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Tesis Doctoral

NANOVACUNAS: DISEÑO DE NANOESTRUCTURAS PARA INMUNIZACIÓN

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INFORMAN:

Que la presente Memoria Experimental titulada: "Nanovacunas: Diseño de nanoestructuras para inmunización", elaborada por Jorge Filipe Correia Pinto, ha sido realizada bajo su dirección en el Departamento de Farmacia y Tecnología Farmacéutica y, hallándose concluida, autorizan su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que conste, expiden y firman el presente certificado en Santiago de Compostela, el 24 de Abril de 2015.

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Aos meus pais Joaquina e Leopoldino

e a minha irmã Nuchinha

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RESUMEN

En la presente tesis doctoral se plantea el diseño y desarrollo racional de nanoestructuras como vehículos para la liberación de antígenos. Para ello, y con el objetivo de incrementar la respuesta inmune a antígenos de distinta naturaleza se eligieron biomateriales tales como polisacáridos, poli aminoácidos y lípidos, con propiedades inmunoestimulantes y/o inmunoadyuvantes. A partir de ellos, se desarrollaran tres tipos de nanoestructuras: i) Sistemas basados en el recubrimiento de antígeno particulados mediante biopolímeros tales como la protamina, la poliarginina, el sulfato de dextrano, el alginato o el polinucleótido, poli (I:C); ii) Nanopartículas de quitosano conteniendo moléculas inmunoestimulantes poly (I:C) encapsulado y asociadas a un péptido T-Helper; iii) Nanoemulsiones y nanoemulsiones recubiertas (nanocápsulas) basados en la lisofosfatidilcolina, el ácido linoléico, el escualeno y el quitosano.

Estas nanoestructuras fueran constituidos por nanotecnologías sencillas y fácilmente escalables, tales como la complejación iónica, gelificación iónica y el desplazamiento del solvente según las características de los biomateriales utilizados y de la naturaleza del antígeno a que se destina. Los sistemas desarrollados presentan en general una adecuada estabilidad en suspensión y pudieron ser liofilizados sin alterar sus propiedades fisicoquímicas originales. A estos sistemas se asociaron diferentes antígenos tales como el antígeno recombinante particulado de la hepatitis B y un antígeno peptídico derivado del virus del papiloma humano.

Las formulaciones resultantes fueron analizadas mediante una variedad de técnicas *in vitro* y/o *in vivo*. En general, las nanoestructuras desarrolladas demostraron tener un interesante potencial como adyuvantes de vacunas, habiendo logrado modular la respuesta inmune frente a antígenos de distinta naturaleza tras su administración parenteral y/o mucosa. Más allá de estos resultados, el trabajo recogido en esta tesis pone de manifiesto el valor que la nanotecnología puede aportar como herramienta excepcional para el desarrollo de nuevos sistemas adyuvantes para vacunas más eficaces y seguras.

ABSTRACT

The main goal of this thesis has been the design and development of a variety of antigen nanocarriers made of different biomaterials, such as polysaccharides, polyamino acids, and lipids, intended to enhance the immune response against antigens of different nature. From these materials, three different types of nanostructures were developed: i) Systems based on the multi-enveloping of virus-like particle antigens using protamine, polyarginine, dextran sulfate, alginate or poly (I:C); ii) Chitosan nanoparticles incorporating poly (I:C) and a T-Helper peptide; iii) Nanoemulsions and coated nanoemulsions (nanocapsules) based on lysophosphatidylcholine, linoleic acid, squalene and chitosan.

These nanostructures were produced through simple and easily scalable nanotechnology methods as ionic complexation and solvent displacement technique, according the characteristics of the biomaterials and the nature of the target antigen. The developed systems presented, in general, an adequate stability and could be freeze dried without losing the original physicochemical properties. Different model antigens were associated to the different nanosystems including the recombinant hepatitis B surface antigen virus-like particle and a peptide-based antigen derived from the human papillomavirus.

The resulting formulations were characterized with regard to their biologic activity using a range of *in vitro* and *in vivo studies* techniques. Overall, the nanostructures developed were shown to have a potential for potentiating and modulating the immune response against different types of antigen, following either parenteral and/or mucosal administration. Beyond these specific data, the work presented here highlights the potential of nanotechnology for the development of new safer and more effective vaccines.

INTRODUCCIÓN

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INTRODUCCIÓN

La mayoría de las vacunas administradas en la actualidad se basan en el uso de microorganismos vivos atenuados o inactivados. Estas vacunas han permitido algunos de los mayores logros de la medicina, como es la erradicación del viruela o la disminución de la incidencia de enfermedades tales como la poliomielitis [1] y el rotavirus [2]. A pesar de estos extraordinarios avances, estas vacunas llevan asociado el peligro de reversión del organismo, particularmente en individuos inmunodeprimidos, pudiendo provocar reacciones adversas graves [1, 3]. La seguridad y efectividad de las vacunas es, como para cualquier producto farmacéutico, una característica esencial, pero en este campo se reviste de particular importancia pues está destinada, en general, a individuos saludables. La preocupación frente a potenciales efectos adversos unida a determinadas creencias y aspectos socio-culturales ha conllevado una cierta resistencia hacia la vacunación [4], permitiendo así el resurgimiento de enfermedades anteriormente consideradas controladas [5]. Además, la aparente ineficacia de algunas vacunas clásicas ha contribuido al escepticismo actual en relación con su capacidad para prevenir enfermedades [6, 7].

Por otra parte, además de la necesidad de desarrollar vacunas para enfermedades emergentes, tales como el ébola, siguen existiendo enfermedades infecciosas de las cuales se destacan el sida, la tuberculosis y la malaria [8, 9], para las que aún no se ha podido desarrollar una vacuna eficaz. Así por ejemplo, en el caso del sida, los últimos ensayos clínicos de vacunas candidatas para prevenir esta enfermedad han resultado decepcionantes [10, 11]. Dichos resultados subrayan la necesidad de recurrir a estrategias innovadoras para lograr el desarrollo de vacunas efectivas. Además de estas enfermedades de gran impacto en un contexto global, existen otras enfermedades que carecen de una vacuna efectiva, y que están asociadas a importantes tasas de morbilidad y mortalidad, como el virus sincitial respiratorio humano, que en EEUU provoca 90.000 hospitalizaciones y 4.500 muertes al año; el citomegalovirus, particularmente peligroso para recién nacidos y inmunodeprimidos [3]; y la hepatitis C, causa de cirrosis y cáncer hepático [12].

Por todo ello, la producción de nuevas vacunas, seguras y con inequívoca eficacia, es una necesidad fundamental y una condición determinante para mantener la cobertura sanitaria necesaria para la prevención de brotes infecciosos. Esta necesidad ha

estimulado el desarrollo de vacunas más seguras y especificas basadas en antígenos recombinantes, entre las cuales podríamos destacar las denominadas VLP (*virus-like particles*) como son, por ejemplo el HBV y el HPV [13, 14]. Sin embargo, la reducción de sustancias que en las vacunas clásicas actuaban como señal de peligro [15], asociado al incremento de pureza de estos antígenos, se ha traducido en la necesidad de utilizar agentes adyuvantes. La aparición más reciente de vacunas basadas en péptidos sintéticos [16] que mimetizan epítopos específicos de los microorganismos, ha afianzado más la diferencia entre el componente antigénico y el componente inmunoestimulante, ambos imprescindibles para lograr vacunas protectoras. Estas vacunas basadas en péptidos sintéticos ofrecen una precisión y seguridad inigualables pero también dependen de adyuvantes particularmente potentes para generar una respuesta inmune efectiva.

DESARROLLO DE RESPUESTAS INMUNES FRENTE A ANTÍGENOS: EL CONCEPTO DE LA VACUNACIÓN

En general, en el curso de una infección, se produce una atracción de leucocitos hacia la región infectada motivada por las denominadas moléculas PAMP (*Pathogen-associated molecular pattern*) y/o DAMP (*Damage-associated molecular pattern*). Las primeras son moléculas con patrones moleculares característicos de agentes infecciosos, mientras que las segundas son moléculas endógenas liberadas por stress o muerte celular. Estas moléculas son reconocidas por receptores PRR (*Pattern Recognition Receptors*) presentes en las células presentadoras de antígeno y provocan la activación de la célula y su posterior migración hacia el origen de esas moléculas [17]. Entre las células presentadoras de antígeno, cabe destacar las células dendríticas, que son parte esencial en la generación de la respuesta adaptiva (**Figura 1**). Estas células captan los antígenos, los degradan en péptidos y los presentan en su superficie por medio de receptores específicos del complejo mayor de histocompatibilidad (MHC) I o II. En general esta presentación depende del origen del antígeno. Los antígenos procedentes del medio extracelular -antígenos extracelulares-, son presentados normalmente por medio del MHC tipo II. Por el contrario, los antígenos presentes en células infectadas por patógenos, son denominados antígenos intracelulares y son presentados por MHC tipo I. Excepcionalmente puede ocurrir que un antígeno extracelular sea presentado por el MHC tipo I, lo que se designa como presentación cruzada del antígeno.

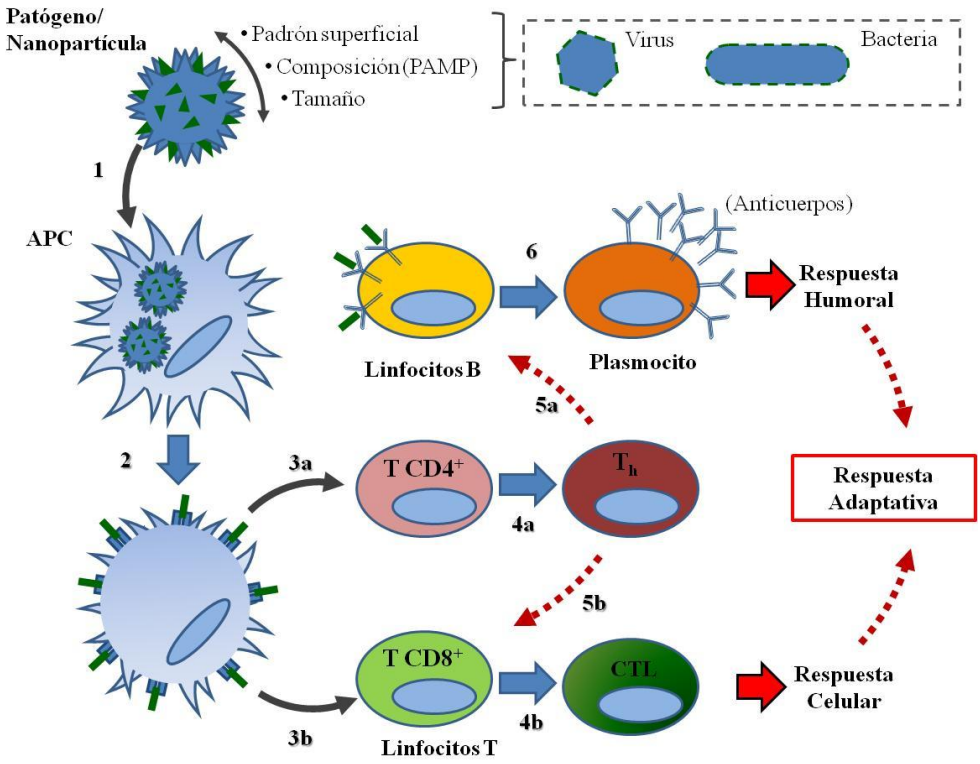

Figura 1. Esquema resumido del proceso de inmunización. *Un patógeno libre, una célula infectada o una nanopartícula provocan la migración de los APC por medio de señales de peligro PAMP y/o DAMP. Las células presentadoras de antígeno (APC) y en particular las células dendríticas, son estimuladas a fagocitar el microorganismo/nanopartícula (**1**) debido a su composición, tamaño y/o patrón superficial. Mientras migra hacia los nódulos linfáticos, el APC sufre un proceso de maturación y procesa el antígeno, presentándolo en un complejo MHC I o II segundo la naturaleza del antígeno (**2**). En general, los antígenos extracelulares son presentados por el MHC II que es reconocido por Linfocitos T CD4+ (**3a**) mientras los antígenos intracelulares son presentados por el MHC I que es reconocido por los Linfocitos T CD8+ (**3b**). Los linfocitos T CD4+ maduran en linfocitos T helper (T_h) (**4a**) mientras los linfocitos T CD8+ maduran en células citotóxicas (**4b**). Los linfocitos T helper, por medio de citoquinas especificas, estimulan tanto a la maduración de los linfocitos B activados (**5a**) como de los linfocitos citotóxicos (CTL) (**5b**). Con el refuerzo positivo de los linfocitos T_h, los linfocitos B maduran a plasmocitos, secretando anticuerpos específicos contra los antígenos inicialmente identificados por las células dendríticas (**6**) generando la respuesta inmune humoral. Los CTLs por su vez destruyen las células infectadas que presenten a su superficie el antígeno identificado, produciéndose la respuesta inmune celular.*

Mientras procesan los antígenos, las células dendríticas inician su proceso de maduración y migran a los nódulos linfáticos en donde presentan, conjuntamente, los antígenos (señal 1) y señales coestimulantes (señal 2) a linfocitos T [18]. Más específicamente, la señal 1 consiste en la presentación del antígeno por el MHC de la célula dendrítica al receptor de las células T (TCR) mientras la señal 2 consiste en la interacción de los correceptores CD80 y CD86 de las células dendríticas con el receptor CD28 de la célula T. La célula dendrítica solo expresa los correceptores CD80 y CD86, cuando capta el antígeno asociado a señales de peligro como PAMP y DAMP, que comprueban la peligrosidad asociada a ese antígeno. En el caso de que la célula dendrítica presente el antígeno a la célula T sin estos correceptores, ocurre la anergia y tolerancia de la célula T a ese antígeno [19].

Los antígenos presentados por las células dendríticas a través del MHC I son reconocidos por linfocitos T CD8+ que se convierten en linfocitos T citotóxicos, dando origen a una respuesta inmune de tipo celular. Una vez activados, los linfocitos T citotóxicos se encargan de lisar las células infectadas por el patógeno, bloqueando así la multiplicación del microorganismo. Por otro lado, los antígenos presentados a través del MHC II son reconocidos por linfocitos T CD4+, los cuales, en virtud de ese reconocimiento, se diferencian en linfocitos T helper [20]. Estas células promueven, por medio de citoquinas especificas, tanto la acción citotóxica de los linfocitos T citotóxicos (respuesta celular) como la producción de anticuerpos (respuesta humoral) por parte de los linfocitos B [21]. A su vez, los linfocitos B reconocen los antígenos extracelulares a través de inmunoglobulinas (Ig) en su superficie, lo que provoca su activación. Junto con el refuerzo positivo de los linfocitos T helper, los linfocitos B se multiplican y diferencian en plasmocitos que secretan anticuerpos específicos para el antígeno reconocido.

Durante este proceso se generan también las células T memoria y las células B memoria, las cuáles se mantienen en estado de alerta ante una posible reinfección. En el caso de que esto ocurra, estas células se multiplican rápidamente y se diferencian en células efectoras, que actúan contra el agente invasor impidiendo su propagación y promoviendo su rápida eliminación. Este es el fundamento inmunológico del proceso de vacunación [17]. Es interesante observar que las células presentadoras de antígenos solo activan las células T (a través de la presentación de la señal 2) si adquieren el antígeno en presencia de una señal de peligro que compruebe que dicho antígeno está relacionado

con algo pernicioso al organismo. La presentación del antígeno sin la señal 2 conlleva la inhibición de la célula T, generándose así un proceso de tolerancia del sistema inmune contra ese antígeno. Este mecanismo evita que se generen respuestas contra proteínas endógenas y, con ello, el desarrollo de enfermedades autoinmunes, pero también supone una barrera a la generación de una respuesta efectiva por medio de una vacuna y obliga a la asociación a los antígenos, de moléculas o sistemas capaces de activar las células dendríticas.

En definitiva, del análisis de este proceso de desarrollo de la respuesta inmune se desprende la necesidad de señales coestimulantes asociadas al antígeno, lo que obliga a la utilización de adyuvantes en vacunas más puras (recombinantes y péptidos sintéticos) para generar una respuesta inmune efectiva [22].

La especificidad de cada MHC por determinadas secuencias de aminoácidos y la variabilidad de alelos de MHC dentro de la especie humana, conlleva el hecho de que no todos los epítopos puedan ser reconocidos por los MHC de un individuo. Como consecuencia, no todos los antígenos pueden ser presentados convenientemente a los linfocitos T y, en definitiva, no todos los antígenos son capaces de generar una respuesta adaptativa inmune efectiva contra un epítopo especifico [21].

Por otra parte, aunque un epítopo sea bien reconocido por el sistema inmune puede ocurrir que se encuentre en una proteína no presente en todas las estirpes del patógeno. En este caso, y aunque se produzca una respuesta inmune efectiva frente a ese epítopo, el individuo no estará protegido contra todas las estirpes del patógeno. Ejemplo de esta situación es la vacuna frente al virus del papiloma humano (HPV), compuesta por la proteína L1 del virus. Esta proteína es muy inmunogénica pero está poco conservada dentro de la especie del papiloma humano, por lo que la vacuna comercial solo es eficaz frente a un espectro limitado de estirpes (aunque cobre las estirpes más peligrosas). A pesar del éxito de esta vacuna, la referida limitación ha motivado el desarrollo de nuevas vacunas dirigidas frente a nueve estirpes distintas del microorganismo [13]. Otra estrategia para lograr una vacuna universal frente al HPV podría estar asociada a la proteína HPV L2, que en el virus se encuentra enmascarada por la proteína L1 y la cual se encuentra mucho más conservada dentro la especie. Sin embargo, esta estrategia está limitada por el hecho de que este antígeno es mucho menos inmunogénico debido a su incapacidad en organizarse en estructuras VLP [13]. La misma situación se presenta en el caso de las vacunas diseñadas frente al virus influenza, de las que forman parte las

proteínas hemaglutinina y neuraminidase. Estas proteínas son inmunogénicas pero su estructura varia dentro de la especie del virus, lo que obliga, todos los años, a una adaptación de la vacuna a la estirpe relevante [23].

