and Riguera [71]. These micelles are envisioned as attractive delivery systems for low-molecular weight drugs, proteins, nucleic acids and imaging agents.

In addition, similar to conventional micelles, the surface of PIC micelles has been modified with ligands for targeting. Lactose [72] and a cyclic arginine–glycine–aspartic acid peptide [73] have been recently introduced into PIC micelles for active gene delivery.

2.2.3 *Polyelectrolyte complexes*

Polyelectrolyte complex (PEC) dispersions result from strong electrostatic interactions between charged microdomains of at least two oppositely charged polyelectrolytes. These systems can generally be obtained by simple mixing of polyanions and polycations, leading to the spontaneous formation of stable PEC under certain conditions [74,75]. Although electrostatic interaction is the main driving force in the formation of PEC and PIC micelles, they differ in their supramolecular structure (**Figure 4**). As detailed above, PIC micelles are organized in a core-shell structure, which resembles polymeric micelles, and their hydrophilic corona renders them stable even under charge stoichiometric conditions. By contrast, stoichiometric mixtures of oppositely charged polyelectrolytes lead to precipitation in aqueous media (**Figure 4**). Therefore, PEC needs to be formed with an excess of one of the charged species in order to confer water solubility to the complex. Thus, for instance, in the case

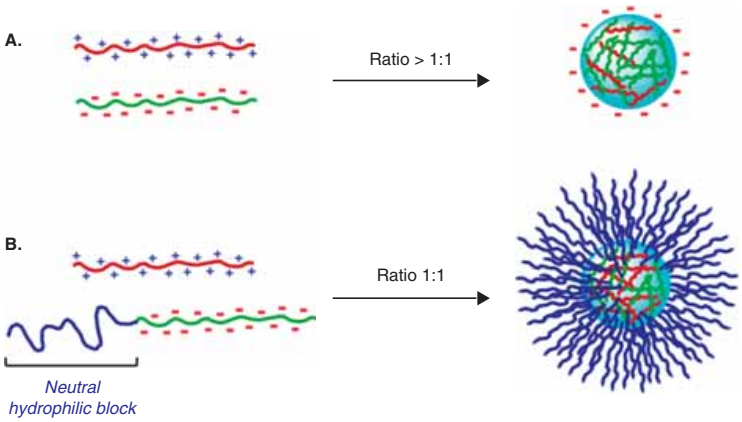


Figure 4. Schematic representation of (a) polyelectrolyte complex (PEC) and (b) polyion complex (PIC) micelle formation.

of PEC composed of polycations and nucleic acids, a large excess of the polycation is generally employed.

Although there have been a few reports on PEC for the delivery of therapeutic molecules or vaccines, this type of delivery systems has been mainly explored for gene delivery due to their capacity of binding and condense nucleic acids reversibly. Some of these materials also combine this capacity with i) cellular and/or tissue specificity (essential for *in vivo* gene delivery), ii) membrane fusogenic or disruptive activities and/or with iii) the promotion of nuclear translocation [76]. Due to these characteristics, a great number of polypeptides and PAA have been investigated for the formation of PEC complexes for gene delivery. In particular, polypeptides and PAA rich in basic residues such as lysine, arginine or histidine have received most attention as these are able to efficiently condense genetic materials into small, stable and compact PEC systems [77].

One of the first and most investigated cationic PAA for complexing nucleic acids has been PLL. PLL may be synthesized with different molecular weights (typically from 1 up to 300 kDa). Its cationic structure is able to protect the associated genetic materials against nuclease digestion and enhance their cellular uptake via nonspecific endocytosis. Nevertheless, its significant molecular weight-dependent cytotoxicity has led to intensive studies in order to achieve effective gene transfer combined with low cytotoxicity and to balance the polymer cationic density with endosomal escape moieties [78].

A relevant example of current polylysine-based gene carriers is those developed by Dr. Hilton Levy for delivery of dsRNA (e.g., polyI:C) for the induction of interferon production in antiviral and anticancer treatments [79]. As shown in **Table 1**, some of these formulations have already entered clinical evaluation for the treatment of malignant brain tumors [80-82].

Other efforts have been directed to enhance the specific cellular uptake of PLL-based complexes through the attachment of diverse ligands to the polymer backbone. Relevant examples of such targeting moieties include asialoorosomucoid proteins, carbohydrates such as fucose, mannose and galactose, epidermal growth factor, folic acid, transferrin, steroids and viral or bacterial proteins.

The use of PEGylated PLLs has also been widely studied with the aim of providing better physical and biological stability to the complexes. It is possible to bind to the PLL–PEG other molecules and form thereby new copolymers with further improved characteristics (e.g., lower cytotoxicity, better endosomolytic capacity or better ability to condense pDNA) and mediate effective gene transfection in various cell lines [83]. Relevant examples of these combinations include materials as polyethylenimine, polylactic acid, lactose or galactose, among others.

Another interesting example of such combined approaches is the modification of PLL/DNA complexes with PEG and the tripeptide (Glu-Lys-Glu), and by incorporating folate moieties [84]. These new complexes showed extended systemic circulation times following intravenous administration to mice with up to 2000-fold more DNA measured in the bloodstream compared with simple PLL/DNA complexes. This study also showed that DNA uptake via the folate receptor is dependent on PEG spacer length, with the transgene expression relatively independent of the level of internalized DNA [84].

More recently, other PAA similar to PLL have also been explored as vehicles for gene delivery. One relevant example is polyornithine (PLO), a polycation that differs from PLL in a methylene (-CH2-) unit in its side chain. This difference affects the interaction with pDNA and the stabilization of the PAA/DNA complex with profound effects on the processing of the associated pDNA molecules. These include cellular uptake, intracellular trafficking and nuclear localization, and as such may well contribute to the disparity in cell transfection efficiency observed between these complexes [85], indicating

that a relatively slight change in the structure of such linear polymers may have a significant effect on their efficacy [86,87].

Arginine-rich compositions represent another important group of biomaterials for nucleic acid complexation [88]. Polyarginine (PArg) itself is a cationic polymer that has shown an ability to translocate through mammalian cell membranes. These cell-penetrating properties of PArg have been attributed to the presence of the guanidine moiety in its side chain, which interacts directly with cell surface domains and subsequently facilitates cellular internalization [89]. This interesting feature has been the rationale of its use in gene therapy. Recently, PArg has also been explored for siRNA delivery in the form of polyionic complexes with hyaluronic acid [90] and as a covalent conjugate with cholesterol. In this latter case, the complexation of siRNA with this new entity resulted in an effective inhibition of VEGF production in colon adenocarcinoma cells (CT-26) *in vitro* and it was able to suppress tumor growth *in vivo* following local administration [91]. PArg also forms stable complexes by electrostatic interaction with CpG ODNs, resulting in complexes with potential interest in immunization strategies as vaccine adjuvants [92].

Other arginine-rich compositions have also been extensively studied for gene delivery due to their potential fusogenic or penetration-enhancing properties. Protamine is a naturally occurring substance synthesized in late-stage spermatids of many animals, with the physiological function of condensing the spermatid genome into an inactive state [93]. Due to this natural affinity for nucleic acids, protamine has been investigated in a great number of different gene therapy-related applications [94-96]. For example, this polypeptide in combination with antisense ODN spontaneously forms compact nanoparticulate complexes called 'proticles,' and this specific association increases the cellular uptake of the ODN (up to eightfold) compared with the free ODN, showing very low cytotoxicity at the same time [97]. Other studies have shown that RNA condensed on protamine is protected from RNase-mediated degradation. In particular, the complexation of messenger RNA with protamine seems to be a promising strategy for genetic vaccination approaches as this complex efficiently activates immune cells and stimulates the secretion of cytokines such as TNF-α and IFN-α [98].

The detailed analysis of binary protamine-nucleic acid nanosystems revealed two major disadvantages: i) aggregation of particles within a few minutes in the presence of salt and ii) low intracellular dissociation between protamine and ODN. To resolve these problems, a ternary system of albumin–protamine–ODN has been developed. This system showed better stability under isotonic conditions and facilitated the intracellular dissociation between ODN and protamine [99].

It is also possible to conjugate protamine with polysaccharides (e.g., dextran) for enhanced transfection properties [100] or to conjugate the ODN first with a polysaccharide and then form the complexes with protamine [101].

Other arginine- and histidine-rich molecules such as TAT, KALA or LAH peptides have also been widely used for their cell penetration enhancer and fusogenic properties. The potential interest of these short sequences for DD and targeting is revised elsewhere [102-104].

As it can be concluded from this section, most PAA and polypeptides used in gene delivery are characterized by a high number of positively charged amino acid monomers (lysine, arginine, histidine, etc.). In fact, a serious drawback associated to their use is that the strong positive charge of the complexes may lead to hemolysis and toxic effects after their intravenous administration. Interestingly, the well-known biocompatibility shown by another, negatively charged polyaminoacid PGA has been the rationale behind its use as an additional coating agent for previously described cationic complexes with nucleic acid [105]. The PGA coating was meant to shield the positive charge of the system and efficiently decreased its hemotoxicity compared with uncoated systems while maintaining high transfection efficacy (**Figure 5**).