La especificidad de los MHC y variabilidad de los antígenos de los microorganismos son problemas particularmente relevantes en vacunas con limitado número de epítopos, como es el caso de las vacunas basadas en péptidos sintéticos, donde cada péptido representa un epítopo especifico. Una estrategia actual para abordar esta limitación, consiste en el diseño de vacunas constituidas por un conjunto de péptidos sintéticos, cada uno de los cuáles representando un epítopo distinto del virus, incrementando así la posibilidad de reconocimiento por los MHC. Al mismo tiempo, los distintos epítopos también pueden cubrir distintas estirpes del microorganismo infeccioso y originar una respuesta múltiple hacia el microorganismo previniendo su adaptación [24]. Alternativamente, se pueden utilizar epítopos altamente conservados del microorganismo y asociar un péptido que sea reconocido por un largo espectro de MHC, como son los péptidos de la familia PADRE (Pan HLA DR) [25], incrementando así la probabilidad de reconocimiento en la población humana.

En resumen, el desarrollo de vacunas eficientes está altamente condicionado por la disposición de adyuvantes capaces de movilizar y activar las células presentadoras de antígenos, estimulando así la proliferación y diferenciación de células T y con ello, la generación de respuestas inmunes. La utilización de antígenos basados en péptidos sintéticos que mimeticen epítopos presentes en las varias estirpes de los microorganismos pueden originar vacunas más seguras y que originen una protección más amplia que las actuales. Dichos antígenos serán posiblemente poco inmunogénicos pero su potencial clínico es extraordinario. Para contrarrestar esta limitación, los adyuvantes cobran un papel fundamental.

AGENTES ADYUVANTES DE VACUNAS

Un adyuvante es una substancia que, al ser administrada en combinación con un antígeno permite incrementar su respuesta inmune [26]. Con excepción de las vacunas atenuadas, el uso de un agente adyuvante es esencial para lograr una respuesta inmunitaria efectiva [27, 28]. Además, el adyuvante puede también modular la respuesta inmune de modo a lograr una respuesta humoral, celular o una respuesta

combinada, bien sea por su interacción con receptores distintivos específicos de ambos tipos de respuesta, o a través del proceso de presentación cruzada [29, 30].

En la actualidad existen agentes adyuvantes de muy distinta composición y naturaleza. Con frecuencia, en el ámbito de la tecnología farmacéutica hacemos la distinción entre moléculas activas inmunoestimulantes, cuya acción resulta de la interacción con receptores específicos, y agentes adyuvantes, cuya acción puede estar condicionada no solo a la presencia de ciertas sustancias químicas sino también a la forma física en la que se presentan. Aquí incluimos entre otros las sales metálicas, determinados aceites y biomateriales. Dependiendo de la organización de estos ingredientes, pueden o no constituir lo que se llama sistemas de liberación de antígenos, ya que promueven de una forma programada, la cesión gradual en el tiempo [8]. A continuación se describen brevemente estas diferentes categorías de agentes adyuvantes.

MOLÉCULAS INMUNOESTIMULANTES

Se consideran moléculas inmunoestimulantes aquéllas que presentan ligandos reconocibles por los PRRs, siendo los agonistas de los receptores Toll-like (TLRs), los más investigados. Los TLR son proteínas transmembranales y en el ser humano constituyen una familia de 10 receptores distintos, que se localizan en la superficie de la célula (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) o en la superficie de los endosomas en el citoplasma de la célula (TLR3, TLR7, TLR8 and TLR9). Cada uno de estos receptores reconoce ligandos específicos [31] (**Tabla 1**).

La identificación de estos receptores permitió el desarrollo de moléculas sintéticas y derivados que actúan como inmunoestimulantes específicos para determinados TLR como el Poly (I:C) (TLR3), el MPL (TLR4), el Imiquimod y el Resiquimod (TLR7/8) y el CpG ODN (TLR 9). Mientras el MPL actúa en un receptor extracelular, los restantes actúan en receptores intracelulares [31], por lo que, supuestamente su acción se podría ver beneficiada de estrategias que faciliten su liberación dentro de las células inmunes (**Figura 2**).

El Poly (I:C), por ejemplo, es un derivado sintético de ARN de cadena doble, compuesto por una cadena de ácido polinosinico asociada a una cadena de ácido policitidilico y es capaz de potenciar la respuesta humoral y celular [32, 33], inclusive por ruta intranasal [34]. Sin embargo, está también asociado a toxicidad sistémica [35,

36]. La asociación de este poderoso inmunoestimulante a un sistema de liberación de antígenos capaz de liberarlo en las células inmunes ofrece la posibilidad de reducir la dosis necesaria y su toxicidad, y de potenciar su efecto [37].

Tabla 1. Resumen de los receptores Toll-like (TLR) conocidos, sus ligandos naturales y los inmunoestimulantes sintéticos desarrollados para el respectivo receptor [31].

Receptor	Ligando natural	Inmunoestimulante
TLR2/1	• Lipopeptidos bacterianos triacilados	Pam3Cys
TLR2/6	• Lipopeptidos bacterianos diacilados • Zimosano • LTA	N/A
TLR3	• dsRNA	Poly (I:C)
TLR4	• LPS	MPL
TLR5	• Flagelina	N/A
TLR7/8	• ssRNA	Imiquimod; Resiquimod
TLR 9	• DNA Viral y Bacteriano	CpG-ODN
TLR11	• Profilina	N/A

Pam3cys: *tri-palmitoyl-S-glyceryl cysteine*; **LTA:** *Lipoteichoic acid*; **dsRNA:** *Double Stranded RNA*; **ssRNA:** *Single stranded RNA*; **Poly (I:C):** *Polyinosinic:polycytidylic acid*; **MPL:** *Monophosphoryl Lipid A*; **LPS**: *Lipopolysaccharide*; **CpG-ODN**: *CpG Oligodeoxynucleotide*; **N/A:** *Not available.*

El CpG ODN, por su parte, es un derivado sintético de ADN de cadena simples compuesto por la repetición del dinucleótido citosina y guanina no metilados, en un padrón característico del DNA bacteriano y viral. Diversos estudios han demostrado la necesidad de entregar simultáneamente el CpG ODN y el antígeno a las células inmunes para lograr una respuesta inmune optima contra ese antígeno [38] y la capacidad de este inmunoestimulante en modular la respuesta inmune hacia el antígeno [39]. Además, el

CpG ODN es fácilmente degradado por las nucleases presentes en el suero, presentando tiempos de media vida muy cortos. Por todo ello, la asociación del CpG ODN a un sistema capaz de protegerlo y liberarlo en las células dendríticas se presenta como un requisito fundamental a su utilización como inmunoadyuvante [40]. En el caso del Imiquimod y el Resiquimod, consisten en imidazoquinolinas con acción inmunoestimulante por interacción con el receptor TLR7 y TLR8 cuyo ligando natural es el ARN de cadena simples. Estas moléculas estimulan una intensa secreción de citoquinas pro-inflamatorias y, como tal, su administración sistémica puede originar fenómenos de toxicidad [41]. La encapsulación de estas moléculas en sistemas de liberación de vacunas puede disminuir la toxicidad asociada y mejorar su acción inmunoadyuvante [41-43]. Por último, el MPL es un derivado de los lipopolisacáridos bacterianos de las bacterias Gram-negativas, que presenta menor toxicidad que la molécula original, aunque conserva su efecto inmunoestimulante. Debido a su carácter liposoluble, esta molécula ha sido asociada a liposomas, habiendo logrado mejorar su perfil de seguridad. Al contrario de los demás agonistas de TLR referidos, en el caso del MPL, es importante que esté expuesto en la superficie de la nanoestructura una vez que el TLR4 es extracelular (**Figura 2**). Al actuar sobre este receptor provoca la producción de citoquinas y el incremento en la presentación de antígenos por los complejos MHC, resultando en un incremento de la respuesta inmune hacia los antígenos asociados [44].

AGENTES ADYUVANTES Y SISTEMAS DE LIBERACIÓN DE ANTÍGENOS

Existen numerosos compuestos y excipientes farmacéuticos cuya capacidad inmunoestimulante, además de ser inherente a su estructura y composición molecular, está reforzada por el hecho de presentarse bajo una forma física específica. En esta categoría podemos incluir los geles de aluminio, las emulsiones y también los sistemas de liberación de antígenos de forma micro- o nanoparticulada. Estos últimos sistemas, adquieren en la actualidad especial relevancia ya que, según se comentó en el apartado anterior, permiten incluir en su estructura junto con los antígenos, moléculas de inmunoestimulantes específicos, tales como los agonistas de TLR e inespecíficos tales como aceites, lípidos y biopolímeros.

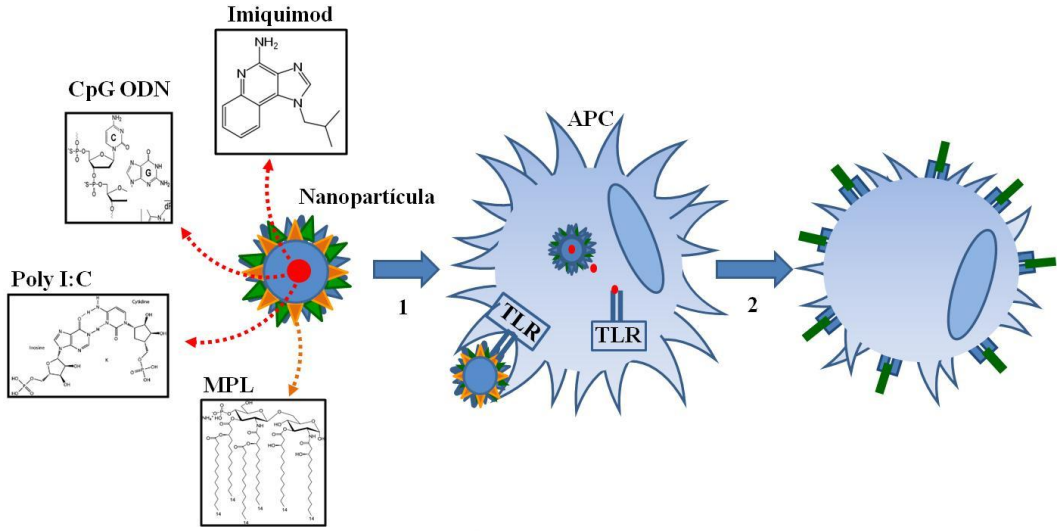

Figura 2. Estrategia sinérgica de la asociación de inmunoestimulantes con nanopartículas. *Los agonistas de TLR deben ser asociados a las nanopartículas de acuerdo con la localización de sus receptores. Así, para los agonistas de receptores intracelulares, la encapsulación permite protegerlos de factores de degradación externos como nucleases y concentrar su acción en las células inmunes mientras para los agonistas de receptores extracelulares es importante que quede expuesto en la superficie de la nanopartícula de forma que pueda interaccionar con su receptor sin limitaciones. La asociación de los inmunoestimulantes y antígenos a nanopartículas permite su más eficaz fagocitosis por parte de los APC (**1**). Una vez las partículas captadas, ocurre su degradación liberándose inmunoestimulantes y antígenos. La interacción de los inmunoestimulantes con sus respectivos receptores TLR provoca la maduración de los APC, estimulando entre otras cosas, el procesamiento y presentación de los antígenos en complejos MHC a los linfocitos T (**2**).*

ADYUVANTES UTILIZADOS EN CLÍNICA

Las sales de aluminio, cuya capacidad adyuvante fue descubierta en 1926, representan el único adyuvante universalmente utilizado en humanos [31, 45]. Sin embargo, estas sales presentan limitaciones importantes, que han estimulado la búsqueda de nuevos adyuvantes. Concretamente, la congelación de las vacunas con sales de aluminio conlleva la inactivación de la vacuna [45]. Además, estas sales resultan ineficaces para antígenos poco inmunogénicos, así como para generar respuestas inmunes de tipo celular [46]. Finalmente, su acción está limitada a la ruta parenteral y puede generar reacciones adversas locales como eritemas y nódulos subcutáneos [45, 46].

Para mejorar la eficacia de las sales de aluminio se ha optado por asociarlo a otras moléculas inmunoestimulantes tales como el MPL [47], constituyendo ambos el adyuvante AS04. Asimismo, se ha procedido al desarrollo de sistemas lipídicos nanométricos que se describen en el apartado siguiente.

NANOTECNOLOGÍA Y VACUNAS

Las limitaciones asociadas a las sales de aluminio han llevado al desarrollo de adyuvantes innovadores basados en el uso de la nanotecnología (**Figura 3**) [48]. Entre dichos adyuvantes se encuentran los virosomas, el AS03 y el MF59 [47, 49], cuyas características y utilidad se describen a continuación.

Los virosomas son liposomas de 150 nm con componentes virales en la membrana lipídica [50], cuya estructura y composición facilita la presentación del antígeno a las células inmunes. Los virosomas fueron comercializados como vacunas para la hepatitis A y la influenza en 1996 y 1997, respectivamente [49, 51]. Su eficacia es incuestionable, habiendo sido contrastada incluso en poblaciones con un sistema inmune débil como ancianos [52] y niños y bebes [53].

El AS03 y el MF59 son nanoemulsiones (tamaño entre 150 y 160 nm) constituidas por un núcleo oleoso (escualeno más tocoferol y escualeno solo, respectivamente) estabilizado por surfactantes (Polisorbato 80 y Polisorbato 80 más Trioleato de sorbitano, respectivamente) [49]. El primero ha demostrado ser un adyuvante muy potente para la vacuna del influenza, al permitir la generación de niveles más elevados de anticuerpos que la vacuna inactivada con 20 veces menos antígeno [54]. Se cree que tiene una acción pro-inflamatoria, promoviendo la llegada y activación de las células inmunes [49]. El MF59 es utilizado como adyuvante en la vacuna de influenza, porque tiene la capacidad de generar una respuesta humoral más completa que el Alum haciendo uso de una dosis inferior de antígeno [27]. El mecanismo de acción del MF59 aun no es claro pero se cree que actúa como sistema reservatorio de antígenos, a la vez que estimula la producción de citoquinas pro-inflamatorias por las células inmunes [46]. El éxito del MF59 y el AS03 llevó a su aplicación como adyuvantes en estudios clínicos para vacunas para el virus sincitial respiratorio humano (RSV) [55] y el citomegalovirus (CMV) [56] o para el Dengue [57], respectivamente.

Otros sistemas nanométricos que debido a su elevado potencial inmunoadyuvante están siendo evaluados en ensayos clínicos son el AS01, AS02, ISCOM e ISCOMATRIX.

El AS01 y AS02 son sistemas adyuvantes de la misma familia de adyuvantes nanométricos que el AS03 (pertenecientes al GlaxoSmithKline) y que consisten en uno liposoma y una emulsión con núcleo oleoso de escualeno, respectivamente, que se caracterizan por tener en su composición el inmunoestimulante MPL. Estas estructuras ya demostraran su potencial como sistemas adyuvantes en estudios clínicos para el HIV, en el cual fueran capaces de estimular respuestas inmunes intensas del tipo linfocito CD4+ [58]. Su potencial inmunoadyuvante ha llevado más recientemente, a su utilización, en estudios clínicos, como adyuvantes para una vacuna contra el malaria [59, 60].

Los ISCOM (*Immunostimulatory complexes*) son sistemas nanométricos (40 nm) en el cual el antígeno se encuentra encapsulado en una matriz compuesta por colesterol, fosfolípidos y la saponina Quil A. La saponina Quil A es un triterpenoide obtenido de la cascara del árbol *Quillaja saponaria* y es un fuerte inmunoestimulante aunque su mecanismo de acción es aún desconocido [46]. Desafortunadamente, presenta además una fuerte acción hemolítica, por lo que es considerada demasiado toxica para uso humano. Una estrategia para reducir su toxicidad ha consistido en la formación de un complejo con el colesterol y determinados fosfolípidos. De este modo, la saponina queda menos disponible para interaccionar con las membranas plasmáticas y, como tal, se reduce drásticamente su efecto hemolítico aunque retiene su efecto inmunoestimulante. Esta mejora en las propiedades del Quil A ilustra la aplicabilidad de la nanotecnología con vista a la obtención de sistemas adyuvantes seguros y eficaces [46]. Por último y para solventar la limitación de los ISCOM a antígenos hidrofóbicos, se han desarrollado los ISCOMATRIX, en los cuales el antígeno en vez de encapsulado en la matriz, se encuentra asociado al superficie del sistema. Debido a la capacidad de generar fuerte respuestas humorales y celulares con diversos antígenos y en distintos modelos animales [61], el ISCOM actualmente está siendo testado en ensayos clínicos para el Herpes (HSV) [62], Malaria [63] y Influenza [64] mientras el ISCOMATRIX es evaluado como adyuvante para una vacuna terapéutica para el cáncer [65].

MICRO Y NANOPARTÍCULAS POLIMÉRICAS COMO SISTEMAS DE LIBERACIÓN DE ANTÍGENOS Y ADYUVANTES

El desarrollo de nuevos adyuvantes ha estado también asociado a los avances en el diseño de micro y nanopartículas poliméricas [49]. Estas partículas ofrecen, además de su capacidad adyuvante, la ventaja, con relación a las sales de aluminio, de poder ser liofilizadas y almacenadas bajo la forma de polvo seco [66]. A continuación, se describen brevemente los avances más significativos en este ámbito de conocimiento, entre los que se centran el desarrollo de vacunas mono-dosis y también de vacunas no inyectables destinadas a ser administradas por vía nasal.

Partiendo del trabajo seminal publicado en 1976 por Spiel y Kruger [67], en el que proponían el uso de nanopartículas de polimetilmetacrilato (PMMA) como vehículos adyuvantes de vacunas, han sido numerosos los sistemas poliméricos testados en el desarrollo de sistemas adyuvantes de vacunas. Dentro de los polímeros sintéticos destacan en particular los polímeros derivados del ácido láctico y del ácido glicólico (PLGA) debido a su biodegradabilidad y capacidad para controlar la liberación de antígenos [68] y incrementar la respuesta inmune hacia el antígeno asociado [69, 70], con reacciones locales mínimas por ruta subcutánea al reverso de los sales de aluminio [71]. Nuestro grupo ha realizado un trabajo pionero en el desarrollo de micro y nanopartículas de PLGA como sistemas de liberación controlada de proteínas y antígenos [72-74], así como en la identificación de la PEGylación de las nanopartículas de PGA, como propiedad esencial para facilitar el paso de antígenos a través de barreras mucosas [75, 76]. Trabajos posteriores nos permitieron demostrar la influencia simultanea del tamaño y de la densidad de PEG en la superficie de las nanopartículas en su capacidad para atravesar la mucosa nasal [77, 78], y generar una respuesta inmune hacia el antígeno encapsulado, el toxoide tetánico [79, 80]. Sin embargo, a pesar del gran potencial de los sistemas de liberación de antígenos basados en PLGA, nuestra experiencia en el uso de estos biomateriales nos llevó a concluir que parte del antígeno encapsulado era degradado por la acumulación de productos resultantes de la degradación del proprio polímero en la matriz polimérica [81]. Este problema pudo ser parcialmente soslayado a través de diferentes estrategias que incluyen desde la constitución de un núcleo oleoso [82] o de gelatina [83] hasta la introducción de aditivos como el poloxamer [84, 85]. Además del toxoide tetánico, este sistema fue utilizado para la asociación de antígenos más complejos, como el antígeno

recombinante de la hepatitis B (rHBsAg), que presenta una estructura semejante a virus (*virus-like particle*) [86]. En conjunto, más allá del uso que nuestro grupo ha hecho de los polímeros PLGA y sus copolímeros con el PEG, existe una abundante bibliografía que, sin duda, ha influido en el desarrollo clínico de vacunas basadas en los mismos y particularmente vacunas frente al cáncer [87] y también una vacuna para la prevención del tabaquismo [88].

Como alternativa a los polímeros sintéticos tipo PLGA, también han recibido una extraordinaria atención los polisacáridos y, de forma particular el quitosano. El quitosano es un polímero compuesto por una cadena linear de D-glucosamina y N-acetil-D-glucosamina obtenido de la deacetilación de la quitina presente en el exosqueleto de los crustáceos y en la pared de los hongos. El quitosano es un polímero soluble y catiónico, capaz de constituir sistemas nanométricos en virtud de su complejación con polímeros negativos o gelificación con agentes entrecruzantes como el tripolifosfato. Estas características, junto con su adecuada biocompatibilidad y mucoadhesividad, hacen del quitosano una de las moléculas mas estudiadas en el desarrollo de sistemas adyuvantes para vacunas [89]. En esta área, nuestro grupo también ha contribuido de forma decisiva al desarrollar por la primera vez las nanopartículas de quitosano como sistemas de liberación de proteínas y antígenos [90, 91]. El uso de las mismas permitió la administración efectiva del toxoide tetánico (TT) por vía nasal [92, 93], así como también ejercer un efecto adyuvante tras la administración de antígenos más complexos, tales como el antígeno VLP de la hepatitis B (rHBsAg) [66]. Esta capacidad adyuvante ha sido posteriormente confirmada por otros autores, no solo para antígenos virales como el antígeno rHBsAg [94] y la hemaglutinina del influenza [95], sino también para antígenos bacterianos, como el extracto proteico del *Streptococcus equi* [96].

El carácter catiónico y flexibilidad de la cadena del quitosano, ha permitido, más recientemente, su utilización como recubrimiento de nanoemulsiones para la constitución de nanocápsulas como sistemas adyuvantes de vacunas [97]. La asociación de la mucoadhesividad del quitosano con la capacidad adyuvante intrínseca de las nanoemulsiones, identificada en adyuvantes como el MF59, permitió el desarrollo de sistemas nanocapsulares capaces de asociar eficazmente el antígeno rHBsAg y generar una respuesta humoral efectiva tras su administración por vía intranasal [42]. Asimismo, estas nanocápsulas han permitido la inmunización frente a HB tras la inyección de una

sola dosis del antígeno [98], mostrando así su potencial en el desarrollo de vacunas mono-dosis. Estudios posteriores de biodistribución han revelado la capacidad de las nanocápsulas de CS de formar un reservatorio en el local de administración y presentar un drenaje sostenido hasta los nódulos linfáticos, en donde se concentran una gran población de células inmunes. Es posible que esta estimulación sostenida del sistema inmune sea la causa de la capacidad adyuvante observada con estas nanocápsulas [99].

Las excepcionales características del CS como materia prima para el desarrollo de sistemas de liberación de vacunas ha estimulado igualmente la combinación con otros polímeros así como el desarrollo de derivados sintéticos para la constitución de sistemas adyuvantes nanoparticulados. Asimismo, estudios con nanopartículas de quitosano cargadas con el rHBsAg pero recubiertas con el polisacárido alginato, demostraran la capacidad de estos sistemas en generar respuestas inmunes por la ruta subcutánea [100], nasal [101] y oral [102], lo que comprueba la versatilidad de estos sistemas. Dentro de los derivados sintéticos cabe destacar el N,N,N-trimetilquitosano (TMC), que producto de la metilación de los grupos amino del quitosano, presenta una solubilidad acrecida pero que mantiene la capacidad de constituir sistemas nanométricos por gelificación iónica [103]. Dicho sistema ha demostrado su capacidad adyuvante con el antígeno H3N2 del influenza A, a través de la vía nasal [104] y estudios posteriores con el antígeno modelo ovalbumina y el toxoide diftérico confirmaron la capacidad inmunoadyuvante de este sistema tanto por la vía nasal como por la vía intramuscular [105] y intradermal [106].

En general, los sistemas poliméricos permiten una liberación prolongada en el tiempo del antígeno asociado, extendiendo así el proceso de estimulación del sistema inmune y, consecuentemente, la generación de células inmunes de memoria [107]. Por otra parte, la elevada superficie específica de las nanostructuras permite la adsorción y exposición de los antígenos asociados, algo que ha sido relacionado con una mayor respuesta inmune [108]. Además, el propio carácter particulado que estos sistemas confieren a los antígenos solubles, facilita su reconocimiento por las células inmunes y, por tanto, una más eficiente captación de los antígenos [109]. Dicho efecto puede resultar de la similitud de las nanopartículas con los virus, que las células dendríticas reconocen como partículas para fagocitar y contra las cuales activarse (**Figura 1**) [109, 110]. Asimismo, se ha observado que los sistemas especialmente pequeños (<100 nm) pueden llegar a alcanzar directamente los nódulos linfáticos, modificando así el perfil de respuesta

inmune [111]. Además del tamaño, otros factores como la carga superficial, la hidrofobicidad del sistema nanométrico o su geometría contribuyen también a la respuesta inmune [109, 112]. Los avances logrados en cuanto al comportamiento biológico de estos sistemas aparece reflejado en diversas revisiones relacionadas con este tema [8, 49, 113].

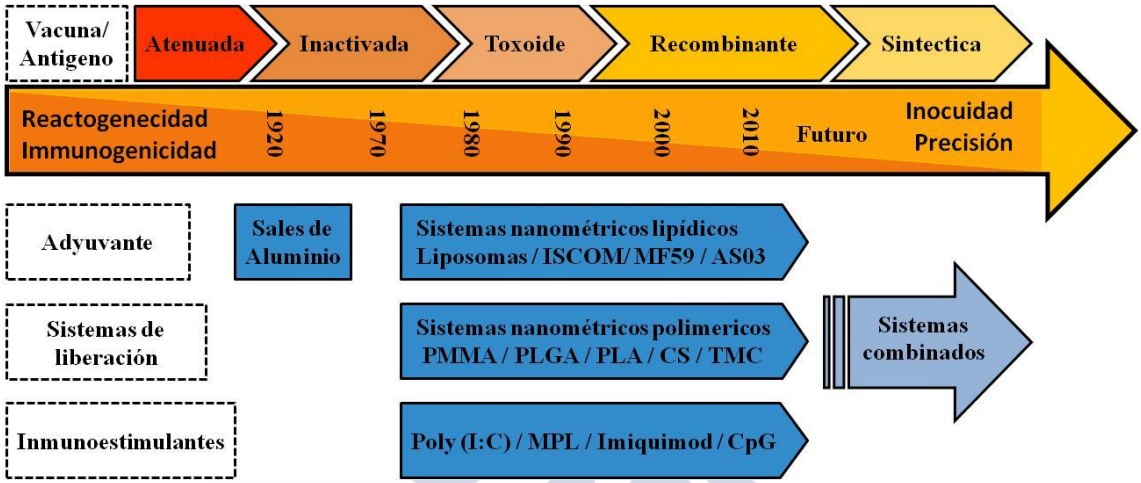

Figura 3. Progresión histórica de las vacunas y de los sistemas adyuvantes

La administración de la vacuna atenuada de la viruela por Jenner en 1768, marca el inicio de la investigación en el campo de las vacunas y desde entonces diversas vacunas atenuadas han sido desarrolladas y aplicadas en la clínica. El peligro de reversión del patógeno de estas vacunas llevó a la busca de vacunas más seguras basadas en microorganismos inactivados o en sus toxoides y posteriormente, en antígenos recombinantes y péptidos sintéticos debido a su inigualable precisión y seguridad. El surgimiento de vacunas no basadas en organismos atenuados obligó al desarrollo de sistemas adyuvantes capaces de incrementar la respuesta inmune de la vacuna. Así, poco después de desarrolladas las primeras vacunas inactivadas, se introduce el primero sistema adyuvante, los sales de aluminio. La progresiva pureza de las vacunas y las limitaciones de los sales de aluminio llevó al desarrollo de sistemas adyuvantes lipídicos como los liposomas, las nanoemulsiones y los sistemas poliméricos y a la identificación de moléculas con acción inmunoestimulante especifica como el Poly (I:C) [114]. Una tendencia actual y que se prevé incrementar en el futuro es la combinación de los sistemas nanométricos con los inmunoestimulantes con vista a un efecto sinérgico capaz de garantizar una efectiva inmunización con antígenos peptídicos sin efectos adversos asociados.

Como se comentó anteriormente, en la actualidad se está investigando el beneficio de la combinación de sistemas nanoparticulados con inmunoestimulantes [9, 46] con el objetivo de lograr la liberación simultánea de antígenos e inmunoestimulantes a las células dendríticas (**Figura 2**). El sinergismo potencial entre estos dos elementos podría resultar particularmente interesante para conseguir la potenciación de la respuesta inmune frente a antígenos poco inmunogénicos como os antígenos peptídicos (**Figura 3**). Finalmente, esta asociación puede permitir también la modulación de la respuesta inmune hacia respuestas inmunes más equilibradas [42] y una protección más completa.

CONCLUSIONES

El innegable impacto de las vacunas en la salud y el mejor conocimiento del sistema inmune han estimulado el trabajo orientado a la optimización de vacunas ya establecidas. Paralelamente, la mayor inocuidad de los nuevos antígenos y las limitaciones de los adyuvantes clásicos han propiciado la búsqueda de sistemas adyuvantes capaces de promover la generación de respuestas inmunes más robustas y duraderas. En este sentido, los sistemas de liberación de antígenos podrían suponer un gran progreso al permitir no únicamente fortalecer y prolongar la respuesta inmune frente al antígeno asociado, sino también su administración por rutas no parenterales, tales como la vía nasal. Los avances en esta línea han llevado al concepto de vacuna mono-dosis y vacuna no inyectable. Además, la posibilidad de liofilizar estas partículas podría conferir a estas nanovacunas una mayor estabilidad que las vacunas convencionales. Estos avances, en gran medida asociados a los hallazgos de nuestro grupo de investigación, ponen de manifiesto el gran potencial de los sistemas de liberación de antígenos en el desarrollo de nuevas vacunas. El éxito de sistemas nanométricos comercializados, tales como el MF59 o el AS03, así como los desarrollos clínicos de nanosistemas poliméricos permiten suponer que, en un futuro cercano, la nanotecnología puede contribuir de forma decisiva a desarrollo de nuevas vacunas contra patógenos para los cuales no existe aún una vacuna efectiva y la mejoría de vacunas ya existentes, permitiendo así una mejor cobertura sanitaria frente a las enfermedades infecciosas.

REFERENCES

1. Huang, D.B., J.J. Wu, and S.K. Tyring, *A review of licensed viral vaccines, some of their safety concerns, and the advances in the development of investigational viral vaccines.* J Infect, 2004. **49**(3): p. 179-209.

2. Richardson, V., et al., *Effect of Rotavirus Vaccination on Death from Childhood Diarrhea in Mexico.* New England Journal of Medicine, 2010. **362**(4): p. 299-305.

3. Huang, D.B., J.J. Wu, and S.K. Tyring, *A review of licensed viral vaccines, some of their safety concerns, and the advances in the development of investigational viral vaccines.* Journal of Infection, 2004. **49**(3): p. 179-209.

4. Poland, G.A. and R.M. Jacobson, *Understanding those who do not understand: a brief review of the anti-vaccine movement.* Vaccine, 2001. **19**(17-19): p. 2440-5.

5. Gupta, R.K., J. Best, and E. MacMahon, *Mumps and the UK epidemic 2005.* Bmj, 2005. **330**(7500): p. 1132-5.

6. Dayan, G.H., et al., *Recent Resurgence of Mumps in the United States.* New England Journal of Medicine, 2008. **358**(15): p. 1580-1589.

7. Winter, K., et al., *California Pertussis Epidemic, 2010.* The Journal of Pediatrics, 2012. **161**(6): p. 1091-1096.

8. Sahdev, P., L.J. Ochyl, and J.J. Moon, *Biomaterials for nanoparticle vaccine delivery systems.* Pharmaceutical Research, 2014. **31**(10): p. 2563-82.

9. Perrie, Y., et al., *Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens.* Int J Pharm, 2008. **364**(2): p. 272-80.

10. Hammer, S.M., et al., *Efficacy Trial of a DNA/rAd5 HIV-1 Preventive Vaccine.* New England Journal of Medicine, 2013. **369**(22): p. 2083-2092.

11. McCarthy, M., *HIV vaccine fails in phase 3 trial*: Lancet. 2003 Mar 1;361(9359):755-6.

12. Halliday, J., P. Klenerman, and E. Barnes, *Vaccination for hepatitis C virus: closing in on an evasive target.* Expert Rev Vaccines, 2011. **10**(5): p. 659-72.

13. Dochez, C., et al., *HPV vaccines to prevent cervical cancer and genital warts: an update.* Vaccine, 2014. **32**(14): p. 1595-1601.

14. Keating, G. and S. Noble, *Recombinant Hepatitis B Vaccine (Engerix-B®).* Drugs, 2003. **63**(10): p. 1021-1051.

15. Janeway, C.A., Jr., *Approaching the asymptote? Evolution and revolution in immunology.* Cold Spring Harb Symp Quant Biol, 1989. **1**: p. 1-13.

16. Yang, D., et al., *Peptide Vaccines.* New Vaccine Technologies, 2001: p. 214.

17. Tak W. Mak, Mary E. Saunders, and Bradley D. Jett, *Chapter 1 - Introduction to the Immune Response*, in *Primer to the Immune Response (Second Edition)*2014, Academic Cell: Boston. p. 3-20.

18. Siegrist, C.-A., *Vaccine immunology.* Vaccines. Saunders, 2008.

19. Kapsenberg, M.L., *Dendritic-cell control of pathogen-driven T-cell polarization.* Nat Rev Immunol, 2003. **3**(12): p. 984-993.

20. Lappin, M.B. and J.D. Campbell, *The Th1-Th2 classification of cellular immune responses: concepts, current thinking and applications in haematological malignancy.* Blood Rev, 2000. **14**(4): p. 228-39.

21. Lanzavecchia, A., *Identifying strategies for immune intervention.* Science, 1993. **260**(5110): p. 937-44.

22. Claire-Anne Siegrist, *Vaccine immunology*, in *Vaccines (Sixth Edition)*, Offit Stanley A., Plotkin Walter A., and Orenstein Paul A., Editors. 2013, W.B. Saunders: London. p. 14-32.

23. Palese, P., *Making better influenza virus vaccines?* Emerg Infect Dis, 2006. **12**(1): p. 61-5.

24. Luo, M., et al., *A novel HIV vaccine targets the 12 protease cleavage sites.* Retrovirology, 2012. **9**(Suppl 2): p. P304.

25. Alexander, J., et al., *Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides.* Immunity, 1994. **1**(9): p. 751-761.

26. Edelman, R., *The development and use of vaccine adjuvants.* Mol Biotechnol, 2002. **21**(2): p. 129-48.

27. Khurana, S., et al., *Vaccines with MF59 Adjuvant Expand the Antibody Repertoire to Target Protective Sites of Pandemic Avian H5N1 Influenza Virus.* Science Translational Medicine, 2010. **2**(15): p. 15ra5.

28. Friede, M., et al., *WHO initiative to increase global and equitable access to influenza vaccine in the event of a pandemic: Supporting developing country production capacity through technology transfer.* Vaccine, 2011. **29, Supplement 1**(0): p. A2-A7.

29. Coffman, R.L., A. Sher, and R.A. Seder, *Vaccine adjuvants: putting innate immunity to work.* Immunity, 2010. **33**(4): p. 492-503.