2.2.4 *Other self-assembled nanostructures*

This section describes further self-assembled delivery systems incorporating polypeptides, PAA and peptides. PGA and PAsp have been the subject of many strategies for the design of new nanoparticulate entities. Among these, it is worth to highlight the Medusa® technology developed by Flamel Technologies. This company has developed a whole pool of products that are currently in the pipeline for various applications such as diabetes, hepatitis or cancer. The basis of the Medusa technology is PGA, optionally further modified with L-leucine in order to obtain amphiphilic block polymers, or with hydrophobic molecules such as α-tocopherol (vitamin E) to produce randomly grafted polymers [106]. Both generations of polymers are known to self-assemble in aqueous media into nanoparticles, exposing the hydrophilic glutamate chains to the outer media, while the hydrophobic domains constitute the core of the structure. Therapeutic molecules such as proteins and peptides can be associated within the polymer matrix and subsequently delivered in a controlled manner after subcutaneous injection [107].

Another interesting system has been developed by Deming and coworkers, who obtained polymeric vesicles from a self-assembling polymer formed by PArg and polyleucine segments. In this study, PArg performs the synergistic role of being a structural component and promoting the cellular uptake of the system by its cell-penetrating properties [108]. A recent improvement of these PAA-based vesicles is the achievement of covalent cross-linking by the incorporation of oxidatively cross-linkable residues, in an attempt to enhance the stability of the system [109]. Similar vesicles have also been obtained by combining PAA with binding agents for biological targets, which also work as hydrophilic blocks constituting the nanostructure. This is illustrated in the work published by Lecommandoux and coworkers by the formation of DOX-loaded poly(γ-benzyl glutamate)-*block*-hyaluronan polymersomes [110].

Self-assembly is also a very attractive strategy to construct nanoscale materials for application in regenerative medicine

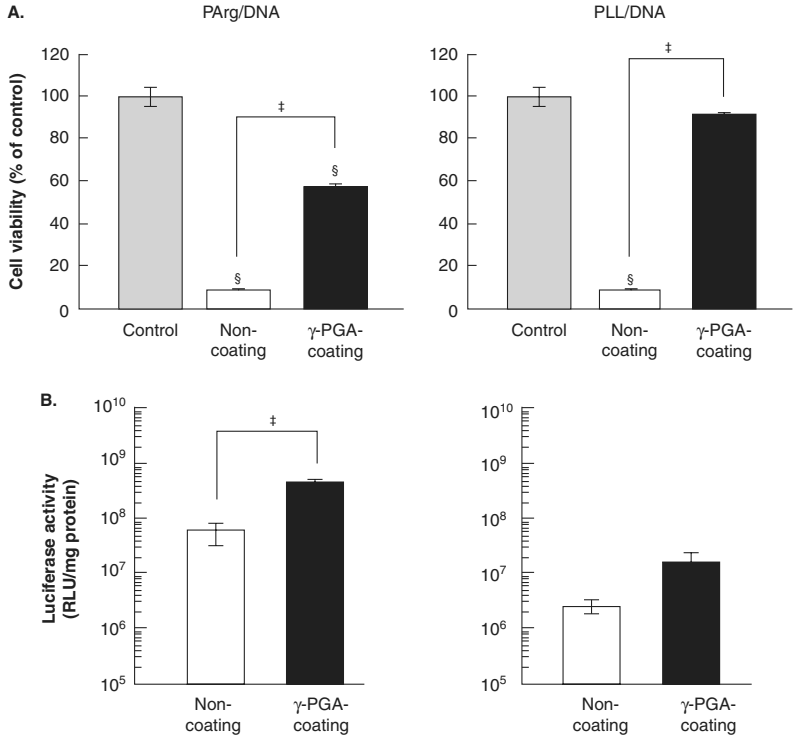


Figure 5. Effect of polyglutamic acid coating on the cytotoxicity (a) and transfection efficiency (b) of polyarginine and polylysine-based polyplexes.

Reproduced with permission from Elsevier [105].

§=statistical differences with the control at $P < 0.01$.

‡=statistical differences between the formulations at $P < 0.01$.

due to its simplicity in application and its unique capacity to produce a variety of diverse nanostructures [111]. In this specific area, it is necessary to produce biologically compatible scaffolds that might be readily adopted by the body without harm and be suitable platforms for cell growth, including stem cells or primary cells that can effectively replace damaged tissues [112]. In this context, self-assembling peptides have been studied for diverse purposes such as cartilage tissue engineering [113], substrate for neurite outgrowth and synapse formation in nerve regeneration [114], functionalized with bone marrow homing peptides for stimulating neural stem cell adhesion and differentiation [115], for the formation of confluent cell monolayers of human aortic endothelial cells [116], as hydrogel scaffolds for controlled release of functional proteins [117].

Polypeptide-based nanofibers are also of great interest in tissue engineering and wound repair. Among the different materials that have been used for the formation of nanofibers, polypeptides and peptides are particularly promising as they resemble extracellular matrix proteins. More information on these applications can be found in the work of Barnes *et al,* [118] Silva *et al.* [119] or Ellis-Behnke *et al.* [120].

2.3 Particulate supramolecular DDS

DDS are extremely versatile carriers with respect to their composition and structure. This facilitates their formulation process by adjustment to the specific requirements. The following subsections will review further PAA and polypeptide-based structures applied in DD. In contrast to the others, self-assembled nanostructures disclosed in the previous section, the systems below are characterized by a more complex arrangement, in terms of their structure and/or shape, generally as a result of diverse methods applied for material processing (melt or solvent casting, mechanical forces, etc.).

2.3.1 Liposomes

Liposomes are spherical structures formed by one or several concentric lipid bilayers comprising inner aqueous phases.

From the early first attempts to the present times, liposome research has considerably evolved toward more optimized and sophisticated structures. One of the main drawbacks that DDS faced at their beginnings was their recognition by the cells of the reticuloendothelial system and their subsequent elimination as foreign to the organism. This limitation was successfully overcome by the design of PEG shell that covered the liposomes, enhancing their circulation times [121]. PEG has since then widely been used as a golden standard for many years for giving stealth properties to nanostructures. Nevertheless, there are some concerns about the use of PEG, arising from possible side effects such as hypersensitivity reactions or altered pharmacokinetics [122]. Consequently, different polymers such as PAA have been investigated as alternatives for PEG. Surface modification of liposomes with poly(hydroxyethyl L-glutamine) and poly(hydroxyethyl L-asparagine) has effectively prolonged liposome circulation times achieving similar levels to the ones obtained with PEG [123]. This prolonged circulation behavior can be adjusted by modifying the physicochemical characteristics of the liposomes, an attractive feature to achieve the optimization of the system [124]. Despite this promising research, there are some aspects like the activation of the complement system after intravenous administration or the application of other PAA for liposome coating that will need further study [125]. By coating liposomes with PAA, it is also possible to decrease the toxicity of the formulation. Despite their efficient transfection, cationic liposomes may induce cytotoxicity and hemagglutination due to their strong positive charge. A recent work of Sasaki and coworkers describes an approach to decrease the toxicity of cationic liposomes by coating with PGA. These authors have shown a significant reduction in the toxicity profile and a high transfection efficacy in coated liposomes compared with non-coated ones [105]. Another interesting approach has been reported by Li and Huang, entrapping protamine/DNA and protamine/siRNA complexes in the interior of liposomes. These liposomes were further modified with anisamide, a molecule with affinity for sigma receptors, which are overexpressed in a variety of human tumors [126]. The biological evaluation of the system showed significant accumulation of siRNA in tumors (up to 70 – 80%), obtaining large differences with the amount that could be found in liver and lung (10 and 20% respectively). The efficiency of the system was evaluated by monitoring the tumor growth; the treatment with the liposomes achieved a 40% tumor growth inhibition, which was even enlarged by the coadministration of the anticancer drug cisplatin. The results exposed provide the first evidence of such high tumor delivery efficiency reported so far in the literature [127].

Protamine has also been used as adjuvant in liposome-based vaccine formulations. The hybrid composition of liposome–protamine–DNA particles developed by Sloat and Cui carrying a protective anthrax antigen achieves strong mucosal and systemic immune responses in mice following nasal immunization. These responses are comparable with subcutaneously injected protective antigen adjuvanted with aluminum hydroxide (a classical adjuvant used in commercial formulations) [128].