30. Uto, T., et al., *Comparative activity of biodegradable nanoparticles with aluminum adjuvants: Antigen uptake by dendritic cells and induction of immune response in mice.* Immunology Letters, 2011. **140**(1–2): p. 36-43.

31. Olive, C., *Pattern recognition receptors: sentinels in innate immunity and targets of new vaccine adjuvants.* Expert Review of Vaccines, 2012. **11**(2): p. 237-256.

32. Durand, V., et al., *Shaping of adaptive immune responses to soluble proteins by TLR agonists: a role for IFN-alpha/beta.* Immunol Cell Biol, 2004. **82**(6): p. 596-602.

33. Sloat, B. and Z. Cui, *Nasal Immunization with Anthrax Protective Antigen Protein Adjuvanted with Polyriboinosinic–Polyribocytidylic Acid Induced Strong Mucosal and Systemic Immunities.* Pharmaceutical Research, 2006. **23**(6): p. 1217-1226.

34. Ichinohe, T., et al., *Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection.* J Virol, 2005. **79**(5):

p. 2910-9.

35. Sobel, D.O., et al., *Poly I:C Induces Development of Diabetes Mellitus in BB Rat.* Diabetes, 1992. **41**(4): p. 515-520.

36. Cunningham, C., et al., *The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C).* Brain, Behavior, and Immunity, 2007. **21**(4): p. 490-502.

37. Hafner, A.M., B. Corthesy, and H.P. Merkle, *Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant.* Adv Drug Deliv Rev, 2013. **65**(10): p. 1386-99.

38. Sacks, T. and D.M. Klinman, *Long-term effect of primary immunization on subsequent immune responsiveness.* Cell Immunol, 1997. **177**(2): p. 162-8.

39. Bal, S.M., et al., *Co-encapsulation of antigen and Toll-like receptor ligand in cationic liposomes affects the quality of the immune response in mice after intradermal vaccination.* Vaccine, 2011. **29**(5): p. 1045-1052.

40. Shirota, H. and D.M. Klinman, *Recent progress concerning CpG DNA and its use as a vaccine adjuvant.* Expert Rev Vaccines, 2014. **13**(2): p. 299-312.

41. Ilyinskii, P.O., et al., *Adjuvant-carrying synthetic vaccine particles augment the immune response to encapsulated antigen and exhibit strong local immune activation without inducing systemic cytokine release.* Vaccine, 2014. **32**(24): p. 2882-95.

42. Vicente, S., et al., *Co-delivery of viral proteins and a TLR7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy.* Journal of Controlled Release, 2013. **172**(3): p. 773-781.

43. Primard, C., et al., *Multifunctional PLGA-Based Nanoparticles Encapsulating Simultaneously Hydrophilic Antigen and Hydrophobic Immunomodulator for Mucosal Immunization.* Molecular Pharmaceutics, 2013. **10**(8): p. 2996-3004.

44. Alving, C.R., et al., *Liposomes containing lipid A: an effective, safe, generic adjuvant system for synthetic vaccines.* Expert Review of Vaccines, 2012. **11**(6): p. 733-744.

45. Clements, C.J. and E. Griffiths, *The global impact of vaccines containing aluminium adjuvants.* Vaccine, 2002. **20, Supplement 3**(0): p. S24-S33.

46. Reed, S.G., et al., *New horizons in adjuvants for vaccine development.* Trends Immunol, 2009. **30**(1): p. 23-32.

47. Verstraeten, T., et al., *Analysis of adverse events of potential autoimmune aetiology in a large integrated safety database of AS04 adjuvanted vaccines.* Vaccine, 2008. **26**(51): p. 6630-6638.

48. Etheridge, M.L., et al., *The big picture on nanomedicine: the state of investigational and approved nanomedicine products.* Nanomedicine, 2013. **9**(1): p. 1-14.

49. Correia-Pinto, J.F., N. Csaba, and M.J. Alonso, *Vaccine delivery carriers: insights and future perspectives.* Int J Pharm, 2013. **440**(1): p. 27-38.

50. Glück, R. and I.C. Metcalfe, *New technology platforms in the development of vaccines for the future.* Vaccine, 2002. **20, Supplement 5**(0): p. B10-B16.

51. Herzog, C., et al., *Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine.* Vaccine, 2009. **27**(33): p. 4381-7.

52. Gluck, R., et al., *Immunogenicity of new virosome influenza vaccine in elderly people.* Lancet, 1994. **344**(8916): p. 160-3.

53. Kanra, G., et al., *Comparison of immunogenicity and tolerability of a virosome-adjuvanted and a split influenza vaccine in children.* Pediatr Infect Dis J, 2004. **23**(4): p. 300-6.

54. Chu, D.W.-S., et al., *Immunogenicity and tolerability of an AS03A-adjuvanted prepandemic influenza vaccine: A phase III study in a large population of Asian adults.* Vaccine, 2009. **27**(52): p. 7428-7435.

55. Novartis. *To Evaluate the Safety and Ability of the Medicine to Induce Antibodies Against the Respiratory Syncytial Virus in Healthy Adults*. 2014 22/02/2015]; Available from: https://clinicaltrials.gov/ct2/results?term=NCT02298179 NLM Identifier: NCT02298179

56. (NIAID), N.I.o.A.a.I.D. *gB/MF59 Vaccine in Preventing Cytomegalovirus Infection in Healthy Adolescent Females*. 2006 22/02/2015]; Available from: https://clinicaltrials.gov/ct2/results?term=NCT00133497&show_flds=Y&submit_fld_opt= NLM Identifier: NCT00133497.

57. GlaxoSmithKline. *A Two-dose Primary Vaccination Study of a Tetravalent Dengue Virus Purified Inactivated Vaccine vs. Placebo in Healthy Adults (in Puerto Rico)*. 2012 22/02/2015]; Available from: https://clinicaltrials.gov/ct2/results?term=NCT01702857&show_flds=Y NLM Identifier: NCT01702857.

58. Leroux-Roels, I., et al., *Strong and persistent CD4+ T-cell response in healthy adults immunized with a candidate HIV-1 vaccine containing gp120, Nef and Tat antigens formulated in three Adjuvant Systems.* Vaccine, 2010. **28**(43): p. 7016-24.

59. GlaxoSmithKline. *Safety and Preliminary Efficacy of the Malaria Vaccine Candidates Falciparum Merozoite Protein-1 (FMP1) and SmithKlineBeecham (SKBB) Candidate Malaria Vaccine RTS,S*. 2001; Available from: https://clinicaltrials.gov/ct2/results?term=NCT01556945&show_flds=Y NLM Identifier: NCT01556945.

60. GlaxoSmithKline. *An Extended Follow up of a RTS,S/AS01E Malaria Vaccine Trial (MAL059)*. 2009 22/02/2015]; Available from: https://clinicaltrials.gov/ct2/show/NCT00872963?term=NCT00872963&rank=1 NLM Identifier: NCT00872963.

61. Sun, H.-X., Y. Xie, and Y.-P. Ye, *ISCOMs and ISCOMATRIX™*. Vaccine, 2009. **27**(33): p. 4388-4401.

62. GenoceaBiosciences. *Dose Ranging Safety and Efficacy of Therapeutic HSV-2 Vaccine*. 2014 23/02/2015]; Available from: https://clinicaltrials.gov/ct2/results/displayOpt?flds=a&flds=b&flds=c&flds=n&submit_fld_opt=on&term=NCT02114060&show_flds=Y NLM Identifier: NCT02114060.

63. University of Oxford. *Adjuvanting Viral Vectored Malaria Vaccines With*

Matrix M. 2012 23/02/2015]; Available from: https://clinicaltrials.gov/ct2/results?term=NCT01669512&Search=Search NLM Identifier: NCT01669512.

64. Novavax and Department of Health and Human Services. *A(H7N9) VLP Antigen Dose-Ranging Study With Matrix-M1™ Adjuvant*. 2014; Available from: https://clinicaltrials.gov/ct2/results?term=NCT02078674&Search=Search NLM Identifier: NCT02078674.

65. National Cancer Institute (NCI) and National Institutes of Health Clinical Center (CC). *Adjuvant Tumor Lysate Vaccine and Iscomatrix With or Without Metronomic Oral Cyclophosphamide and Celecoxib in Patients With Malignancies Involving Lungs, Esophagus, Pleura, or Mediastinum*. 2014 23/02/2015]; Available from: https://clinicaltrials.gov/ct2/results/displayOpt?flds=a&flds=b&submit_fld_opt=on&term=NCT02054104&show_flds=Y NLM Identifier: NCT02054104.

66. Prego, C., et al., *Chitosan-based nanoparticles for improving immunization against hepatitis B infection.* Vaccine, 2010. **28**(14): p. 2607-14.

67. Kreuter, J. and P.P. Speiser, *New adjuvants on a polymethylmethacrylate base.* Infect Immun, 1976. **13**(1): p. 204-10.

68. Aguado, M.T., *Future approaches to vaccine development: single-dose vaccines using controlled-release delivery systems.* Vaccine, 1993. **11**(5): p. 596-7.

69. Alonso, M., et al., *Determinants of Release Rate of Tetanus Vaccine from Polyester Microspheres.* Pharmaceutical Research, 1993. **10**(7): p. 945-953.

70. Alonso, M.J., et al., *Biodegradable microspheres as controlled-release tetanus toxoid delivery systems.* Vaccine, 1994. **12**(4): p. 299-306.

71. Gupta, R.K., et al., *Chronic local tissue reactions, long term immunogenicity and immunologic priming of mice and guinea pigs to tetanus toxoid encapsulated in biodegradable polymer microspheres composed of poly lactide-co-glycolide polymers.* Vaccine, 1997. **15**(16): p. 1716-1723.

72. Blanco, M.D. and M.J. Alonso, *Development and characterization of protein-*

loaded poly(lactide-co-glycolide) nanospheres. European Journal of Pharmaceutics and Biopharmaceutics, 1997. **43**(3): p. 287-294.

73. Sánchez, A., J. Vila-Jato, and M.J. Alonso, *Development of biodegradable microspheres and nanospheres for the controlled release of cyclosporin A.* International Journal of Pharmaceutics, 1993. **99**(2–3): p. 263-273.

74. Blanco, D. and M.a.J. Alonso, *Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants.* European Journal of Pharmaceutics and Biopharmaceutics, 1998. **45**(3): p. 285-294.

75. Tobio, M., et al., *Stealth PLA-PEG nanoparticles as protein carriers for nasal administration.* Pharmaceutical Research, 1998. **15**(2): p. 270-275.

76. Tobio, M., et al., *The role of PEG on the stability in digestive fluids and in vivo fate of PEG-PLA nanoparticles following oral administration.* Colloids Surf B Biointerfaces, 2000. **18**(3-4): p. 315-323.

77. Vila, A., et al., *Transport of PLA-PEG particles across the nasal mucosa: effect of particle size and PEG coating density.* Journal of Controlled Release, 2004. **98**(2): p. 231-244.

78. Vila, A., et al., *PLA-PEG particles as nasal protein carriers: the influence of the particle size.* Int J Pharm, 2005. **292**(1-2): p. 43-52.

79. Vila, A., et al., *PEG-PLA nanoparticles as carriers for nasal vaccine delivery.* J Aerosol Med, 2004. **17**(2): p. 174-85.

80. Vila, A., et al., *PLA-PEG nanospheres: new carriers for transmucosal delivery of proteins and plasmid DNA.* Polymers for Advanced Technologies, 2002. **13**(10-12): p. 851-858.

81. Tobio, M. and M. Alonso, *Study of the inactivation process of the tetanus toxoid in contact with poly (lactic/glycolic acid) degrading microspheres.* STP pharma sciences, 1998. **8**(5): p. 303-310.

82. Sanchez, A., et al., *Pulsed controlled-released system for potential use in vaccine delivery.* J Pharm Sci, 1996. **85**(6): p. 547-52.

83. Tobío, M., et al., *Improved immunogenicity of a core-coated tetanus toxoid delivery system.* Vaccine, 1999. **18**(7–8): p. 618-622.

84. Tobío, M., et al., *A Novel System Based on a Poloxamer/ PLGA Blend as a Tetanus Toxoid Delivery Vehicle.* Pharmaceutical Research, 1999. **16**(5): p. 682-688.

85. Sánchez, A., et al., *Formulation strategies for the stabilization of tetanus toxoid in poly(lactide-co-glycolide) microspheres.* International Journal of Pharmaceutics, 1999. **185**(2): p. 255-266.

86. Paolicelli, P., et al., *Surface-modified PLGA-based nanoparticles that can efficiently associate and deliver virus-like particles.* Nanomedicine, 2010. **5**(6): p. 843-53.

87. University of Washington. *Vaccine Therapy Plus Sargramostim in Treating Patients With Stage III or Stage IV Cancer*. 1999 23/02/2015]; Available from: https://clinicaltrials.gov/ct2/results?term=NCT00005023&show_flds=Y NLM Identifier: NCT00005023.

88. Kim, M.-G., et al., *Nanotechnology and vaccine development.* Asian Journal of Pharmaceutical Sciences, 2014. **9**(5): p. 227-235.

89. Garcia-Fuentes, M. and M.J. Alonso, *Chitosan-based drug nanocarriers: where do we stand?* J Control Release, 2012. **161**(2): p. 496-504.

90. Calvo, P., et al., *Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers.* Journal of Applied Polymer Science, 1997. **63**(1): p. 125-132.

91. Calvo, P., et al., *Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines.* Pharmaceutical Research, 1997. **14**(10): p. 1431-6.

92. Vila, A., et al., *Design of biodegradable particles for protein delivery.* Journal of Controlled Release, 2002. **78**(1–3): p. 15-24.

93. Vila, A., et al., *Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice.* Eur J Pharm Biopharm, 2004. **57**(1): p. 123-31.

94. Lugade, A.A., et al., *Single low-dose un-adjuvanted HBsAg nanoparticle vaccine elicits robust, durable immunity.* Nanomedicine, 2013. **9**(7): p. 923-34.

95. Sawaengsak, C., et al., *Chitosan Nanoparticle Encapsulated Hemagglutinin-Split Influenza Virus Mucosal Vaccine.* AAPS PharmSciTech, 2014. **15**(2): p. 317-325.

96. Figueiredo, L., et al., *Intranasal immunisation of mice against Streptococcus equi using positively charged nanoparticulate carrier systems.* Vaccine, 2012. **30**(46): p. 6551-6558.

97. Prego, C., D. Torres, and M. Alonso, *Chitosan nanocapsules: a new carrier for nasal peptide delivery.* Journal of drug delivery science and technology, 2006. **16**(5): p. 331-337.

98. Vicente, S., et al., *A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach.* PLoS ONE, 2013. **8**(4): p. e62500.

99. Vicente, S., et al., *Biodistribution and lymph node retention of polysaccharide-based immunostimulating nanocapsules.* Vaccine, 2014. **32**(15): p. 1685-92.

100. Borges, O., et al., *Alginate coated chitosan nanoparticles are an effective subcutaneous adjuvant for hepatitis B surface antigen.* International Immunopharmacology, 2008. **8**(13–14): p. 1773-1780.

101. Borges, O., et al., *Immune response by nasal delivery of hepatitis B surface antigen and codelivery of a CpG ODN in alginate coated chitosan nanoparticles.* European Journal of Pharmaceutics and Biopharmaceutics, 2008. **69**(2): p. 405-416.

102. Borges, O., et al., *Evaluation of the immune response following a short oral vaccination schedule with hepatitis B antigen encapsulated into alginate-coated chitosan nanoparticles.* Eur J Pharm Sci, 2007. **32**(4-5): p. 278-90.

103. Amidi, M., et al., *Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system.* Journal of Controlled Release, 2006. **111**(1): p. 107-116.

104. Amidi, M., et al., *N-trimethyl chitosan (TMC) nanoparticles loaded with*

influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model. Vaccine, 2007. **25**(1): p. 144-53.

105. Slütter, B., et al., *Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: Nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen.* Vaccine, 2010. **28**(38): p. 6282-6291.

106. Bal, S.M., et al., *Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations.* Journal of Controlled Release, 2010. **142**(3): p. 374-383.

107. Demento, S.L., et al., *Role of sustained antigen release from nanoparticle vaccines in shaping the T cell memory phenotype.* Biomaterials, 2012. **33**(19): p. 4957-4964.

108. Jegerlehner, A., et al., *Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation.* Eur J Immunol, 2002. **32**(11): p. 3305-14.

109. Bachmann, M.F. and G.T. Jennings, *Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns.* Nat Rev Immunol, 2010. **10**(11): p. 787-796.

110. Scheerlinck, J.P. and D.L. Greenwood, *Virus-sized vaccine delivery systems.* Drug Discov Today, 2008. **13**(19-20): p. 882-7.

111. Reddy, S.T., et al., *Exploiting lymphatic transport and complement activation in nanoparticle vaccines.* Nat Biotech, 2007. **25**(10): p. 1159-1164.

112. Foged, C., et al., *Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model.* International Journal of Pharmaceutics, 2005. **298**(2): p. 315-322.

113. Zhao, L., et al., *Nanoparticle vaccines.* Vaccine, 2014. **32**(3): p. 327-337.

114. Ott, G. and G. Van Nest, *Development of Vaccine Adjuvants: A Historical Perspective*, in *Vaccine Adjuvants and Delivery Systems*2006, John Wiley & Sons, Inc. p. 1-31.

CAPÍTULO 2

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CAPÍTULO 2

VACCINE DELIVERY CARRIERS: INSIGHTS AND FUTURE PERSPECTIVES

Adapted from "Vaccine delivery carriers: Insights and future perspectives"

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ABSTRACT

Vaccination is undoubtedly the most effective health intervention for disease prevention and eradication. Nevertheless, currently there is still a need for improving immunization coverage worldwide. A promising strategy to achieve this goal nowadays relies on the use of delivery carriers capable of inducing an effective immune response and providing improved stability, safety and cost effectiveness. This article focuses on analyzing the critical aspects in the design of these carriers, and reviewing the state of the art of currently marketed formulations and those in advanced clinical development. These vaccine delivery carriers include emulsions, liposomes and polymeric particulate carriers. Finally, particular attention is given to the evolution in the design of polymeric nanocarriers, which have been receiving increasing attention and hold promise to generate novel platforms for needle-free administration and single-dose vaccination.