2.3.2 *Particulate polymeric micro- and nanostructures*

These delivery systems include micro/nanospheres and micro/nanocapsules with a matrix or vesicular structure, respectively, and a size typically in the range from 0.1 to 250 μm. This difference in size between nanoparticles and microparticles entails variations in parameters such as drug encapsulation efficiency, release rates, stability issues, administration route and cellular recognition and processing [129]. These parameters can also been controlled by the nature and structure of the matrix as it will be illustrated in the following examples. Due to their size, in general, microspheres can be taken up by only phagocytic cells, which entail them as excellent vehicles for passive targeting in vaccine delivery to antigen-presenting cells. Surface modification of biodegradable poly(D,L-lactic-co-glycolic acid) and non-biodegradable polystyrene microspheres with PAA and polypeptides has been extensively studied for this purpose [130]. A recent work of Merkle and coworkers highlights the potential of PLL–PEG-modified microspheres for the delivery poly(I:C) as a potent immunoadjuvant. These results show that poly(I:C) exhibits a strongly enhanced immune cell maturation and activation when assembled on the surface of these microparticles [131].

Surface modification of microparticles with protamine has also been reported, showing that the presence of this polypeptide (also see Section 2.2.3.) promotes the stimulation of stronger and more specific immune responses and enhances cytokine secretion in a dose-dependent manner. The success of this system may rely on combining the prolonged antigen release from the microparticles with the internalization properties of protamine [132].

Protamine microspheres have also been successfully tested for tissue engineering applications by Nakamura and coworkers; the results show that fragmin/protamine microspheres promote vascularization and fibrous tissue formation after their *in vivo* administration. The role of protamine in the microspheres is to diminish the anticoagulant effect of fragmin, a heparinoid used to stabilize and promote the activity of encapsulated growth factors [133]. Protamine and PArg have also been used in combination with polysaccharides (e.g., dextran sulfate or sodium alginate) for the formulation of multilayered microcapsules. These microcapsules are constituted by successive adsorption of the two oppositely charged polymers onto the surface of a preformed particle core that can subsequently be dissolved under mild conditions [134,135]. The benefit of such multilayered microcapsules is the controlled release of the entrapped molecules as a function of the coating nature and thickness. This aspect has attracted much interest from the pharmaceutical industry, as it is illustrated by the ongoing research of Flamel Technologies on microparticle formulations for the oral delivery of

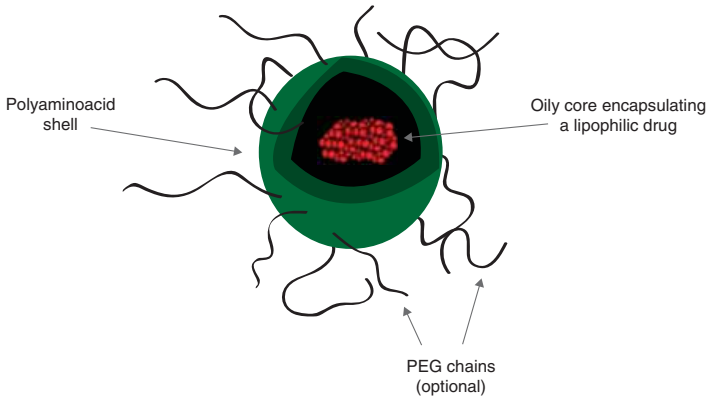


Figure 6. Schematic representation of polyaminoacid nanocapsules.

peptide drugs based on the Medusa technology, which has been commented in the previous section.

On the other hand, nanometric size is a powerful feature with important consequences in the properties of DDS and their activity. Indeed one of their main characteristics is a high surface/volume ratio, which makes them highly reactive, clearly affecting the performance of the system. In addition to the great number of nanostructures described in the previous sections, recent work performed in our research group indicates that PAA are promising biomaterials for the formulation of nanocapsules. The additional values that nanocapsules hold are i) great versatility due to the wide range of polymers that can be used as shell, from polysaccharides to polyesters [136,137], allowing the adaptation of prototypes as a function of the biological barriers to overcome [138]; ii) enhanced physicochemical stability due to the presence of the polymer corona [139]; and iii) co-encapsulation of different types of drugs in the inner core and the surrounding polymeric shell (**Figure 6**) [140]. During the last years, our group has intensively worked in the development of new formulations of nanocapsules, relying on the potential of PAA and polypeptides as polymeric coating materials. PGA and polyasparagine (PAsn) were selected as examples of negatively charged or neutral coating materials due to their biocompatibility and long-circulating properties. Moreover, PAsn nanocapsules may also benefit from the high PAsn demand of solid tumors that could be potentially used as an active targeting strategy. Based on this rationale, we have extensively investigated PAA-based nanocapsules as novel DDS for cancer treatment [141]. The versatility of this nanocapsule technology has been also proved by the formulation of positively charged, PArg nanocapsules. This nanosystem gathers the cell-penetrating properties of PArg [142] with the ability for co-encapsulation of drugs with different properties, such as docetaxel (in the inner core) and plasmid DNA (assembled onto the cationic surface), which seems to be a promising combination therapy in cancer [140,143]. *In vivo* experiments are currently ongoing with the aim of further exploring the potential of these PAA nanocapsules for cancer therapy.

3. Conclusions

The results exposed throughout this work provide a brief overview of the most important achievements of PAA and polypeptide research in DD. Synthesis methods have been improved from the early solid-phase technique toward new synthesis pathways that yield optimized products with improved purity. Several novel formulations based on these biomaterials are currently in clinical trials, followed by many others with interesting results. Altogether, the information collected herein illustrates the potential of these polymers and predicts exciting advances for clinical applications in the future.

4. Expert opinion

During the last two decades, PAA and polypeptides have emerged as interesting building blocks for the design of functional materials thanks to their well-defined secondary structure, biocompatibility and lack of toxicity. They have demonstrated great capacity for self-assembling into well-defined supramolecular structures and show promising applications in DD. These interesting applications have been fueled by recent advances in the synthesis of PAA by ROP of NCAs. The use of initiators based on transition metal complexes and amine hydrochloride salts, or high-vacuum/low-temperature polymerizations, represents breakthroughs in the synthesis of PAA and their block copolymers with precise molecular weight and low polydispersity.

PAA and polypeptides have demonstrated a great versatility as polymeric materials for DD, with applications ranging from polymer therapeutics (covalently bound drugs) to

various types of self-assembled nanostructures (micelles, vesicles, capsules, fibers) with the ability to encapsulate a plethora of different drugs. The possibility of locating positive and negative charges on the amino acid side chains results in PAA and polypeptides with polyelectrolyte character that have found application in the delivery of oppositely charged biopharmaceuticals such as nucleic acids and proteins. Also advantage has been taken from the low pKa of some PAA (PGA, PAsp, polyhistidine) for the preparation of block copolymers with PEG that have found application in the preparation of pH-sensitive DDS.

In particular, the application of polypeptides and PAA to cancer therapy has been attracting great interest and is yielding highly original and promising results. The appealing properties of these polymers, such as biodegradability or their versatility for chemical modifications, turn them into suitable candidates for the design of novel delivery systems for cancer treatment and diagnosis. Interestingly, the nanometric size of these constructs benefits from a tendency to accumulate in solid tumors thanks to the EPR effect. The potential of these polymers in cancer research is reflected by the increasing number of formulations in preclinical and clinical studies.

Among the different systems mentioned above, polymer therapeutics and micelles have shown to be so far the most relevant concerning their clinical development for cancer therapy. Accordingly, as shown in **Table 1**, more than 50% of the formulations in clinical trials belong to these types of structures carrying antitumor agents. In general, the data collected from the clinical evidence show improved pharmacokinetic profiles compared with well-established reference formulations in use. Most of the formulations are well tolerated after their administration to the patients, although some adverse reactions such as hypersensitivity could be observed in some cases. This and other issues related to toxicity and others regulatory considerations have been carefully revised elsewhere.

From the perspective of new nanotherapeutics, novel DDS are proposed by merging the appealing properties of polypeptides and PAA with already known possibilities offered by classical DDS. An interesting example of these new strategies is the recent development of PAA-based nanocapsules. These nanosystems provide a flexible platform with adjustable composition, size, surface charge, according to specific needs. The promising results obtained during the past few years indicate that possible applications of these novel nanosystems include i) encapsulation of hydrophobic drugs in the inner oily core of the system (e.g., anticancer drugs, lipophilic immunoadjuvants), ii) delivery of hydrophilic macromolecules embedded in the surrounding PAA shell (e.g., nucleic acids, antigens) and (iii) the co-delivery of different therapeutic molecules within the same system.

Nevertheless, and despite all the exciting findings obtained so far, it has to be taken into account that these strategies are in their early development stage and, therefore, their potential will have to be fully assessed in subsequent studies. In addition, the vast majority of the delivery systems described in this review have been conceived for parenteral administration. Taking into account the accumulated knowledge, we believe that PAA and polypeptides could also be interesting biomaterials for alternative routes such as the oral or nasal administration of drugs and vaccines. Within this regard, it is clearly necessary to gather more information on the *in vivo* behavior of these biomaterials. In particular, their interaction with the biological environment as well as their biodistribution and immunotoxicology profile should be properly evaluated for different administration routes in order to fully explore the potential of polypeptide and PAA-based systems for DD applications.

Declaration of interest

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ANEXOS

PATENTE: NANOCAPSULAS DE PROTAMINA

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Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

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NANOCÁPSULAS DE PROTAMINA

Campo de la técnica

La presente invención se refiere a un sistema para la administración de principios activos que comprende nanocápsulas a base de protamina de tamaño nanométrico, así como a las composiciones farmacéuticas que comprenden los mismos y procedimientos para su elaboración.

Antecedentes de la invención

La incorporación de ingredientes activos en sistemas de tamaño nanométrico ha ayudado a solventar las limitaciones de formulación que presentan estas moléculas, incrementando adicionalmente su potencial en terapéutica. Mejoras en la solubilidad, protección frente a la degradación o mayor penetración de los ingredientes activos son algunas de las ventajas que ofrecen la nanoencapsulación de moléculas activas o la adsorción de estas mismas en la cubierta polimérica. Asimismo, es también conocido que la capacidad de estos sistemas para atravesar las barreras externas y acceder al interior del organismo depende tanto de su tamaño como de su composición. Partículas de pequeño tamaño aumentarán el grado de transporte respecto a las de un mayor tamaño: los nanosistemas, de diámetro inferior a 1 μm, responden a este criterio.

Además, es posible recubrir estas nanocápsulas con materiales poliméricos. Así un posible material polimérico es la protamina, la cual pertenece a una familia de polipéptidos naturales, ricos en arginina que se sintetizan en la última etapa de espermátida de muchos animales y plantas, en el proceso de la espermatogénesis. Si bien su estudio comienza en los año 1868 por Friedrich Miescher, muchos son los trabajos que se han realizados para caracterizar a este conjunto de péptidos alifáticos, fuertemente básicos, de peso molecular aproximado entre los 4000 – 10000 Da.

La Protamina ha sido aprobada como excipiente farmacéutico y su principal aplicación hoy en día, se encuentra en la formulación de liberación sostenida de insulina: NPH (del ingles: Neutral Protamine Hagedorn), aparte de tener registro sanitario como principio activo por ser el antídoto para intoxicaciones por heparina.

Muchos y diversos son los estudios en los que se han utilizado este excipiente principalmente en combinación con liposomas para la liberación de DNA al interior de las células (Gene Ther. 1997. 4, 961–968), conjugadas con algún oligonucleótido; nanopartículas llamadas proticles (Nucleic Acids Res. 2000. 15;28(10):E45.), para la liberación de péptido intestinal vasoactivo (J Control Release. 2008. 10;130(2):192-8), aprovechando su supuesta actividad antimicrobiana (J Antimicrob Chemother. 2008 Mar;61(3):651-7), entre otras.

En los estudios mencionados anteriormente, por su actividad intrínseca, la protamina ha sido ampliamente utilizada e investigada para formar complejos con material genético mediante interacciones electrostáticas entre su elevada carga positiva, dada por los grupos argininas que interaccionan con los grupos aniónicos de los ácidos nucleicos; por ende es capar de unir y precipitar el DNA dentro de las estructuras que sirven como vehículos en su administración. También se ha demostrado la actividad adyuvante de este polipéptido, incrementando la secreción de interleukina 2 e interferón gama, así como la proliferación de células T (J Control Release. 2008; 10;130(2):161-7).

No obstante se hace imposible su utilización en la formulación de nanocápsulas de protamina conteniendo un nucleo oleoso, ya que este polipéptido desestabiliza las emulsiones aceite/agua. Teniendo esto en consideración se hace necesario diseñar nuevos sistemas nanocapsulares que no sean desestabilizados por la protamina y se beneficien de sus propiedades.

Breve descripción de la invención

Los autores de la presente invención han desarrollado un sistema nanocapsular que incorpora protamina y no se desestabiliza por su presencia. Este sistema nanocapsular es estable, fácil de obtener y además permite asociar eficazmente ingredientes activos de diferente naturaleza, tanto hidrofílicos como lipofílicos. El tamaño reducido de dichas nanocápsulas (diámetro inferior a 1 μm) y la carga superficial positiva de protamina, permite la interacción con superficies biológicas del organismo cargadas negativamente, como lo son las mucosas y posibilita su paso a través de estas y que sean internalizadas por las células. Asimismo, la presencia de una cubierta polimérica le confiere mayor estabilidad a las nanocápsulas y le proporciona características beneficiosas propias de la protamina.

Por lo tanto, un primer aspecto de la presente invención se refiere a un sistema nanocapsular (de aquí en adelante "sistema nanocapsular de la invención") adecuado para la administración de principios activos, donde las nanocápsulas del sistema comprenden:

a. Una capa superficial constituida por el polipéptido protamina ó que comprende el polipéptido protamina;

b. Un núcleo oleoso;

c. Un tensoactivo caracterizado por tener una relación hidrofílica-lipofílica (balance hidrófilico-lipofílico (HLB)) superior a 8; y

d. opcionalmente al menos un principio activo,

con la condición de que el tensoactivo no es un fosfolípido.

Ejemplos de tensoactivos adecuados para poner en práctica la presente invención son: monooleato de sorbitán poli oxietilénico (Tween 80®), monoláurato de sorbitan poli oxietilénico (Tween 20®), monoestearato de polioxietilensorbitano (Tween 61®), monooleato de polioxietilensorbitano (Tween 81®), triestearato de polioxietilensorbitano (Tween 65®), trioleato de polioxietilensorbitano (Tween 85®), monolaurato de polioxietilensorbitano (Tween 21®), monoestearato de polietilenglicol, estearato de polietilenglicol, dilaurato de polietilenglicol, monopalmitato de polietilenglicol, polietilenglicol estearato, Poloxamer 124, Poloxamer 188, Poloxamer 237, Poloxamer 338, Poloxamer 407, Solutol HS15 ®, TPGS, oleato de trietanolamonio, oleato sódico, colato de sodio, deoxicolato de sodio, lauril sulfato sódico, oleato de irietanolamina, goma tragacanto y dodecilsulfato sódico ó cualquier combinación de los tensoactivos citados. Preferentemente el tensoactivo se selecciona de la lista que consiste en colato de sodio, polietilenglicol estearato, Solutol HS15 ®, TPGS, monooleato de sorbitán poli oxietilénico (Tween 80®) y monoláurato de sorbitan poli oxietilénico (Tween 20®) ó cualquier combinación de estos tensoactivos.

Ejemplos de componentes del núcleo oleoso adecuados para poner en práctica la presente invención son: aceite de cacahuete, algodón, oliva, ricino, soja, cártamo, palma, α tocoferol (vitamina E), miristato de isopropilo, escualeno, Miglyol®, Labrafil®, Labrafac®, Peceol® y Maisine® o cualquier combinación de estos aceites. Preferentemente el núcleo lipófilo oleoso se selecciona de la lista que consiste en Miglyol®, escualeno ó α tocoferol ó cualquiera de sus combinaciones.

En una realización particular del primer aspecto de la invención, el sistema nanocapsular de la invención comprende:

a. Una capa superficial constituida por el polipéptido protamina ó que comprende el polipéptido protamina;

b. Un núcleo oleoso seleccionado de la lista que consiste en Miglyol®, escualeno ó α tocoferol o cualquiera de sus combinaciones;

c. Un tensoactivo caracterizado por tener una relación hidrofílica-lipofílica (balance hidrófilico-lipofílico (HLB)) superior a 8, seleccionado de la lista que consiste en colato de sodio, polietilenglicol estearato, Solutol HS15 ®, TPGS, monooleato de sorbitán poli oxietilénico (Tween 80®) y monoláurato de sorbitan poli oxietilénico (Tween 20®) ó cualquiera de sus combinaciones; y

d. opcionalmente al menos un principio activo.

En otra realización del primer aspecto de presente invención el sistema nanocapsular de la invención se caracteriza por estar liofilizado.

Un segundo aspecto de la invención se refiere a un procedimiento para obtener el sistema nanocapsular de la invención (de aquí en adelante "procedimiento de obtención de la invención"). Así, para lograr la formación de nanocápsulas en un rango de tamaños deseado, se procede a la formación de los núcleos oleosos que comprenden un aceite y uno o más tensoactivos, en cuya superficie se une el polímero de recubrimiento a través de diferentes tipos de interacción. Se trata, por tanto, de un proceso de difusión de solventes, que ocurre de manera controlada y proporciona estabilidad al sistema, sin que exista la necesidad de crear enlaces covalentes entre los componentes.

Por tanto, en una realización preferida del segundo aspecto de la invención, el procedimiento de obtención de la invención es un procedimiento de difusión de disolvente en una etapa que comprende los siguientes pasos:

a. preparar una disolución acuosa que comprende protamina;

b. preparar una disolución orgánica que comprende los componentes del núcleo oleoso y uno o más tensoactivos caracterizados por tener una relación hidrofílica-lipofílica superior a 8;

c. mezclar bajo agitación las disoluciones preparadas en las etapas a) y b) para obtener las nanocápsulas; y

d. opcionalmente, evaporar total o parcialmente los disolventes orgánicos de la mezcla obtenida en la etapa anterior hasta volumen constante,
con la condición de que el tensoactivo no es un fosfolípido.