1. INTRODUCTION

The impact of vaccines in global health is beyond question. Since Jenner in 1796 to the present, vaccines have permitted the control of many infectious diseases [1] and even the eradication of smallpox [2]. Not limited to prophylactic treatment, vaccines have recently started to be applied with success also as therapeutic agents in allergy, autoimmune treatment, cancer therapy and infectious diseases [3-6].

While important achievements in vaccination have relied on the use of attenuated vaccines or whole killed pathogens, nowadays research is mostly focused on subunit vaccines. This is because subunit vaccines are purer, safer and easier to produce than classical vaccines [7]. Unfortunately, the high purity of the subunit vaccines often renders them poorly immunogenic [8, 9] and, thus, dependent on the use of effective adjuvants that can assist them in generating an effective immune response.

An adjuvant is by definition any molecule, macromolecular structure or system capable of amplifying an immune response against a specific antigen [10]. Fundamental for this response are the antigen presenting cells (APC), especially the dendritic cells (DC). These cells capture the antigen, process it and modulate the cellular and humoral immune response [11]. While antibody titers have been the classical parameter used to evaluate the relevance of a specific adjuvant, in recent years there is an increasing interest in achieving strong cellular responses to overcome challenging intracellular pathogens, such as malaria or HIV or to develop effective therapeutic vaccines against tumor cells [12-14].

According to the above definition, immune adjuvants are a large and heterogeneous group, which could be divided into two subgroups: immunostimulants and delivery systems [10, 13]. The first category of adjuvants stimulates the immune system by interacting with specific receptors, while the second type can increase the immune response by multiple mechanisms, depending on their particular characteristics [15]. In this review we pay particular attention to antigen delivery systems especially those having a size in the micro/nanometer range.

Besides the potential of antigen delivery carriers as adjuvants, there is increasing body of evidence supporting their potential as single-dose and needle-free vaccine delivery strategies. Indeed, major entities in global health such as the World Health Organization (WHO) and the Center for Disease Control and Prevention (CDC) have claimed the

necessity to invest research efforts in developing advanced delivery technologies, which could simplify immunization schedules [16-18]. The accomplishment of these objectives would generate an improvement in the vaccination coverage for several infectious diseases, especially in developing countries.

Taking this into account, this review article is intended to highlight and analyze the most relevant advances achieved so far in the development of antigen delivery carriers. Furthermore, this article aims to provide the reader with the understanding of the basis for future improvements in the design of antigen delivery systems.

2. CRITICAL ASPECTS IN THE DESIGN OF ANTIGEN DELIVERY CARRIERS

As indicated above, antigen delivery carriers not only have the possibility to facilitate the antigen uptake by the immunocompetent cells and provide controlled antigen delivery, but also the capacity to overcome mucosal barriers. This section describes the main factors and mechanisms governing such special features.

2.1. Parameters affecting the ability of delivery carriers to transport antigens across mucosal barriers

Vaccine delivery systems have been shown to improve antigen passage through relevant biological barriers, such as the intestinal and nasal mucosa and hold some promise towards the generation of needle-free vaccines. Regarding the oral route, the size of the delivery carrier is a fundamental parameter for adequate immune stimulation. It has been known for more than two decades that small microspheres are preferable to the large ones for oral immunization [19, 20]. However, the issue of optimal size is still a controversial matter. For example, using BSA as a model antigen, Gutierro et al. [21], observed a better immunostimulating ability for microspheres of 1 μm than for those of 0.2 – 0.5 μm. The same group found that the oral administration of SPf66 antigen (a synthetic malaria peptide) encapsulated into 1- μm PLGA microparticles generated a strong immune response, similar to the one achieved following subcutaneous administration of the antigen adjuvanted with alum. The authors attributed these positive results to the ability of microparticles to be taken up by the Peyer's patches [21]. Interestingly, over the last decade the trend observed is that the smaller particle size, the greater their ability to transport antigens across the intestinal barrier [22-24].

Consequently, it could be argued that the ideal size for an oral vaccine carrier would be within the nanometer range, although affected by other characteristics such as surface composition and release properties. In this sense, our group found that providing PLA nanoparticles with a protecting PEG coating helps in maintaining their stability in gastrointestinal fluids and facilitates the transport of the associated antigen (tetanus toxoid) across the intestinal mucosa [25].

The nasal route is another promising alternative for needle-free administration due to its particular physiological characteristics and immunological features. Indeed, despite the limitations associated to the mucociliarly clearance, the nasal mucosa displays several characteristics that make it attractive for antigen delivery. Namely, it has a limited enzymatic activity and a relatively leaky epithelium accompanied by specialized cells (M cells) that harvest the antigens present in the mucosa and deliver them to associated lymphoid tissues [26].

The first work showing the potential of nanoparticles for nasal antigen delivery was reported by Almeida et al. [27]. In this work, tetanus toxoid (TT) was adsorbed onto PLA nanoparticles of 800 nm size and administered to guinea-pigs by the nasal route. The immune response achieved with the adsorbed antigen was about 10 times stronger than the free antigen administered by the same route, evidencing the potential of nanoparticles for an effective nasal vaccination. A few years later, using PLA-PEG nanoparticles, our group demonstrated the importance of size and surface hydrophilicity on the ability of particles to efficiently deliver antigen through the nasal route. As indicated for the oral route, the idea of using PEGylated PLA came from the observation that PLA nanoparticles aggregated upon contact with mucosal fluids and, therefore, PEGylation was conceived as a way to preserve the stability of nanoparticles [28]. The results of the absorption and biodistribution of TT associated to these nanoparticles clearly evidenced the positive effect of the PEGylation (**Figure 1**). In fact, it was observed that the antigen could penetrate through the nasal mucosa much more efficiently when encapsulated into PLA-PEG particles. These results were corroborated by a work of Vila et al. [29] in which it was found that both particle size and PEGylation degree influenced the uptake by the nasal mucosa and the subsequent biodistribution of the associated antigen. The same authors found that this improved transport of the tetanus toxoid antigen was the explanation for the important and long-

lasting response [30]. These results emphasize the importance of an adequate design of the delivery system to achieve an effective vaccine formulation.

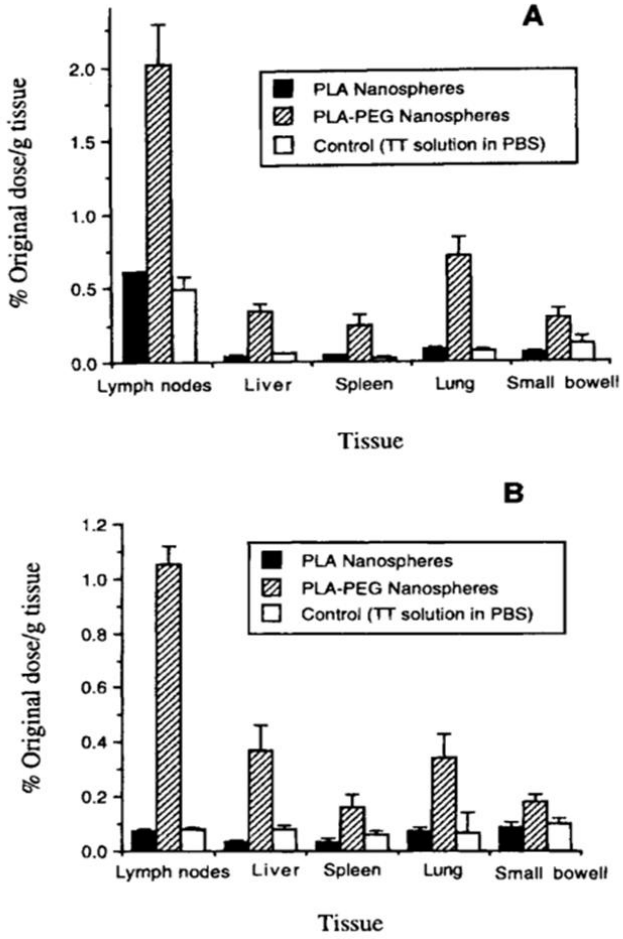

Figure 1. *Tissue distribution of 125I-labelled tetanus toxoid at 24 h (A) and 48 h (B) after nasal administration of 125I-TT-loaded PLA and PLA-PEG nanospheres. Adapted from [28] with permission.*

Besides the mucosal routes, the transdermal route is also progressively becoming a feasible option for vaccination. This method takes advantage of the high concentration of immune cells in the epidermal and dermal tissues [31] and the ability of nanoparticles to cross the epidermal barrier by the hair follicles pathway. This ability has been found to be related to particle size. For example Vogt et al. [32] compared the skin penetration of fluorescent nanoparticles of 40, 750 and 1500 nm size and observed that only the 40 nm particles could penetrate deeply through the follicular epithelium and interact with the epidermal Langerhans cells. Later Mahe et al., reported also the greater penetration of 40-nm particles as compared to that of 200-nm particles [33]. Other groups tried to

increase the flexibility of liposome carriers in order to improve their ability to penetrate the skin and generate an immune response [34, 35]. This area has been gaining much interest in more recent years and it is possible to find complete and up to date information regarding this field in the reviews of Combadiére and Mahé et al. [36] and Li et al. [37].

2. 2. Parameters affecting the biodistribution of antigen delivery systems and their ability to target antigen presenting cells (APC)

The adequate control of size of a delivery system can also influence its biodistribution and consequently, also that of the antigen. When administered by intramuscular or subcutaneous route, particles with a size of 20–100 nm can penetrate the extracellular matrix and enter directly into the lymphatic vessels. Once in the lymph, the particles travel to the lymphatic nodes where they are captured by the dense population of immune cells, mostly by dendritic cells (DC), and generate effective immune responses [38, 39]. These mechanisms have been summarized in **Figure 2**.

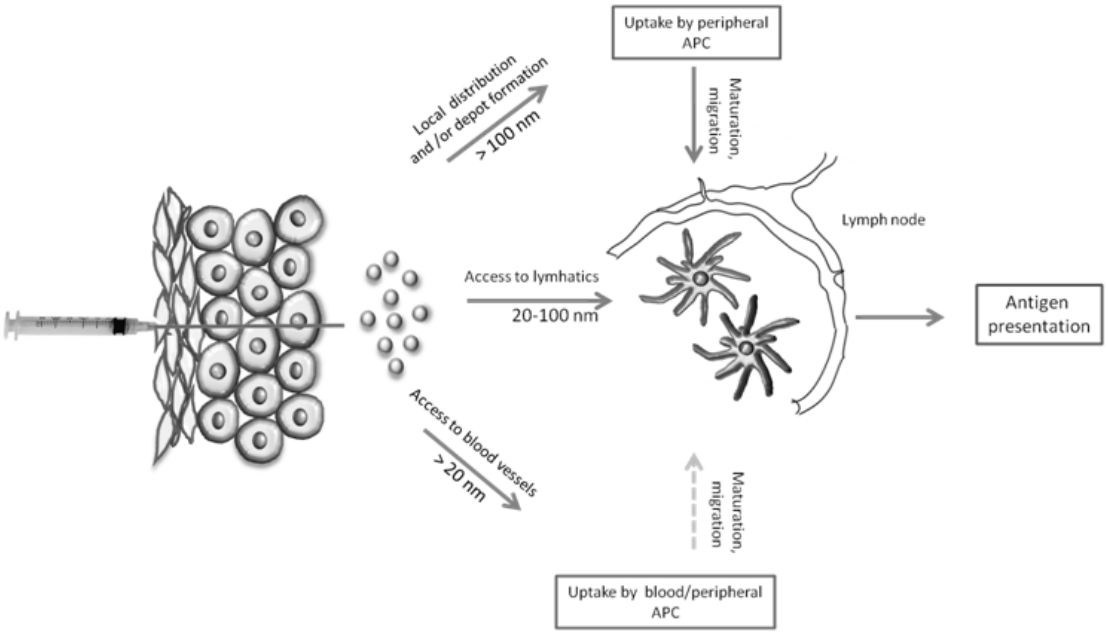

Figure 2. *Schematic representation of size dependent particle processing following s.c./i.m. administration.*

The size of the polymeric particles seems to influence the distribution of the particles after subcutaneous, intradermal or intramuscular administration. Several authors seem

to agree that polymeric particles larger than 100 nm, will mainly linger at the administration point as they cannot pass through the extracellular matrix´s pores and reach the lymph directly [38, 40-43]. These particles can still raise an effective immune response as they are subsequently scavenged by peripheral DC. After capturing the particles, these DC migrate to the lymphatic nodes and initiate a systemic immune response [44]. As for particles smaller than 20 nm, they will drain mainly to the blood capillaries and suffer elimination from the organism, although a fraction can eventually also reach the lymph nodes [38, 45].

In addition to the critical role of the particle size, some authors observed in vitro that the uptake of model polystyrene nanoparticles by macrophages and DC can be strongly enhanced if the particles have a cationic surface [46, 47]. Interestingly, Foged et al., when comparing the dendritic cell uptake of polystyrene spheres of 100 nm and 1000 nm with different surface charge, observed that the cationic surface increased the uptake of larger particles but smaller particles seem to undergo equal uptake independently to their surface charge [47].

Altogether, irrespective of the influence of the size and surface charge, there is no doubt that the association of antigens to micro- or nanosized particulate carriers facilitates their uptake by DC [48]. A possible explanation for such behavior could be found on the resemblance between the particles and the bacteria and virus that the APC are programmed to capture [44, 49]. Moreover, it has been suggested that the particulate character given to the antigen not only facilitates its uptake by immune cells but also induces their activation and can further assist to the so called cross presentation phenomenon [50]. As a result, particulated antigens do not only stimulate humoral but also cellular responses [51].

2. 3. Improving the stability and providing controlled antigen release

An advantage of vaccine delivery carriers over immunostimulant molecules is their ability to protect the associated antigen from adverse physiologic conditions such as enzyme degradation or non specific interaction with other molecules in the extracellular matrix and provide a prolonged release profile, more similar to a real infection. The antigen (generally a protein or peptide), is susceptible to chemical and enzymatic degradation as well as physical alteration like aggregation or precipitation [52]. Besides, it can establish nonspecific interactions with extracellular components, leading to the

inefficient stimulation of the immune system. All these problems can be highly attenuated when the antigen is incorporated into an adequate vaccine delivery system [53, 54].

Vaccine delivery carriers can be designed to offer sustained release of the antigen and thus, to extend the time available for immune stimulation. Some authors have found that this continuous stimulation provided by controlled release systems may even surpass the effect of strong immunostimulants such as lipopolysaccharide (LPS). This has been, for example, the case of polyanhydride nanoparticles containing OVA when administered by the oral route [24].

In addition to the value of controlled release systems in terms of enhancing the immune response, their ability to extend antigen release for prolonged periods of time has been regarded as a promising strategy for single-shot immunization protocols [18]. As discussed later in section 4, the idea of using controlled release technologies to simplify vaccination schedules was first proposed by Preis and Langer [55]. After a few decades of work in this field it has become clear that antigen release mainly depends on the way it is incorporated within the delivery carrier, i.e. encapsulated/entrapped into or simply adsorbed/associated onto the polymer. In the first case, the release rate depends on the degradation, erosion or dissolution of the polymer surrounding the antigen. In the second case, antigen release is governed by the interaction forces between the polymer and the antigen. Frequently, there is a combination of associated and encapsulated antigen in the particles, generating a characteristic release profile with an initial burst release due to the release of the more loosely associated antigen and a subsequent more sustained release of the encapsulated protein [56-58]. However, as important as the release profile is the fact that the loaded antigen should be delivered in its antigenically active form, capable of generating an adequate immune response. Regarding this point, the formulation conditions are of critical importance since the use of organic solvents, extreme temperatures or high energy inputs can degrade or aggregate the proteic material. In addition, the materials themselves used in the elaboration of the delivery systems or their degradation products can be an important source of protein deterioration [59-62].

Finally, an important additional advantage of controlled release carriers relies on the possibility of co-delivering an immunostimulant agent, thus providing means to further enhance the stimulation of the immune system [63].

3. CURRENT ANTIGEN DELIVERY SYSTEMS ON THE MARKET OR UNDER CLINICAL DEVELOPMENT

Currently, the most widely used immune adjuvants are the alum salts. The traditional explanation for their adjuvant action is the formation of a depot structure at the injection site, from which the antigen is slowly released [64]. However, more recently, the adjuvant ability of the alum salts has also been attributed to their ability to trigger the so-called "inflammasome" mechanism in the cells. According to this mechanism, the alum salts would be recognized as a danger signal or would promote the release of danger signals from the cells, thereby generating a pro-inflammatory environment, which leads to the activation of the immune system [65].

These adjuvants have been successfully used since 1926 [66, 67] and have an impressive record of safe administrations and robust immunizations rates [64]. Nevertheless, they have some limitations, such us their inability to stimulate cellular immune responses [14, 63], their potential adverse local reactions [67] and their degradation upon freeze-drying [68]. In addition, this type of adjuvant requires a multiple administration schedule to guarantee long lasting protection. Altogether, the limitations of alum salts have stimulated the search for new adjuvants and particularly, for novel vaccine delivery systems with the potential to solve the limitations of present vaccines. Those currently on the market (**Table 1**) or under clinical investigation (**Table 2**) will be described in this section.