En otra realización preferida del segundo aspecto de la invención, el procedimiento de obtención de la invención es un procedimiento de difusión de disolvente en dos etapas que comprende los siguientes pasos:

a. preparar una disolución orgánica que comprende los componentes del núcleo oleoso y uno o más tensoactivos caracterizados por tener una relación hidrofílica-lipofílica superior a 8;

b. añadir la disolución obtenida en la etapa a) sobre una fase acuosa que opcionalmente contiene un tensoactivo soluble en agua y que está bajo agitación para formar una nanoemulsión;

c. opcionalmente, evaporar total o parcialmente los disolventes orgánicos hasta volumen constante; y

d. recubrir la nanoemulsión obtenida en la etapa anterior mediante un proceso de incubación con una disolución acuosa que comprenda protamina, con la condición de que el tensoactivo no es un fosfolípido.

En el supuesto que el sistema nanocapsular de la invención comprendiese un principio activo el procedimiento de la invención incluiría la adición de éste a la disolución orgánica si es éste tiene carácter lipófilo ó a la disolución acuosa si éste tiene carácter hidrófilo.

Un tercer aspecto de la presente invención, se refiere al sistema nanocapsular de la invención para su uso en terapia.

En una realización preferida del tercer aspecto de la invención, el sistema nanocapsular de la invención comprende como principio activo docetaxel y se usa para el tratamiento del cáncer, preferentemente del cáncer de pulmón o páncreas.

En otra realización preferida del tercer aspecto de la invención, el sistema nanocapsular de la invención comprende como principio activo el antígeno recombinante de la hepatitis B (rHBsAg) y se usa para el tratamiento o prevención de la hepatitis B.

En aún otra realización preferida del tercer aspecto de la invención, el sistema nanocapsular de la invención comprende como principio activo el antígeno recombinante de la influenza H1N1 (HI) y se usa para el tratamiento o prevención de la influenza del tipo H1N1.

Un cuarto aspecto de la invención, se refiere a una composición farmacéutica que comprende el sistema nanocapsular de la invención y opcionalmente uno o más excipientes farmacéuticamente aceptables.

BREVE DESCRIPCIÓN DE LAS FIGURAS

Figura 1: Viabilidad de la línea celular de cáncer A549 tras 24 y 48 horas de contacto con nanocápsulas de protamina cargadas con DCX, nanocápsulas blancas de protamina, disolución de DCX en etanol y etanol.

Figura 2: Viabilidad de la línea celular de cáncer H460 tras 24 y 48 horas de contacto con nanocápsulas de protamina cargadas con DCX, nanocápsulas blancas de protamina, disolución de DCX en etanol y etanol.

Figura 3: Viabilidad de la línea celular de cáncer MiaPaCa 2 tras 24 y 48 horas de contacto con nanocápsulas de protamina cargadas con DCX, nanocápsulas blancas de protamina, disolución de DCX en etanol y etanol.

Figura 4: Viabilidad de la línea celular de cáncer BxPC3 tras 24 y 48 horas de contacto con nanocápsulas de protamina cargadas con DCX, nanocápsulas blancas de protamina, disolución de DCX en etanol y etanol.

Figura 5: Niveles de anticuerpo IgG en sangre de ratóntras la administración de nanocápsulas de protamina con aceite escualeno (SQL) y α tocoferol (TCPH) cargadas con el antígeno de la hepatitis B (10 μg) por vía nasal (3 dosis), intramuscular (2 dosis), y un calendario combinado (1 dosis intramuscular y 2 nasal); comparados con el antígeno adsorbido en hidróxido de aluminio administrado por vía intramuscular(2 dosis).

Figura 6: Niveles de absorbancia producido por los anticuerpos IgG en sangre de ratón tras la administración de nanocápsulas de protamina con núcleo de α tocoferol (TCPH) cargadas con el antígeno de la influenzaen dosis de 2 y 7,5 μg, por vía subcutanea.

Figura 7: Wesern blot del antígeno recombinante de la hepatitis B adsorbido en las nanocápsulas de protamina tras el proceso de liofilización.

Figura 8: Viabilidad de la línea celular de macrófagos RAW 264,7 tras 24 y 48 horas de contacto con nanocápsulas de protamina y núcleo de escualeno (SQL) o α tocoferol (TCPH).

Figura 9: Imágenes de microscopia electrónica de transmisión (TEM) de nanocápsulas de protamina preparadas con la combinación de tensoactivos PEG estearato/colato de sodio y aceite Miglyol®.

Figura 10: Imágenes de microscopia confocal de nanocápsulas de protamina preparadas con el tensoactivo PEG estearato/colato de sodio y aceite α tocoferol. Como molécula activa están cargadas con el antígeno de la influenza (H1N1).

DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

La presente invención se dirige al diseño y desarrollo de sistemas de nanocápsulas para la administración de principios activos, en donde las nanocápsulas del sistema tienen un diámetro medio inferior a 1 μm y se caracterizan por comprender (a) una cubierta de protamina (b) un núcleo oleoso y uno o más tensoactivos caracterizados por tener una relación hidrofílica-lipofílica superior a 8, con la condición de que dicho tensoactivo no es un fosfolípido.

Resulta conocido que la protamina desestabiliza las emulsiones aceite/agua. De hecho, ni siquiera la utilización de tensoactivos tales como lipoproteínas u otros que posean estructura fosfolipídica, como lecitina, fosfatidilglicerol, fosfatidilserina, fosfatidilinositol, difosfatidilglicerol, ácido fosfatídico, fosfatidilcolina y fosfatidiletanolamina son capaces de lograr estabilizar dichas emulsiones aceite/agua en las que la protamina está presente. Este hecho se confirma en el ejemplo 1 de la presente invención donde los autores han intentado la formación de sistemas de nanocápsulas con un diámetro medio inferior a 1 μm caracterizadas por comprender (a) una cubierta de protamina y (b) un núcleo oleoso utilizando como tensoactivos fosfolípidos como la lecitina, lecitinas modificadas o la lisofosfatidilcolina sin éxito.

Por lo tanto se hace imposible la utilización de la protamina en este tipo de formulaciones. Por ello y con el objeto de solventar este problema los autores de la presente invención han diseñado nuevos sistemas nanocapsulares que no sean desestabilizados por la protamina y se beneficien de sus propiedades.

En este sentido, los autores de la presente invención han descubierto como a través de la utilización de ciertos tipos de tensoactivos caracterizados por tener una relación hidrofílica-lipofílica (balance hidrófilico-lipofílico (HLB)) superior a 8, preferentemente superior a 12, se puede observar la obtención de sistemas nanocápsulares de protamina caracterizadas por comprender (a) una cubierta de protamina y (b) un núcleo oleoso. Así, en los ejemplos 2.1 a 2.18 se ilustra como con diferentes surfactantes, núcleos oleosos y métodos de preparación se pueden obtener este tipo de sistemas, siempre y cuando los tensoactivos utilizados estén caracterizados por tener un HLB > 8 y no sean fosfolípidos. Asimismo, estos ejemplos demuestran la versatilidad del sistema respecto a sus características fisicoquímicas.

Por lo tanto, un primer aspecto de la presente invención se refiere a un sistema nanocapsular ("sistema nanocápsular de la invención) adecuado para la administración de principios activos, que comprende:

a. Una capa superficial constituida por el polipéptido protamina ó que comprende el polipéptido protamina;

b. Un núcleo oleoso;

c. Un tensoactivo caracterizado por tener una relación hidrofílica-lipofílica (balance hidrófilico-lipofílico (HLB)) superior a 8; y

d. opcionalmente al menos un principio activo, con la condición de que el tensoactivo no es un fosfolípido.

La ventaja de este sistema concreto de nanocápsulas con respecto a otros sistemas conocidos ya sean nanocapsulares o de nanoemulsiones es la presencia del polipéptido protamina en la cubierta. Este polipéptido brinda, entre otras propiedades, estabilidad a la nanocápsula así como protección, capacidad de penetración y especificidad en su interacción con determinadas células diana. La presencia de protamina en la superficie de las nanocápsulas además brinda una mayor respuesta por parte de las células inmunes por tener una comprobada actividad adyuvante. La protamina es un compuesto natural, lo que presenta ventajas frente a otros polímeros con alto contenido de argininas como por ejemplo la poliarginina que es una molécula sintética. De este modo, la presencia de protamina permite que las nanocápsulas de la invención sean un vehículo seguro capaz de metabolizarse y excretarse rápidamente evitando así llegar a concentraciones tóxicas y acumulación en el organismo. Además, para las nanocápsulas de la invención con cubierta de protamina no es

necesario un crioprotector para la liofilización ni elevadas diluciones para evitar agregaciones indeseadas.

Adicionalmente, se hace notar que en el contexto de la presente invención el término protamina incluye las sales solubles en agua de protamina así como derivados hidrosolubles de protamina.