3.1. Emulsions

The investigation of emulsions as vaccine adjuvants started in the late 30's with Freunds Complete Adjuvant (FCA), (a water-in-oil (w/o) emulsion of a mineral oil, paraffin and killed mycobacteria) [69]. Although capable of generating high immunization titers, this emulsion led to strong adverse reactions, which hampered its clinical use. A less toxic version, the Freunds Incomplete Adjuvant (FIA) which lacks the mycobacterial component, was used instead until the mid-1960s, when it was finally abandoned in favor of the safer alum-based adjuvants [70]. Since those early days, there has been an

intensive research leading to other w/o formulations such as the Montanide Series, Adjuvant 65™ and Lipovant™. These formulations share the same mechanism of action, which is the formation of a depot at the injection site capable of attracting immune cells. The most intensively studied among them is the Montanide series, particularly the Montanide™ ISA51. This w/o emulsion has squalene as the oil fraction and mannide-mono-oleate as the surfactant and has been used as an adjuvant in an epidermal growth factor (EGF) protein vaccine for lung cancer treatment [71]. Unfortunately, the adverse reactions associated to this adjuvant and its questionable success make the future for this adjuvant uncertain [72, 73]. Oil-in-water (o/w) emulsions have been presented as interesting alternative vaccine adjuvants [74, 75]. MF59™ was the first o/w emulsion approved in 1997 for human use in Europe as an adjuvant in an influenza vaccine (**Table 1**) [76]. This emulsion is composed of squalene oil, dispersed in the form of 160 nm droplets, conveniently stabilized with a mixture of a high HLB (polysorbate 80) and a low HLB surfactant (sorbitan trioleate) [77, 78]. This adjuvant renders the vaccine more efficacious than other commercially available influenza vaccines [79] specially in elderly people [80]. Equally important, is the good safety profile shown in several clinical trials, where no major adverse effects were detected [77]. Regarding its mechanism of action, besides a depot effect, MF59™ is able to activate the immune cells directly [81]. Currently, this adjuvant is being tested in clinical trials as a vaccine against Hepatitis Virus C (HCV) and Cytomegalovirus (CMV) (**Table 2**). Another o/w emulsion (AS02™) is composed of squalene and two hydrophobic immune adjuvants, MPL1™, a synthetic derivative of LPS, and QS-21, a purified saponin plant extract. While the first adjuvant is able to promote immune cell recruitment and activation [82], the second one stimulates strong antibody responses against the associated antigens [83]. AS02™ is capable of inducing both strong humoral and cellular responses, making it a promising adjuvant. It has shown remarkable results when tested in infants as an adjuvant for the RTS malaria antigen [84] constituting, currently, the leading vaccine candidate against this disease. The same adjuvant has also been evaluated with tuberculosis [85] and HIV antigens [86], showing promising immunization results.

Table 1. Delivery systems present in marketed human vaccines

Delivery System	Antigen	Vaccine	Disease	Reference
AS03	HA	Arepanrix ™ (GSK) *[1]	Influenza	[87]
	HA	Pandemrix™ (GSK)	Influenza	[88]
Virosomes	Inactivated virus	Epaxal ™ (Crucell)	Hepatitis A	[89]
	HA and NE	Inflexal ™ V (Crucell)	Influenza	[90]
MF59	HA	Fluad™ (Novartis)	Seasonal influenza	[77]
	HA from influenza A/H1N1	Focetria™ (Novartis)	Influenza	[91]
AF03	HA from H1N1	Humenza™ (Sanofi) *[2]	Influenza	[92]

*HA: Hemagglutinin; NE: Neuraminidase; [*1] Withdrawal of marketing authorization in the European Union at 18/01/2011; [*2] Withdrawal of marketing authorization in the European Union at 30/06/2011.*

A similar emulsion formulation named as AS03™ is composed of squalene and tocopherol dispersed in the form of nanodroplets (150 nm) thanks to the use of Tween® 80 as the surfactant [78]. This formulation has already been commercialized as an adjuvant for a pandemic influenza vaccine (Pandemrix®), showing an improved response compared to that of the alum adjuvanted vaccine [93]. Another clinical trial is currently in progress to assess the relevance of this adjuvant system in a different influenza vaccine (**Table 2**). The work of Morel et al. suggests that this adjuvant does not act through a depot effect but solely as a danger signal that attracts and activates immune cells [94].

Table 2. Antigen delivery systems in clinical development

Delivery System	Antigen	Disease	Route	Phase	End	Clinical Trial Identifier
MF59	glycoprotein B	CMV	IM	II	Ongoing	NCT00133497
			IM	II	2010	NCT00125502
	glycoprotein gp E1/E2	HCV	n.d.	I	2005	NCT00500747
AS02A	FMP2.1	Malaria	IM	II	2009	NCT00460525
ISCOM	HA	Influenza	IM	I	Ongoing	NCT00868218
ISCOMATRIX™	L1 protein VLP	HPV	IM	I	2009	NCT00851643
PLGA	HER-2 derived peptide	HER-2 positive cancers	IM; SC	I	2009	NCT00005023
Virosome	AMA-1 & CSP peptides	Malaria	n.d.	I	2009	NCT00513669
	n.d.	HIV	IM; IN	I	2010	NCT01084343
	n.d.	Hepatitis A	IM	IV	2011	NCT01349829
	n.d.		IM	II	Ongoing	NCT01405677
	HA	Influenza	IM	III	2010	NCT01229371
	n.d.	Candidiasis	IM; IV	I	Ongoing	NCT01067131

Delivery System	Antigen	Disease	Route	Phase	End	Clinical Trial Identifier
AS01 & AS02	RTS,S	Malaria	IM	II	2006	NCT00197054
	FMP 2.1		IM	I & II	2007	NCT00385047
AS01	RTS,S	Malaria	n.d.	II & III	Ongoing	NCT00872963
	VMP001		IM	I & II	2011	NCT01157897
	FMP011		IM	I & II	2007	NCT00312663
	RTS,S		n.d.	I & II	2006	NCT00075049
	FMP010		IM	I	2009	NCT00666380
	RTS,S		IM	II	2007	NCT00307021
AS03	n.d.	Influenza H5N1	IM	I & II	Ongoing	NCT01353534

CMV: cytomegalovirus; HPV: Human Papilloma Virus; VLP: virus-like particle; AMA-1: apical membrane antigen 1; CSP: circumsporozoite protein; n.d.: not disclosed; FMP011: falciparum malaria protein 11; FMP010: Plasmodium falciparum malaria protein 010. Data retrieved from [95].

In parallel with the development of emulsions, there has been intensive research on several alternative technologies such as liposomes, immunostimulating complexes and polymeric delivery systems, which will be described in the following sections.

3.2. Liposomes

Liposomes are composed of phospholipid bilayers in the form of small vesicles which can efficiently incorporate antigens [96]. Liposomes are among the most promising delivery vehicles and have received an impressive amount of attention from the literature analysis point of view (**Figure 3**). They have been described as antigen carriers which are able to deliver their content to the APCs [97] facilitate the cross presentation of antigens and promote cellular immune responses [98]. These carriers also allow the co-encapsulation of immunostimulants [99, 100]. There are recent review articles describing the specific advances on liposomes as antigen carriers [97, 101, 102]. In the present review we focus on those liposomal compositions that have reached clinical development.

An example of a composition in clinical development is AS01™ which contains two lipophilic immunostimulants, MPL1 and QS-21 [103]. In a clinical trial for a HIV vaccine, this adjuvant system was found to stimulate a higher immune response than the one observed for the o/w emulsion AS02™ [86]. In addition, clinical trials are in progress to evaluate the potential of this adjuvant for a malaria vaccine (**Table 2**).

The most advanced liposomal structures for vaccine application are currently the immunopotentiating reconstituted influenza virosome (IRIV) vaccines, generally denoted as virosomes. These liposomes have integrated into their surface two glycoproteins from influenza, hemagglutinin (HA) and neuraminidase (NA). The antigen associate by covalent or electrostatic interactions with the lipid bilayer [104]. It has been reported that these glycoproteins increase capture by APCs and improve the processing of the antigens. This improvement seems to be related with the ability of the HA protein to fuse with the endosomal membrane after the endocytosis of the virosome. By fusing with the endosomal membrane, the HA facilitates the escape of the virosome, avoiding the destruction of the antigen in the lysosome vesicles, and making it available for class I antigen presentation. The first virosome-based vaccine for human use was licensed in 1996 as a hepatitis A vaccine (Epaxal®) and later on it was also licensed in

an influenza vaccine (Inflexal V ®) [90]. Currently, several clinical trials are in progress to evaluate different vaccines containing this adjuvant (**Table 2**).

Finally, an alternative liposome technology is the Supramolecular Biovector (SMBVe). This liposome has a cross-linked polysaccharide core inside and a global size of about 60 nm [105]. By controlling the nature of the core or the lipid composition, this system can be easily adapted to the association of specific antigens. This composition generated high IgG antibodies levels, cytotoxic T lymphocyte (CTL) responses as well as mucosal IgA antibodies in mice when used as a vaccine carrier for nasal immunization against hepatitis B [106]. More recently, this adjuvant entered in clinical evaluation phase using an influenza antigen [107].

Overall, despite the long time required for antigen-associated liposomes to reach the clinical development stage, it is now clear that they offer great versatility and promising features. At this point, it seems realistic to say that the success of the vaccines recently commercialized or under clinical trials will dictate the way to proceed in this specific adjuvant approach.

3.3. Immunostimulating complexes

The immunostimulating complexes (ISCOMs) are colloidal antigen delivery systems with a 40 nm size and a cage-like structure [108]. They are composed of saponins, cholesterol, phospholipids (traditionally phosphatidylcholine) and an entrapped antigen [109]. Antigens are entrapped by hydrophobic interactions in the interior of the complex. For hydrophilic antigens the ISCOM components are assembled in the absence of the antigen generating the ISCOMATRIX™. As the ISCOMATRIX surface is negative, due to the glucuronic moieties of the saponin, the hydrophilic antigen is then associated to the surface by electrostatic interactions [108].

The adjuvant ability of these delivery systems rises from a combination of factors. Beside the intrinsic immunostimulant ability of saponins, the structure of the complex itself also work as an activation signal for the immune cells [44] and as a promoter for antigen cross presentation [110, 111]. Interestingly, free saponins are known to be toxic compounds [112], however, when they are complexed with cholesterol, their toxicity is significantly reduced and their immune stimulant ability enhanced [113].

The efficacy of ISCOMs has been reported in terms of generating strong antigen-specific cellular and humoral immune responses in animal models following either parenteral [114] or nasal administration [115]. Furthermore, these complexes are currently being evaluated as adjuvants in clinical trials for influenza and Human Papilloma Virus (HPV) vaccines (**Table 2**).

Overall there is clear evidence of the potential of these nanocarriers as adjuvants. The further development of these composite carriers will greatly depend on their toxicity and efficacy profile all along their clinical path towards commercialization. Some authors have suggested that the presence of saponin might pose safety concerns and thereby impede their clinical development [116].

In summary, as a consequence of the significant amount of research devoted to adjuvants and delivery carriers over the last decade we have attractive strategies that warrant clinical evaluation. Clinical trials of these nanocompositions will significantly influence the future scientific developments in this area.

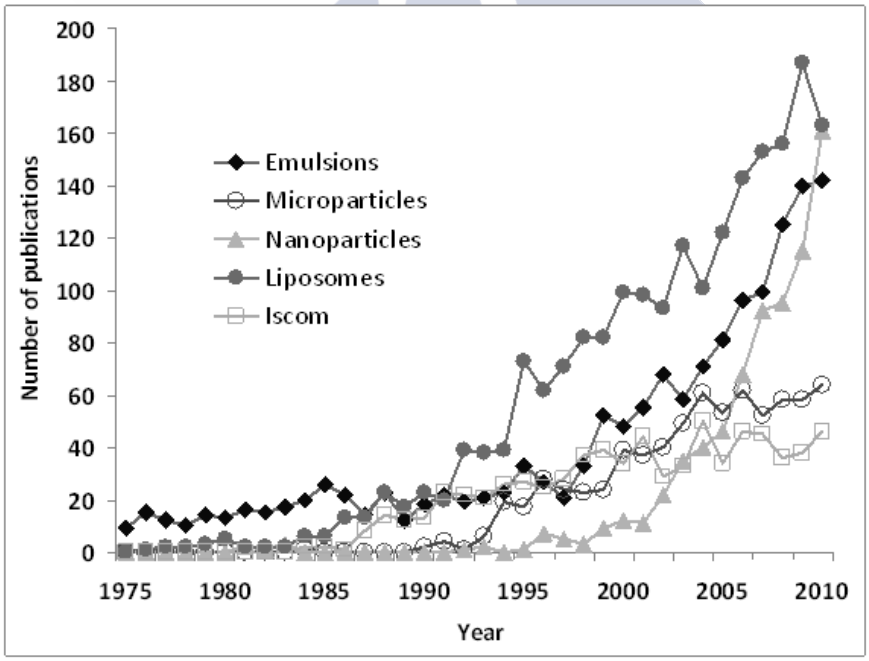

Figure 3. *Evolution of the number of publications related to different vaccine delivery systems from 1975 to 2010. Data retrieved from [117]*

4. ADVANCES IN THE SEARCH FOR NEW ANTIGEN DELIVERY SYSTEMS. A PERSPECTIVE OF POLYMER-BASED NANOCARRIERS

In addition to the advances made over the last decades in the clinical development of emulsions, liposomes and immunostimulating complexes, there are new delivery platforms based on the use of polymers which also hold promise for the future development of new adjuvants and innovative vaccine formulations. As illustrated by **Figure 3** [117], over the last couple of decades there has been a continuous increase in the number of publications regarding antigen delivery carriers. The dramatic increase in the use of nanoparticles as antigen delivery carriers is particularly noteworthy. In fact, yearly rate of publications has been multiplied by 10 (from ~20 to ~200) over the last ten years. Given this tendency, we decided to concentrate on the advances made in the development and application of polymer-based micro and nanoparticles.

The first evidence of the potential of polymeric nanoparticles as vaccine delivery vehicles was reported in 1976 by Birrenbach and Speiser [118] where they demonstrated the ability of polyacrylamide nanostructures to increase immune responses against the associated antigen following subcutaneous administration. Some years later, Preis and Langer proposed the use of ethylene-vinyl acetate beads as a single-dose vaccine delivery strategy [55]. The idea was based on the ability of these polymer matrices to control the release of the associated antigen, thus leading to a prolonged presentation of an antigen to the immune system. Even though none of these delivery vehicles have found their way towards the clinical development stage due to their toxicity, they provided a strong proof-of-principle which has motivated the development of new polymer-based antigen delivery carriers. A summary of the advances made on the use of these carriers is briefly described in the following sections.

4.1. Polyester-based microparticles

The family of polylactic acid and polylactic-co-glycolic acid (PLA/PLGA) is very well-known for their safety profile and ability to control the release of drugs for extended periods of time [119]. The interest in their use for controlled antigen delivery started in the late 70's following their commercialization for the delivery of peptide drugs [120]. Indeed, it was in that period, when the World Health Organization (WHO) proposed the initiative of developing a single-dose vaccine for tetanus toxoid in order to prevent

neonatal tetanus [121]. Overall, the results from several groups working on this initiative showed the potential of these particles in controlling the release of the encapsulated antigen, thus supporting their potential for generating long-lasting responses [57, 122-125]. Unfortunately, despite the initially promising data, we subsequently observed that a large fraction of the antigen was damaged during the process of encapsulation and release from the PLGA and PLA microparticles [60]. The cause for the antigen degradation has been mainly attributed to the drastic decrease of pH inside the particles, due to the accumulation of degradation products of the polymer matrix [61]. To solve this problem, we have developed a number of strategies, which involve the protection of the antigen by preventing its interaction with the polymer's degradation products. One of these strategies involved the use of a protective oil-core surrounded by a PLGA wall. Such microcapsules were able to provide extended antigen delivery for more than two months [126]. An alternative approach to the antigen degradation problem was proposed by Tobío et al. [127]. Through the addition of poloxamer 188 to the formulation, the authors found a way of avoiding unwanted interactions between PLGA and the antigen. I.m. administration of this formulation to mice revealed the efficacy of this approach. Both IgG levels and neutralization titers to tetanus toxoid were higher and much more prolonged than those achieved with the alum-adsorbed antigen formulation. These promising results have motivated the development of new formulations of PLGA/PLA microparticles containing antigens from other diseases such as Rabies [128], Brucellosis [129] Tuberculosis [130] or Malaria [131]. More recent work, using the hepatitis B antigen, has also shown the ability of PLGA microparticles to generate strong cellular immune responses [132]. This response is critical in the case of chronically infected people where the cellular immune response could overcome immune tolerance and lead to the clearance of the persisting virus.

Recently, PLGA microparticles have been tested as adjuvant in a clinical trial for a cancer vaccine using a HER-2 derived peptide as antigen (**Table 2**).

There are also some recent strategies aimed at improving the efficacy of PLGA microparticles as antigen delivery vehicles by coating them with cationic polymers [133, 134]. For example, the polypeptide protamine has been used as a surface coating material due to its ability of increasing cell penetration. When encapsulating the purified phospholipase A2(PLA2) and ovalbumin (OVA) as model antigens, the authors found

that the protamine coating led to an impressive improvement of the humoral responses in mice against both antigens [134].

Another recent trend in this field is the association of immunostimulant molecules with PLGA microparticles in order to obtain a synergistic effect between them. For example, Sharp et al. observed a strong increase in the production of Interleukin-1β, through the incorporation of LPS, CpG or PAM3Csk (a synthetic analogue of a bacterial lipoprotein) into PLGA microparticles [135]. Such synergistic effect of immunostimulant molecules and PLGA microparticles has been further confirmed for CpG [136]. In another work, Kazzaz et al. [137] encapsulated either MPL or its synthetic analogue (RC529) into PLGA microspheres. Antigens from either Neisseria meningitides B or from HIV-1 were associated to the surface of the microspheres. The results showed that immune responses were much superior when the immunostimulants were incorporated into the microparticles instead of being administered separately. Therefore, the conjunct formulation seemed to enhance the adjuvant properties of both the particles and the immunostimulants.