Además, en comparación con otros sistemas como los liposomas o las nanopartículas, que generalmente se ven condicionadas a una limitada carga de fármaco, las nanocápsulas de la presente invención poseen una mayor posibilidad de carga, en particular de fármacos lipofílicos, debido a la presencia del núcleo oleoso. Otra de las grandes ventajas de las nanocápsulas de la presente invención es la capacidad de combinar fármacos de diferente naturaleza, pudiendo estar un fármaco lipofílico encapsulado en el núcleo y un fármaco hidrofílico asociado a la cubierta; asimismo, la cubierta les brinda estabilidad, protección y especificidad.

Estos sistemas presentan además ventajas respecto a otros de mayor tamaño (micropartículas, pellets, films, esponjas…) en cuanto a sus aplicaciones biológicas. De hecho, se sabe que la interacción de un sistema de liberación de fármacos con una superficie biológica está altamente condicionada por su tamaño. Así, las nanocápsulas son capaces de atravesar mucosas y de ser internalizadas por las células actuando como sistemas de transporte de fármacos, mientras que las micropartículas no tienen esa capacidad. Igualmente, la biodistribución de estos sistemas está altamente condicionada por el tamaño. El conocimiento generado en los últimos años en el mundo de la nanomedicina y los nanosistemas de liberación de fármacos ha permitido fijar una frontera claramente definida entre los sistemas nanométricos (que poseen un tamaño inferior a una micra ej. nanopartículas y nanocápsulas) y los sistemas micrométricos (micropartículas y microcápsulas). Además de las diferencias de comportamiento en cuanto a su capacidad para ser internalizados por las células y superar complejas barreras biológicas, en el caso de las formulaciones destinadas a la administración intravenosa de fármacos antitumorales es imprescindible el tamaño nanométrico de los sistemas de liberación a fin de prevenir la obstrucción de los capilares sanguíneos. Asimismo, se sabe que las posibilidades de los nanosistemas para alcanzar el tejido tumoral están estrictamente relacionadas con su tamaño y también por el carácter hidrofílico de su superficie.

Asimismo, es importante destacar la diferencia entre los sistemas de nanocápsulas y los "complejos". Se entiende por "complejos" la nanoestructura formada por la interacción de polielectrolitos o bien por polielectrolitos y tensoactivos de carga opuesta. Los sistemas de nanocápsulas de la presente invención se diferencian de los complejos por tratarse de un sistema transportador nanocapsular, tipo reservorio, en cuyo núcleo se pueden alojar un importante número de moléculas que tengan una mayor o menor afinidad por el núcleo oleoso (encapsulación) y en cuya cubierta pueden incorporarse moléculas hidrofílicas que tengan una cierta afinidad por la misma (adsorción). Estas características permiten mantener la integridad y funcionalidad de la nanoestructura, así como aportar mayor estabilidad en presencia de fluidos biológicos.

Así, el sistema nanocapsular de la invención presentan ventajas en comparación con otros sistemas de administración y/o liberación de fármacos, debido a su comportamiento singular en cuanto a:

- la encapsulación/asociación de principios activos: el sistema puede incluir uno o más principios activos o sustancias adyuvantes, hidrofílicos o lipofílicos, en proporciones superiores a la de las nanopartículas, micelas, complejos, nanogeles.

- la liberación del principio activo: la cubierta ejerce una función en la velocidad de liberación del mismo, permitiendo liberar de forma controlada el principio activo según aplicación y necesidades.

- la estabilidad en fluidos biológicos: la cubierta polimérica confiere a los núcleos oleosos una gran estabilidad, lo que representa una ventaja frente a otros sistemas de micro y nanoemulsiones.

- la interacción específica con determinados superficies biológicas: la cubierta polimérica confiere a los núcleos oleosos la posibilidad de interaccionar con superficies mucosas así como con epitelios y células específicas.

- la rápida metabolización y excreción de la protamina, confiere a este sistema un perfil de seguridad farmacocinético, no pudiéndose demostrar el mismo perfil para otros sistemas nanocapsulares con otra tipo de cubierta, como el de poliarginina.

- la estabilidad durante la liofilización, no es necesario en las nanocápsulas de la invención crioprotectores para ese proceso, ni elevadas diluciones para evitar agregaciones indeseadas.

Las nanocápsulas del sistema de la presente invención presentan un diámetro medio inferior a 1 μm, respondiendo por tanto a la definición de nanosistema, sistema coloidal constituido a base de polímeros con un tamaño inferior a 1 μm, es decir, tienen un tamaño de entre 1 y 999 nm, preferiblemente de entre 30 y 500 nm. Se entiende por diámetro medio aquel medido mediante la técnica Dynamic Light Scattering (DLS) que está definido por el diámetro hidrodinámico de una esfera que difunde a la misma velocidad que las partículas que se están midiendo. El tamaño de las nanocápsulas está influido principalmente por la composición y las condiciones de formación y puede medirse utilizando procedimientos estándar conocidos por el experto en la técnica y que se describen en el apartado de ejemplos. En este sentido, tal y como se puede comprobar el tamaño de las mismas no varía notoriamente al modificar la relación de compuesto de cubierta en la formulación, obteniéndose en todos los casos sistemas de tamaño nanométrico.

Los sistemas de nanocápsulas aquí descritos presentan una estabilidad adecuada tanto en suspensión como bajo forma de liofilizado. Por otra parte, los estudios de estabilidad parecen indicar que tras su administración a organismos, humano o animal, no sufren un proceso rápido de agregación o destrucción, sino que previsiblemente permanecen bajo forma nanocapsular hasta alcanzar el tejido o célula diana.

Por otro lado, como ya se ha comentado, el sistema nanocapsular de la presente invención comprende al menos un tensoactivo. En la presente invención, el término “tensoactivo” alude a un componente que posee estructuras y/o grupos funcionales que les permiten interaccionar simultáneamente con la parte lipófila e hidrófila de la formulación. Para preparar el sistema nanocapsular de la invención resulta necesario tener en consideración el HLB del tensoactivo a utilizar. El concepto HLB se basa en un método experimental que consiste en atribuir un cierto número HLB a los agentes emulsionantes a partir de datos relativos a la estabilidad de una emulsión. Este número HLB representa implícitamente varios parámetros y da cuenta del balance hidrofílico-lipofílico del sistema. El método para calcular el HLB como se define en la presente invención se basa en los grupos funcionales de la molécula estudiada, teniendo en cuenta la fuerza de los grupos hidrofílicos, se calcula de la siguiente manera:

$$HLB = 7 + m * Hh - n * Hl$$

Donde *m* es el número de grupos hidrofilicos, H*h* el valor de los grupos hidrofilicos, *n* el número de grupos lipofilicos y H*l* su valor (Davies, J. T. (1957) "A quantitative kinetic theory of emulsion type. I. Physical chemistry of the emulsifying agent. Gas/Liquid and Liquid/Liquid Interfaces". Proceedings of 2nd International Congress Surface Activity, Butterworths, London, pp. 426-38).

Ejemplos de tensoactivos adecuados para llevar a cabo la presente invención se seleccionan de entre ésteres de sorbitan etoxilados y ésteres de ácidos grasos. En una realización particular, los ésteres de sorbitán se seleccionan de entre monooleato de sorbitán poli oxietilénico (Tween 80®), monoláurato de sorbitan poli oxietilénico (Tween 20®), monoestearato de polioxietilensorbitano (Tween 61®), monooleato de polioxietilensorbitano (Tween 81®), triestearato de polioxietilensorbitano (Tween 65®), trioleato de polioxietilensorbitano (Tween 85®) y monolaurato de polioxietilensorbitano (Tween 21®). En otra realización particular, los ésteres de ácidos grasos se seleccionan de entre monoestearato de polietilenglicol, estearato de polietilenglicol, dilaurato de polietilenglicol, monopalmitato de polietilenglicol, polietilenglicol estearato, Poloxamer 124, Poloxamer 188, Poloxamer 237, Poloxamer 338, Poloxamer 407, Solutol HS15 ®, TPGS. En otra realización particular, el tensoactivo se selecciona de entre el grupo constituido por oleato de trietanolamonio, oleato sódico, colato de sodio, deoxicolato de sodio, lauril sulfato sódico, oleato de irietanolamina, goma tragacanto y dodecilsulfato sódico. En aún otra realización particular el tensoactivo es colato de sodio, estearato de polietilenglicol, TPGS, Solutol HS15 ®, Tween 20, Tween 80 o combinaciones de los mismos.

Adicionalmente, el sistema nanocapsular de la presente invención se caracteriza por tener un nucleo oleoso constituido, entre otros componentes, a base de aceites volátiles o no volátiles. En el contexto de la presente invención se entiende por núcleo oleoso el que constituye la estructura interna de las nanocápsulas de la invención y está compuesto por al menos un aceite y al menos un tensoactivo como se ha descrito anteriormente.Estos aceites se pueden seleccionar entre aceites naturales, semisintéticos y sintéticos de uso farmacéutico tales como aceites de origen animal, vegetal, aceites hidrocarbonados o aceites de silicona. Aceites adecuados para llevar a cabo la presente invención incluyen, pero no se limitan a, aceite mineral, aceite de escualeno, aceites de sabor, aceite de silicona, aceites esenciales, vitaminas insolubles en agua, isopropil estearato, butil estearato, octil palmitato, cetil palmitato, tridecil behenato, diisopropil adipato, dioctil sebacato, mentil antranilato, cetil octanoato, octil

salicilato, isopropil miristato, cetoles de dicarpato de neopentilglicol, Cerafilos®, decil oleato, C_{12}-C_{15} alquil lactatos, cetil lactato, lauril lactato, isostearil neopentanoato, miristil lactato, isocetil estearoil estearato, octildodecil estearoil estearato, aceites de hidrocarburos, isoparafina, parafinas fluidas, isododecano, vaselina, aceite de argán, aceite de colza, aceite de chile, aceite de coco, aceite de maíz, aceite de algodón, aceite de lino, aceite de semilla de uva, aceite de mostaza, aceite de oliva, aceite de palma, aceite de palma fraccionado, aceite de cacahuete, aceite de ricino, aceite de semilla de pino, aceite de semilla de amapola, aceite de semilla de calabaza, aceite de salvado de arroz, cártamo, aceite de té, aceite de trufa, aceite vegetal, aceite de albaricoque, aceite de jojoba, aceite de macadamia, aceite de germen de trigo, aceite de almendra, aceite de soja, aceite de sésamo, aceite de avellana, aceite de girasol, aceite de cáñamo, aceite de bois, aceite de nuez de Kukui, aceite de aguacate, aceite de nuez, aceite de pescado, aceite de baya, aceite de pimienta de Jamaica, aceite de enebro, aceite de semilla, aceite de semilla de almendra, aceite de semilla de anís, aceite de semilla de apio, aceite de semilla de comino, aceite de semilla de nuez moscada, aceite de hoja de albahaca, aceite de hoja de laurel, aceite de hoja de canela, aceite de hoja de salvia común, aceite de hoja de eucalipto, aceite de hoja de limón, aceite de hoja de melaleuca, aceite de oregano, aceite de hoja de pachuli, aceite de hoja de menta, aceite de aguja de pino, aceite de hoja de romero, aceite de menta verde, aceite de hoja del árbol de té, aceite de hoja de tomillo, aceite de hoja de té de Canadá, aceite de flor, aceite de camomila, aceite de salvia romana, aceite de clavo, aceite de flor de geranio, aceite de flor de hisopo, aceite de flor de jazmín, aceite de flor de lavanda, aceite de flor de mauka, aceite de flor de mejorana, aceite de flor de naranja, aceite de flor de rosa, aceite de flor de ylang-ylang, aceite de corteza, aceite de corteza de casia, aceite de corteza de canela, aceite de corteza de sasafrás, aceite de madera, aceite de madera de alcanfor, aceite de madera de cedro, aceite de palo de rosa, aceite de sándalo, aceite de madera de jengibre, aceite de resina, aceite de recino, aceite de mirra, aceite de piel, aceite de piel de Bérgamo, aceite de piel de pomelo, aceite de piel de limón, aceite de piel de lima, aceite de piel de naranja, aceite de piel de mandarina, aceite de raíz, aceite de valeriana, ácido oleico, ácido linoleico, oleil alcohol, alcohol de isostearilo, oleato de etilo, Miglyol®, Labrafil®, Labrafac®, Rylo®, Peceol® y Maisine®, derivados sintéticos o semisintéticos de los mimos y combinaciones de los mismos. Preferentemente el aceite se selecciona de la lista que consiste en aceite de cacahuete, algodón, oliva, ricino, soja, cártamo, palma, α tocoferol (vitamina E), miristato de isopropilo, escualeno, Miglyol®, Labrafil®, Labrafac®, Peceol® y Maisine® o mezclas de los mismos. De forma más preferida los aceites son Miglyol®, escualeno o α tocoferol.

Tal como se ha definido anteriormente, las nanocápsulas de la invención también comprenden de manera opcional al menos un principio activo. El término “principio activo” se refiere a cualquier sustancia que se utiliza en el tratamiento, cura, prevención o diagnóstico de una enfermedad o que se utiliza para mejorar el bienestar físico y mental de seres humanos y animales. El principio activo podrá ser por ejemplo un fármaco, un antígeno, una vitamina, etc. Los sistemas de nanocápsulas objeto de la presente invención son adecuados para incorporar principios activos de naturaleza lipófila o hidrófila. En una realización preferida, los ingredientes activos son antígenos de superficie recombinante de hepatitis B, antígeno de la influenza (H1N1) y docetaxel.

La proporción de principio activo incorporado dependerá en cada caso del ingrediente activo que va a incorporarse, la indicación para la que se utiliza y la eficiencia de administración.

Por otro lado, los procedimientos de obtención de los sistemas de nanocápsulas de la invención son métodos sencillos que evitan condiciones drásticas como altas temperaturas. Además, tampoco es necesario llevar a cabo ningún tipo de reacción química para la obtención de los mismos, ya que según se ha indicado anteriormente la obtención del sistema implica interacciones no covalentes. Por tanto, se preserva así la integridad de las moléculas incorporadas al sistema, susceptibles de ser degradadas. Para lograr la formación de nanocápsulas en un rango de tamaños deseado, se procede a la formación de los núcleos oleosos que comprenden un aceite y uno o más tensoactivos, en cuya superficie se une el polímero de recubrimiento a través de diferentes tipos de interacción. Se trata, por tanto, de un proceso de difusión de solventes, que ocurre de manera controlada y proporciona estabilidad al sistema, sin que exista la necesidad de crear enlaces covalentes entre los componentes.

Un procedimiento particular para la obtención de los sistemas de la invención (denominado en los ejemplos procedimiento de difusión de disolvente en una etapa), comprende:

a) preparar una disolución acuosa que comprende protamina, y opcionalmente un tensoactivo soluble en agua;

b) preparar una disolución orgánica que comprende un aceite y uno o más tensoactivos, caracterizados por tener una relación hidrofílica-lipofílica superior a 8, con la condición de que el tensoactivo no es un fosfolípido;

c) mezclar bajo agitación las disoluciones preparadas en las etapas a) y b), obteniéndose espontáneamente las nanocápsulas; y

d) opcionalmente, evaporar total o parcialmente los disolventes orgánicos de la mezcla obtenida en la etapa anterior hasta volumen constante.

Los sistemas de la presente invención se pueden preparar mediante un procedimiento alternativo (denominado en los ejemplos procedimiento de difusión de disolvente en dos etapas) que comprende recubrir una nanoemulsión con protamina mediante un proceso de incubación con una disolución acuosa del polímero. Asimismo, la formación de la nanoemulsión puede favorecerse mediante ultrasonidos (denominado en los ejemplos procedimiento de sonicación) u homogeneización (denominado en los ejemplos procedimiento de homogeneización).

En una realización particular, el proceso de incubación comprende mezclar la nanoemulsión con una disolución acuosa del polímero de recubrimiento. Dicha nanoemulsión está constituida al menos por un aceite, uno o más tensoactivos, caracterizados por tener una relación hidrofílica-lipofílica superior a 8 y una fase acuosa. La fase acuosa puede contener otros agentes tensoactivos, sales, y otros agentes auxiliares.

Los procedimientos de preparación de dicha nanoemulsión son conocidos en el estado de la técnica, y pueden comprender un proceso de difusión, sonicación u homogeneización (Prego et al. J. Nanosci. Nanotechnol. (2006) 6:1; Tadros et al. Adv. Colloid Interface Sci. (2004) 109:303).

Un procedimiento particular para la obtención de la nanoemulsión (denominado en los ejemplos procedimiento de difusión de disolvente) comprende:

i) preparar una disolución orgánica que comprende un aceite, uno o más tensoactivos, caracterizados por tener una relación hidrofílica-lipofílica superior a 8, con la condición de que el tensoactivo no es un fosfolípido;

ii) añadir la disolución obtenida en la etapa i) sobre una fase acuosa que opcionalmente contiene un tensoactivo soluble en agua y que está bajo agitación para formar una nanoemulsión;

iii) opcionalmente, evaporar total o parcialmente los disolventes orgánicos hasta volumen constante.

Otro procedimiento particular para la obtención de la nanoemulsión (denominado en los ejemplos procedimiento de sonicación) comprende:

i) preparar una disolución orgánica que comprende un aceite, uno o más tensoactivos caracterizados por tener una relación hidrofílica-lipofílica superior a 8, con la condición de que el tensoactivo no es un fosfolípido;

ii) añadir la disolución obtenida en la etapa i) sobre una fase acuosa que opcionalmente contiene un tensoactivo soluble en agua y sonicar;

iii) diluir con agua la emulsión obtenida en la fase ii);

iv) opcionalmente, evaporar total o parcialmente los disolventes orgánicos hasta volumen constante.