4.2. Polyester-based nanoparticles

The size reduction of PLGA microparticles to the nanometer's range has been mainly motivated by the necessity to develop needle-free delivery vehicles and new adjuvants for mucosal immunization. As indicated in section 2.1, there are some pioneering works that have shown the importance of submicron range size and the presence of a PEG coating on the ability of PLGA particles to facilitate the transport of antigens across mucosal barriers [27, 28, 138]. The same nanocarriers were also shown to generate long lasting immune responses against tetanus following intranasal administration [30]. The evidence of the positive effect of a PEG corona represented a major breakthrough because it was generally assumed that hydrophobic particles were preferable over hydrophilic particles as carriers for mucosal antigen delivery [139]. This classical assumption was based upon the idea that hydrophobic particles are preferentially taken up by the APC of the mucosal surfaces [140]. This theory failed to take into consideration the stability problem of hydrophobic particles as well as the fact that small nanoparticles can be internalized in the nasal mucosa not only through direct uptake by the associated lymphoid cells but also by transport into the epithelial cells. Beyond establishing the potential of PLA-PEG as nasal vaccine delivery carriers, the

above results highlight the importance of a rational design of nanocarriers for an optimal delivery of proteins through mucosal tissues.

Another approach chosen by our group for improving the controlled release of macromolecules from PLGA nanoparticles and their delivery across the nasal mucosa, has been the formation of intimate blends of PLGA and surfactants from the poloxamer and poloxamine series [141, 142]. Working with a model genetic vaccine, we observed the possibility of modulating its release and facilitate its transport across the nasal mucosa [143]. More recently, we have applied the same kind of nanostructures for the controlled delivery of recombinant hepatitis B surface antigen (rHBsAg), which assembles into 22 nm particles [144]. A selected prototype was additionally coated with chitosan (CS) in order to further improve the presentation of the nanocarrier to the immunocompetent cells. As shown in **Figure 4**, these nanostructures were able to control the release of rHBsAg without altering its integrity and antigenicity.

PLA/PLGA nanoparticles have also been modified with specific ligands, such as RGD peptide [145] or lectins [146], in order to increase their uptake by the M cells associated to the intestinal mucosa. For example, in the work of Garinot and coworkers, OVA antigen was encapsulated in PLGA nanoparticles surface-modified with RGD. Although an increased uptake of the modified particles *in vitro* and an effective targeting *in vivo* of the M cells were observed, the improvement of immune response was found to be minimal. A possible explanation for these results is the degradation of the ligand by the harsh conditions of the GI tract [145]. On the other hand, Gupta and coworkers modified PLGA nanoparticles with the UEA-1 lectin for the oral delivery of the recombinant hepatitis B surface antigen (rHBsAg). In this case, the surface modification resulted in a significantly enhanced immune response. Nevertheless, carbohydrates present in ingested food can complex with lectins and prevent its targeting activity which can be a source of interference in a clinical application of such systems [146].
In addition to a mucosal route, polyester nanoparticles are also interesting systems for parenteral vaccination. In a work by Ataman-Önal et al. [147], the HIV-1 p24 protein was associated to the surface of PLA nanoparticles and administered subcutaneously to mice, rabbits and macaques. In all animal models, both a strong humoral and a cellular immune response were detected against the presented antigen.

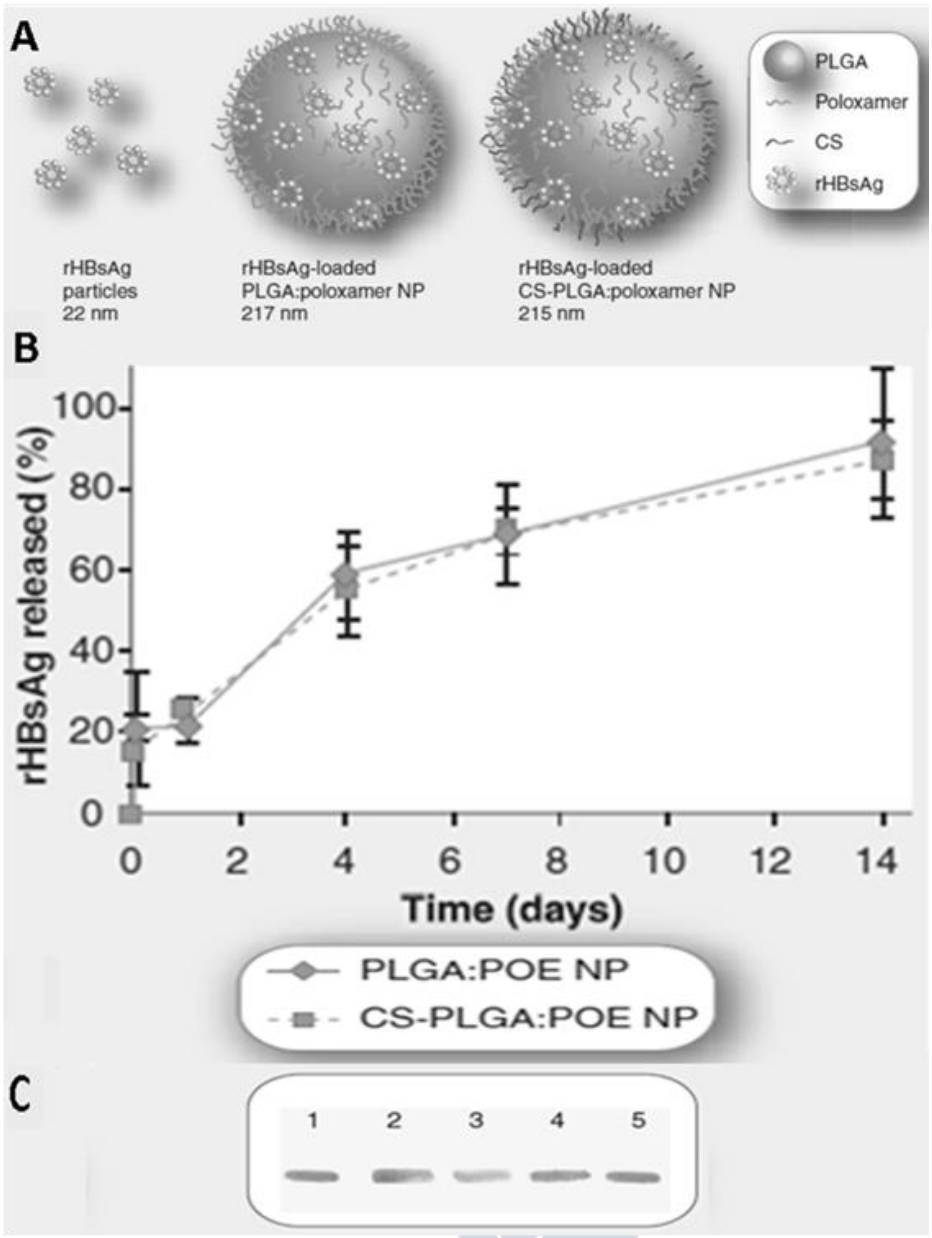

Figure 4. *Schematic representation of PLGA-based nanoparticles for the delivery of rHBsAg (**A**), their corresponding release profiles (**B**) and the analysis of the structural integrity of the antigen (**C**). Lane 1: bulk rHBsAg; lanes 2 and 3: rHBsAg recovered from PLGA:POE NPs after 4 and 7 days; lanes 4 and 5: rHBsAg recovered from CS-coated PLGA:POE NPs after 4 and 7 days. rHBsAg: recombinant hepatitis B surface antigen; PLGA:POE NP: poly(d,l-lactide-co-glycolide):poloxamer nanoparticle; CS: chitosan. Reproduced from [148] with permission.*

More recently, Moon et al. [149] used PLGA nanoparticles as substrates to create pathogen-mimicking polymeric nanoparticles for a malaria vaccine. The PLGA

nanostructure was coated with a lipid membrane integrating MPL and then a malaria recombinant antigen (VMP001) was linked covalently to this membrane. Using this carrier by subcutaneous route in mice, Moon et al. could achieve increased VMP001-specific IgG titers for over 6 months together with enhanced cellular immune responses.

4.3. Polyanhydride-based Nanocarriers

Synthetic polyanhydrides, such as poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA), have been regarded as interesting materials for antigen delivery due to their bioadhesive properties [150] and their ability to specifically interact with immune cells [151], combined with their low oral toxicity [150]. Using ovalbumin (OVA) as a model antigen, Gómez et al., evaluated the behavior of PVM/MA nanoparticles as carriers for intradermal vaccination. The results indicated that the OVA-loaded nanoparticles generated stronger humoral responses (IgG titers) than alum [152]. As noted in the section 2.3., the same particles were also capable of producing high and sustained immune response following oral administration with the model antigen (OVA) [24], showing the versatility of this delivery carrier.

4.4. Polysaccharide-based Nanocarriers

Several polysaccharides, such as inulin [153] alginate [154] and hyaluronic acid [155], have attracted interest as biomaterials for vaccine delivery. However, chitosan (CS) is by far the most intensively investigated polysaccharide for antigen delivery. This biodegradable polymer has interesting mucoadhesive and immune modulating properties [156-158] that make it a promising biomaterial for mucosal antigen delivery. In fact, the early recognition of its potential motivated the pioneering work by our group on the development of CS nanoparticles and their use for nasal immunization [138, 159]. These nanoparticles, formed under very mild conditions by the ionotropic gelation of CS with counter-anions (e.g. tripolyphosphate), have a size in the range of 150-450 nm and are specifically adapted to carry significant amounts of antigens. As a proof-of-concept, we have shown that tetanus toxoid-loaded nanoparticles administered intranasally were able to generate higher antibody levels than the soluble antigen, increasing to 10 times higher values after 24 weeks (**Figure 5**). This long-lasting and increasing response revealed the potential of the formulation for needle-free administration. In a further work, it was shown that these particles are internalized by

the nasal mucosa [160], thus suggesting an alternative pathway, via the nasal-associated lymphoid tissue, for the antigen to reach submucosal immunocompetent cells.

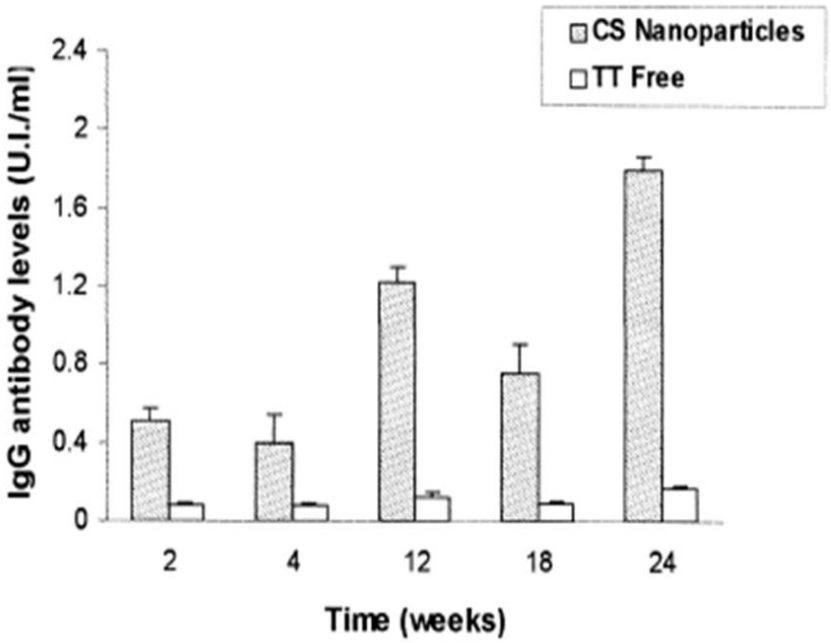

Figure 5. *IgG antibody levels following intranasal administration of TT entrapped in CS nanoparticles (70 kDa) (mean ± SEM, n = 6–9). Reproduced from [161] with permission.*

The promising initial results observed for CS-based nanocarriers has stimulated research intended to further enhance their potential. For example, our group observed that the immunogenicity of CS nanoparticles after the intranasal delivery of diphtheria toxoid could be enhanced using PEGylated chitosan [162]. Other authors have investigated the potential of N-trimethyl chitosan (TMC) in the form of nanoparticles as a delivery vehicle for a influenza subunit antigen following intramuscular administration [163] and also for the model OVA antigen [164]. TMC nanoparticles displayed an interesting adjuvant effect after i.m. injection, but did not show increased immunogenicity after oral administration, as compared to the unmodified CS nanoparticles.

The reliability of chitosan nanoparticles as vaccine delivery vehicles has been further confirmed by our group using the viral protein rHBsAg. Specifically, we observed that rHBsAg-loaded chitosan nanoparticles, with a size of about 180 nm, were able to

control the release of the associated antigen while preserving its integrity [165]. Moreover, following intramuscular administration to mice, these nanoparticles generated immune responses that were higher than those observed for the alum-adjuvanted vaccine. The same nanoparticles have also shown their capacity for generating protective antibody levels following nasal administration. Finally, an additional advantage of this formulation, as compared to the commercial alum-based rHBsAg vaccine, has been found in their possibility to be converted into an easily resuspendable freeze dried powder.

The promising results achieved with these chitosan-based nanoparticles together with the solid antibody titers achieved previously with o/w emulsions, recently motivated the development of a new type of system called chitosan nanocapsules. The rationale for the application of this system in immunization is to take advantage of a potential synergistic effect of an oily core and the chitosan coating. Moreover, the core-shell structure of these nanocapsules permits the co-association of a hydrophobic immunopotentiator (within the core) and a hydrosoluble protein antigen (in the polymer coating). These versatile nanocapsules have been loaded with rHBsAg and administered either intranasally or intramuscularly. The results have provided evidence of their potential either as a needle-free (nasal) vaccine delivery strategy or as a single-dose parenteral formulation [166, 167].

In summary, a number of recent reports illustrate the potential of chitosan nanocarriers as antigen delivery systems. They pave the way for subsequent optimization approaches, which will be mainly determined by the nature of the specific antigen to be delivered.

5. CONCLUDING REMARKS

The limitations of present vaccines have prompted the investigation of novel vaccine delivery technologies with improved efficacy, safety and stability. Among the great number of different strategies under exploration, particulate delivery carriers have emerged as strong and promising candidates. Besides improving antigen stability and its presentation to immunocompetent cells, depending on their specific characteristics (composition, size and surface properties), these vehicles can also offer the ability to overcome biological barriers and provide controlled release of the antigen, properties

that are fundamental for the development of needle-free and/or single dose vaccine formulations.

Despite the intense efforts to develop more efficient vaccines based on the application of micro- and nanometric vaccine delivery systems, there is still a relatively low number of such products in clinical use. The most advanced antigen nanocarriers are those composed on lipid materials containing immunostimulatory molecules. Nevertheless, judged by the large number of recent publications related to this field and the encouraging clinical results reported so far, it is reasonable to presume that, novel vaccine delivery systems will be increasingly applied in the near future, hopefully leading to major improvements in worldwide immunization coverage.

REFERENCES

1. Zepp, F., *Principles of vaccine design--Lessons from nature.* Vaccine, 2010. **28**(Supplement 3): p. C14-C24.

2. Smith, J., et al., *Certification of polio eradication: process and lessons learned.* Bulletin of the World Health Organization, 2004. **82**: p. 24-30.

3. Lake, R.A. and B.W.S. Robinson, *Immunotherapy and chemotherapy - a practical partnership.* Nat Rev Cancer, 2005. **5**(5): p. 397-405.

4. Larche, M. and D.C. Wraith, *Peptide-based therapeutic vaccines for allergic and autoimmune diseases.* Nat Med 11, 2005: p. S69 - S76.

5. Gonzalez, G., et al., *CIMAvax-EGF: A novel therapeutic vaccine for advanced lung cancer.* Biotecnol. Apl., 2009. **26**: p. 345-348.

6. Oxman, M.N., et al., *A Vaccine to Prevent Herpes Zoster and Postherpetic Neuralgia in Older Adults.* N Engl J Med, 2005. **352**(22): p. 2271-2284.

7. Peek, L.J., C.R. Middaugh, and C. Berkland, *Nanotechnology in vaccine delivery.* Adv. Drug Delivery Rev., 2008. **60**(8): p. 915-928.

8. Moser, M. and O. Leo, *Key concepts in immunology.* Vaccine, 2010. **28, 3**(0): p. C2-C13.

9. Matzinger, P., *Tolerance, danger, and the extended family.* Annu Rev Immunol, 1994. **12**: p. 991-1045.

10. Wack, A. and R. Rappuoli, *Vaccinology at the beginning of the 21st century.* Curr. Opin. Immunol., 2005. **17**(4): p. 411-418.

11. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-252.

12. Casares, S., T.-D. Brumeanu, and T.L. Richie, *The RTS,S malaria vaccine.* Vaccine, 2010. **28**(31): p. 4880-4894.

13. Reed, S.G., et al., *New horizons in adjuvants for vaccine development.* Trends Immunol., 2009. **30**(1): p. 23-32.

14. Bramwell, V.W. and Y. Perrie, *Particulate Delivery Systems for Vaccines.* Crit Rev Ther Drug Carrier Syst., 2005. **22**(2): p. 151-214.

15. Basith, S., et al., *Toll-like receptor modulators: a patent review (2006-2010).* Expert Opin. Ther. Pat., 2011. **21**(6): p. 927-944.

16. Giudice, E.L. and J.D. Campbell, *Needle-free vaccine delivery.* Adv. Drug Delivery Rev., 2006. **58**(1): p. 68-89.

17. Neutra, M.R. and P.A. Kozlowski, *Mucosal vaccines: the promise and the challenge.* Nat. Rev. Immunol., 2006. **6**(2): p. 148-158.