Aún otro procedimiento particular para la obtención de la nanoemulsión (denominado en los ejemplos procedimiento de homogeneización) comprende:

i) preparar una disolución orgánica que comprende un aceite, uno o más tensoactivos, caracterizados por tener una relación hidrofílica-lipofílica superior a 8, con la condición de que el tensoactivo no es un fosfolípido;

ii) añadir la disolución obtenida en la etapa i) sobre una fase acuosa que opcionalmente contiene un tensoactivo soluble en agua y homogeneizar;

iii) diluir con agua la emulsión obtenida en la fase ii) y homogeneizar;

iv) opcionalmente, evaporar total o parcialmente los disolventes orgánicos hasta volumen constante.

Según realizaciones particulares de los procedimientos anteriores, si el principio activo es lipofílico o anfifílico, dicho ingrediente activo se añade a la disolución orgánica de la etapa b)

o de la etapa i). Según otras realizaciones particulares, si el ingrediente activo es hidrofílico, dicho ingrediente activo se añade a la disolución de la etapa a) o de la etapa ii). De manera preferida, dicho ingrediente activo hidrófilo se añade disuelto en una disolución acuosa. También es posible incorporar el ingrediente activo hidrófilo mediante adsorción a la suspensión de nanocápsulas obtenidas en la etapa d) o tras el proceso de incubación una vez formadas las nanocápsulas.

En el contexto de la presente invención se entiende por lipofílico a las moléculas, sustancias, principios activos, estructuras o parte de ellas que no son capaces de interaccionar por si sola con moléculas de agua y son principalmente disueltas en solventes apolares.

En el contexto de la presente invención se entiende por anfifílico a las moléculas, sustancias, principios activos, estructuras o parte de ellas que poseen tanto propiedades hidrofóbicas como hidrofílicas.

En el contexto de la presente invención se entiende por hidrofílico a las moléculas, sustancias, principios activos, estructuras o parte de ellas que son atraídas por el agua y son capaces de disolverse en ella o en solventes polares

La formación de las nanocápsulas se produce al mezclar volúmenes de las soluciones mencionadas que contienen la nanoemulsión con disoluciones acuosas del polímero de recubrimiento en diferentes proporciones, variando la relación de polímero de recubrimiento.

El disolvente de la disolución orgánica es preferentemente una mezcla de disolventes polares tales como etanol, isopropanol y acetona pudiendo incluir además disolventes no polares como por ejemplo el diclorometano. En esta fase orgánica se incorpora el aceite y el o los tensoactivos, caracterizados por tener una relación hidrofílica-lipofílica superior a 8. En una composición particular se incorpora igualmente el ingrediente activo.

Un ejemplo particular para la obtención de los sistemas de nanocápsulas de la invención que comprenden protamina siguiendo el primer procedimiento descrito anteriormente comprende:

a) preparar una disolución acuosa de 20 ml al 0,05 % p/v de Protamina;

b) preparar una fase oleosa compuesta por una disolución etanol/acetona un o ambos tensoactivos (Colato de Sodio y/o PEG estearato), a la que se le adiciona Miglyol® 812.

c) mezclar bajo agitación las disoluciones resultantes de las etapas a) y b), obteniéndose espontáneamente las nanocápsulas;

d) opcionalmente, evaporar los disolventes orgánicos de la mezcla obtenida en la etapa anterior hasta volumen constante.

El procedimiento de elaboración de los sistemas de nanocápsulas puede incluir una etapa adicional de liofilización, con el fin de preservarlos durante su almacenamiento para que conserven sus características iniciales. Debido a la naturaleza de la cubierta de las nanocápsulas de la presente invención, así como de las características de las mismas, no es necesario emplear crioprotectores durante la liofilización. Otra ventaja adicional, es que no es necesario diluir el sistema coloidal antes de la liofilización, ya que los sistemas nanocapsulares de la invención no forman agregados durante la reconstitución del liofilizado.

Alternativamente, es posible añadir uno o mas azúcares que ejerzan efecto crioprotector como por ejemplo trehalosa, glucosa, sucrosa, manitol, maltosa, polivinil pirrolidona (PVP). En forma liofilizada, las nanocápsulas pueden ser almacenadas durante largos períodos de tiempo, y ser fácilmente regeneradas, en caso necesario, simplemente añadiendo un volumen de agua óptimo.

De acuerdo con esta etapa adicional, la presente invención se refiere también a los sistemas de nanocápsulas que comprenden una cubierta de protamina bajo forma de liofilizado.

Adicionalmente, la invención en una realización particular se refiere al sistema nanocápsular de la invención para su uso en terapia. Más concretamente, a una composición farmacéutica, que comprende los sistemas de nanocápsulas de la invención, y opcionalmente uno o más excipientes farmacéuticamente aceptables. En particular, la incorporación de principios activos en las nanocápsulas de la invención origina sistemas, cuyas características en cuanto a su composición, propiedades y morfología, les convierte en excelentes candidatos para el área de la terapéutica. El principio activo a incorporar en los sistemas de la invención será aquél con propiedades farmacoterapéuticas adecuadas de acuerdo con la aplicación terapéutica a la

cual sea destinada la formulación. En una realización particular, el principio activo se selecciona entre péptidos, proteínas, compuestos lipídicos o lipofílicos, compuestos sacarídicos, compuestos de ácidos nucleicos o nucleótidos como oligonucleótidos, polinucleótidos o bien combinaciones de las moléculas citadas.

En una realización preferida, el principio activo lipofílico es docetaxel.

En otra realización preferida, el principio activo es de naturaleza hidrofóbica, anfifílica o hidrofílica. Los principios activos de naturaleza hidrofóbica o anfifílica preferentemente son añadidos en la etapa b) del procedimiento de preparación de nanocápsulas de la invención. Los principios activos de naturaleza hidrofílica preferentemente son añadidos en la etapa a) del procedimiento o en una etapa posterior a la d) mediante un proceso de incubación. Sin embargo, la invención también contempla otras realizaciones como por ejemplo añadir en la etapa b) un principio activo hidrófilo disuelto en un pequeño volumen de fase acuosa. A diferencia de los principios activos hidrofóbicos, que son encapsulados dentro de las nanocápsulas, los principio activos de naturaleza hidrofílica se pueden asociar a la superficie de las mismas mediante adsorción.

En una realización preferida, los principios activos hidrofílicos son el antígeno de superficie recombinante de hepatitis B y el antígeno de la influenza (H1N1).

Dichas composiciones farmacéuticas pueden ser administradas por diferentes vías, tales como a través de mucosas, tópica o parenteral.

La proporción de principio activo incorporado en los sistemas puede llegar a ser de hasta aproximadamente el 50% en peso con respecto al peso total, base seca, de los componentes del sistema de nanocápsulas. Sin embargo, la proporción adecuada dependerá en cada caso del ingrediente activo que va a incorporarse, la indicación para la que se utiliza y la eficiencia de administración. En una realización particular, la proporción de principio activo lipofílico puede llegar a ser de hasta aproximadamente el 10% en peso, preferentemente hasta aproximadamente el 5%.

Tal como se ha descrito anteriormente, cabe la posibilidad de que los sistemas de nanocápsulas descritos en la presente invención incorporen más de un principio activo, que podrán estar disueltos en la misma disolución o por separado, dependiendo esto de la naturaleza de las moléculas a incorporar, evitando que exista ningún tipo de interacción, bien sea química o física, entre ellos.

Tal como se ha definido anteriormente, la invención se refiere al uso de dicho sistema en la preparación de un medicamento. En una realización particular, dicho uso está relacionado con el uso de vacunas o el tratamiento del cáncer. En este sentido, en una realización preferida de la invención, el sistema nanocapsular de la invención comprende como principio activo docetaxel y se usa para el tratamiento del cáncer, preferentemente del cáncer de pulmón o páncreas. Alternativamente, la presente invención también se refiere al uso del sistema nanocapsular de la invención comprendiendo como principio activo docetaxel para la elaboración de un medicamento para el tratamiento del cáncer, preferentemente del cáncer de pulmón o páncreas.

En otra realización preferida de la invención, el sistema nanocapsular de la invención comprende como principio activo el antígeno recombinante de la hepatitis B (rHBsAg) y se usa para el tratamiento o prevención de la hepatitis B. Alternativamente, la presente invención también se refiere al uso del sistema nanocapsular de la invención comprendiendo como principio activo el antígeno recombinante de la hepatitis B (rHBsAg) para la elaboración de un medicamento para el tratamiento o prevención de la hepatitis B.

En aún otra realización preferida de la invención, el sistema nanocapsular de la invención comprende como principio activo el antígeno recombinante de la influenza H1N1 (HI) y se usa para el tratamiento o prevención de la influenza del tipo H1N1. Alternativamente, la presente invención también se refiere al uso del sistema nanocapsular de la invención comprendiendo como principio activo el antígeno recombinante de la influenza H1N1 (HI) para la elaboración de un medicamento para el tratamiento o prevención de la la influenza H1N1.