18. Aguado, M.T., *Future approaches to vaccine development: single-dose vaccines using controlled-release delivery systems.* Vaccine, 1993. **11**(5): p. 596-7.

19. Eldridge, J.H., et al., *Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies.* Infect. Immun., 1991. **59**(9): p. 2978-2986.

20. Igartua, M., et al., *Enhanced immune response after subcutaneous and oral immunization with biodegradable PLGA microspheres.* J. Controlled Release, 1998. **56**(1-3): p. 63-73.

21. Gutierro, I., et al., *Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres.* Vaccine, 2002. **21**(1-2): p. 67-77.

22. Oyewumi, M.O., A. Kumar, and Z. Cui, *Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses.* Expert Rev. Vaccines, 2010. **9**(9): p. 1095-1107.

23. Shakweh, M., G. Ponchel, and E. Fattal, *Particle uptake by Peyer's patches: a pathway for drug and vaccine delivery.* Expert Opin. Drug Delivery, 2004. **1**(1): p. 141-163.

24. Gómez, S., et al., *Gantrez® AN nanoparticles as an adjuvant for oral immunotherapy with allergens.* Vaccine, 2007. **25**(29): p. 5263-5271.

25. Tobío, M., et al., *The role of PEG on the stability in digestive fluids and in vivo fate of PEG-PLA nanoparticles following oral administration.* Colloids Surf., B, 2000. **18**(3-4): p. 315-323.

26. Csaba, N., M. Garcia-Fuentes, and M.J. Alonso, *Nanoparticles for nasal vaccination.* Adv. Drug Delivery Rev., 2009. **61**(2): p. 140-157.

27. Almeida, A.J., H.O. Alpar, and M.R.W. Brown, *Immune response to nasal delivery of antigenically intact tetanus toxoid associated with poly(L-lactic acid) microspheres in rats, rabbits and guinea pigs.* J. Pharm. Pharmacol., 1993. **45**(3): p. 198-203.

28. Tobío, M., et al., *Stealth PLA-PEG Nanoparticles as Protein Carriers for Nasal Administration.* Pharm. Res., 1998. **15**(2): p. 270-275.

29. Vila, A., et al., *Transport of PLA-PEG particles across the nasal mucosa: effect of particle size and PEG coating density.* J. Controlled Release, 2004. **98**(2): p. 231-244.

30. Vila, A., et al., *PEG-PLA Nanoparticles as Carriers for Nasal Vaccine Delivery.* J. Aerosol Med., 2004. **17**(2): p. 174-185.

31. Kenney, R.T., et al., *Dose Sparing with Intradermal Injection of Influenza Vaccine.* N. Engl. J. Med., 2004. **351**(22): p. 2295-2301.

32. Vogt, A., et al., *40 nm, but not 750 or 1,500 nm, Nanoparticles Enter Epidermal CD1a+ Cells after Transcutaneous Application on Human Skin.* J Invest Dermatol, 2006. **126**(6): p. 1316-1322.

33. Mahe, B., et al., *Nanoparticle-Based Targeting of Vaccine Compounds to Skin Antigen-Presenting Cells By Hair Follicles and their Transport in Mice.* J Invest Dermatol, 2008. **129**(5): p. 1156-1164.

34. Li, N., et al., *Effective transcutaneous immunization by antigen-loaded flexible liposome in vivo.* Int. J. Nanomed., 2011a. **6**(1): p. 3241-3250

35. Gupta, P.N., et al., *Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study.* Int. J. Pharm., 2005. **293**(1-2): p. 73-82.

36. Combadiére, B. and B. Mahé, *Particle-based vaccines for transcutaneous vaccination.* Comp. Immunol. Microbiol. Infect. Dis., 2008. **31**(2-3): p. 293-315.

37. Li, N., et al., *Transcutaneous vaccines: Novel advances in technology and delivery for overcoming the barriers.* Vaccine, 2011b. **29**(37): p. 6179-6190.

38. Swartz, M.A., *The physiology of the lymphatic system.* Adv. Drug Delivery Rev., 2001. **50**(1-2): p. 3-20.

39. Cubas, R., et al., *Virus-like Particle (VLP) Lymphatic Trafficking and Immune Response Generation After Immunization by Different Routes.* J. Immunother., 2009. **32**(2): p. 118-128.

40. De Temmerman, M.-L., et al., *Particulate vaccines: on the quest for optimal delivery and immune response.* Drug Discovery Today, 2011. **16**(13-14): p. 569-582.

41. Dane, K.Y., et al., *Nano-sized drug-loaded micelles deliver payload to lymph node immune cells and prolong allograft survival.* J. Controlled Release, 2011. **156**(2): p. 154-160.

42. Moghimi, S.M., et al., *Surface engineered nanospheres with enhanced drainage into lymphatics and uptake by macrophages of the regional lymph nodes.* FEBS Letters, 1994. **344**(1): p. 25-30.

43. Reddy, S.T., et al., *Exploiting lymphatic transport and complement activation in nanoparticle vaccines.* Nat Biotech, 2007. **25**(10): p. 1159-1164.

44. Fifis, T., et al., *Size-Dependent Immunogenicity: Therapeutic and Protective Properties of Nano-Vaccines against Tumors.* J. Immunol., 2004. **173**(5): p. 3148-3154.

45. Manolova, V., et al., *Nanoparticles target distinct dendritic cell populations according to their size.* Eur. J. Immunol., 2008. **38**(5): p. 1404-1413.

46. Thiele, L., H.P. Merkle, and E. Walter, *Phagocytosis and phagosomal fate of surface-modified microparticles in dendritic cells and macrophages.* Pharm. Res., 2003. **20**(2): p. 221-228.

47. Foged, C., et al., *Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model.* Int.J.Pharm., 2005. **298**(2): p. 315-322.

48. Bachmann, M.F. and G.T. Jennings, *Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns.* Nat Rev Immunol, 2010. **10**(11): p. 787-796.

49. O'Hagan, D.T. and M. Singh, *Microparticles as vaccine adjuvants and delivery systems.* Expert Rev. Vaccines, 2003. **2**(2): p. 269-283.

50. Scheerlinck, J.P.Y. and D.L.V. Greenwood, *Virus-sized vaccine delivery systems.* Drug Discovery Today, 2008. **13**(19-20): p. 882-887.

51. Kovacsovics-Bankowski, M., et al., *Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages.* Proc. Natl. Acad. Sci. U. S. A., 1993. **90**(11): p. 4942-4946.

52. Manning, M., et al., *Stability of Protein Pharmaceuticals: An Update.* Pharm. Res., 2009. **27**(4): p. 544-575.

53. Pawar, R., A. Ben-Ari, and A.J. Domb, *Protein and peptide parenteral controlled delivery.* Expert Opin. Biol. Ther., 2004. **4**(8): p. 1203-1212.

54. Allémann, E., J.C. Leroux, and R. Gurny, *Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics.* Adv. Drug Delivery Rev., 1998. **34**(2-3): p. 171-189.

55. Preis, I. and R.S. Langer, *A single-step immunization by sustained antigen release.* J Immunol Methods, 1979. **28**(1-2): p. 193-7.

56. Alonso, M.J., et al., *Determinants of Release Rate of Tetanus Vaccine from Polyester Microspheres.* Pharm. Res., 1993. **10**(7): p. 945-953.

57. Kissel, T., et al., *Parenteral protein delivery systems using biodegradable polyesters of ABA block structure, containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethylene oxide) B blocks.* J. Control. Rel., 1996. **39**(2): p. 315-326.

58. Johansen, P., et al., *Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination.* Eur. J. Pharm Biopharm., 2000. **50**(1): p. 129-146.

59. Blanco, M.D. and M.J. Alonso, *Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres.* Eur. J. Pharm. Biopharm., 1997. **43**(3): p. 287-294.

60. Tobío, M. and M.J. Alonso, *Study of the inactivation process of the tetanus toxoid in contact with poly(lactic/glycolic acid) degrading microspheres.* S.T.P. Pharma Sciences, 1998. **8**(5): p. 303-310.

61. Fu, K., A.M. Klibanov, and R. Langer, *Protein stability in controlled-release systems.* Nat Biotech, 2000. **18**(1): p. 24-25.

62. Fu, K., et al., *Visual Evidence of Acidic Environment Within Degrading Poly(lactic-co-glycolic acid) (PLGA) Microspheres.* Pharm. Res., 2000. **17**(1): p. 100-106.

63. Pashine, A., N.M. Valiante, and J.B. Ulmer, *Targeting the innate immune response with improved vaccine adjuvants.* Nat. Med., 2005. **11**(4): p. S63 - S68.

64. HogenEsch, H., *Mechanisms of stimulation of the immune response by aluminum adjuvants.* Vaccine, 2002. **20, 3**(0): p. S34-S39.

65. Marrack, P., A.S. McKee, and M.W. Munks, *Towards an understanding of the adjuvant action of aluminium.* Nat Rev Immunol, 2009. **9**(4): p. 287-293.

66. Vogel, F.R., *Improving Vaccine Performance with Adjuvants.* Clin. Infect. Dis., 2000. **30**(Supplement 3): p. S266-S270.

67. Gupta, R.K. and B.E. Rost, *Aluminum Compounds as Vaccine Adjuvants.* Vaccine Adjuvants : Preparation Methods and Research Protocols Vol.42, 2000: p. 65-89.

68. Zapata, M.I., et al., *Mechanism of freeze-thaw instability of aluminum hydroxycarbonate and magnesium hydroxide gels.* J. Pharm. Sci., 1984. **73**(1): p. 3-8.

69. Casals, J. and J. Freund, *Sensitization and Antibody Formation in Monkeys Injected with Tubercle Bacilli in Paraffin Oil.* J. Immunol., 1939. **36**(5): p. 399-404.

70. Stuart-Harris, C.H., *Adjuvant influenza vaccines.* Bull World Health Organ, 1969. **41**(3): p. 617-21.

71. Vinageras, E.N., et al., *Phase II Randomized Controlled Trial of an Epidermal Growth Factor Vaccine in Advanced Non–Small-Cell Lung Cancer.* J. Clin. Oncol., 2008. **26**(9): p. 1452-1458.

72. Leffers, N., et al., *Immunization with a P53 synthetic long peptide vaccine induces P53-specific immune responses in ovarian cancer patients, a phase II trial.* Int. J. Cancer, 2009. **125**(9): p. 2104-2113.

73. Wu, Y., et al., *Phase 1 Trial of Malaria Transmission Blocking Vaccine Candidates Pfs25 and Pvs25 Formulated with Montanide ISA 51.* PLoS ONE, 2008. **3**(7): p. e2636.

74. Lindblad, E.B., *Safety Evaluation of Vaccine Adjuvants.* Vaccine Adjuvants and Delivery Systems, 2006: p. 421-444.

75. Geert, L.R., *Unmet needs in modern vaccinology: Adjuvants to improve the immune response.* Vaccine, 2010. **28, 3**(Supplement 3): p. C25-C36.

76. Ott, G., et al., *The Adjuvant MF59: A 10-Year Perspective.* Methods Mol. Med. Vol.42, 2000. **42**: p. 211-228.

77. Schultze, V., et al., *Safety of MF59(TM) adjuvant.* Vaccine, 2008. **26**(26): p. 3209-3222.

78. Fox, C.B., *Squalene Emulsions for Parenteral Vaccine and Drug Delivery.* Molecules, 2009. **14**(9): p. 3286-3312.

79. Frey, S., et al., *Comparison of the safety, tolerability, and immunogenicity of a MF59-adjuvanted influenza vaccine and a non-adjuvanted influenza vaccine in non-elderly adults.* Vaccine, 2003. **21**(27-30): p. 4234-4237.

80. De Donato, S., et al., *Safety and immunogenicity of MF59-adjuvanted influenza vaccine in the elderly.* Vaccine, 1999. **17**(23): p. 3094-3101.

81. Ott, G., et al., *MF59: Design and evaluation of a safe and potent adjuvant for human vaccines.* Pharm. Biotechnol., 1995. **6**(Vaccine Design): p. 277-96.

82. Persing, D.H., et al., *Taking toll: lipid A mimetics as adjuvants and immunomodulators.* Trends Microbiol., 2002. **10**(10): p. s32-s37.

83. Kensil, C.R., *QS-21 Adjuvant.* Vaccine Adjuvants : Preparation Methods and Research Protocols, 2000. **42**: p. 259-271.

84. Aide, P., et al., *Four year immunogenicity of the RTS,S/AS02A malaria vaccine in Mozambican children during a phase IIb trial.* Vaccine, 2011. **29**(35): p. 6059-6067.

85. Leroux-Roels, I., et al., *Evaluation of the Safety and Immunogenicity of Two Antigen Concentrations of the Mtb72F/AS02A Candidate Tuberculosis Vaccine in Purified Protein Derivative-Negative Adults.* Clin. Vaccine Immunol., 2010. **17**(11): p. 1763-1771.

86. Leroux-Roels, I., et al., *Strong and persistent CD4+ T-cell response in healthy adults immunized with a candidate HIV-1 vaccine containing gp120, Nef and Tat antigens formulated in three Adjuvant Systems.* Vaccine, 2010. **28**(43): p. 7016-7024.

87. Garcia-Sicilia, J., et al., *Immunogenicity and safety of AS03-adjuvanted H1N1 pandemic vaccines in children and adolescents.* Vaccine, 2011. **29**(26): p. 4353-4361.

88. Roman, F., et al., *Immunogenicity and safety in adults of one dose of influenza A H1N1v 2009 vaccine formulated with and without AS03A-adjuvant: Preliminary report of an observer-blind, randomised trial.* Vaccine, 2010. **28**(7): p. 1740-1745.

89. Usonis, V., et al., *Antibody titres after primary and booster vaccination of infants and young children with a virosomal hepatitis A vaccine (Epaxal).* Vaccine, 2003. **21**(31): p. 4588-92.

90. Gluck, R. and I.C. Metcalfe, *New technology platforms in the development of vaccines for the future.* Vaccine, 2002. **20, 5**(Supplement 5): p. B10-B16.

91. Esposito, S., et al., *Pandemic influenza A/H1N1 vaccine administered sequentially or simultaneously with seasonal influenza vaccine to HIV-infected children and adolescents.* Vaccine, 2011. **29**(8): p. 1677-1682.

92. Girard, M.P., et al., *Report of the 7th meeting on Evaluation of Pandemic Influenza Vaccines in Clinical Trials, World Health Organization, Geneva, 17-18 February 2011.* Vaccine, 2011. **29**(44): p. 7579-7586.

93. Chu, D.W.-S., et al., *Immunogenicity and tolerability of an AS03A-adjuvanted prepandemic influenza vaccine: A phase III study in a large population of Asian adults.* Vaccine, 2009. **27**(52): p. 7428-7435.

94. Morel, S., et al., *Adjuvant System AS03 containing [alpha]-tocopherol modulates innate immune response and leads to improved adaptive immunity.* Vaccine, 2011. **29**(13): p. 2461-2473.

95. , 2011, Last visited at October 2011. U.S. National Institutes of Health.

96. Zasadzinski, J.A., et al., *Novel methods of enhanced retention in and rapid, targeted release from liposomes.* Curr. Opin. Colloid Interface Sci., 2011. **16**(3): p. 203-214.

97. Altin, J.G. and C.R. Parish, *Liposomal vaccines--targeting the delivery of antigen.* Methods, 2006. **40**(1): p. 39-52.

98. Rao, M., et al., *Visualization of peptides derived from liposome-encapsulated proteins in the trans-Golgi area of macrophages.* Immunol. Lett., 1997. **59**(2): p. 99-105.

99. Kedar, E., et al., *Delivery of Cytokines by Liposomes: Hematopoietic and Immunomodulatory Activity of Interleukin-2 Encapsulated in Conventional Liposomes and in Long-Circulating Liposomes.* J. Immunother., 2000. **23**(1): p. 131-145.

100. Sprott, G.D., et al., *Liposome adjuvants prepared from the total polar lipids of Haloferax volcanii, Planococcus spp. and Bacillus firmus differ in ability to elicit and sustain immune responses.* Vaccine, 2004. **22**(17-18): p. 2154-2162.

101. Heurtault, B., B. Frisch, and F. Pons, *Liposomes as delivery systems for nasal vaccination: strategies and outcomes.* Expert Opin. Drug Deliv. , 2010. **7**(7): p. 829-844.

102. Leserman, L., *Liposomes as Protein Carriers in Immunology.* J. Liposome Res., 2004. **14**(3-4): p. 175-189.

103. Garçon, N. and M. Van Mechelen, *Recent clinical experience with vaccines using MPL- and QS-21-containing Adjuvant Systems.* Expert Rev. Vaccines, 2011. **10**(4): p. 471-486.

104. Gluck, R., *Adjuvant activity of immunopotentiating reconstituted influenza virosomes (IRIVs).* Vaccine, 1999. **17**(13-14): p. 1782-1787.

105. De Miguel, I., et al., *Proofs of the Structure of Lipid Coated Nanoparticles (SMBV ^{TM}) Used as Drug Carriers.* Pharm. Res., 2000. **17**(7): p. 817-824.

106. Debin, A., et al., *Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses.* Vaccine, 2002. **20**(21-22): p. 2752-2763.

107. Stephenson, I., et al., *Phase I Evaluation of Intranasal Trivalent Inactivated Influenza Vaccine with Nontoxigenic Escherichia coli Enterotoxin and Novel*

Biovector as Mucosal Adjuvants, Using Adult Volunteers. J. Virol., 2006. **80**(10): p. 4962-4970.

108. Pearse, M.J. and D. Drane, *ISCOMATRIX® adjuvant for antigen delivery.* Adv. Drug Delivery Rev., 2005. **57**(3): p. 465-474.

109. Lovgren, K. and B. Morein, *The requirement of lipids for the formation of immunostimulating complexes (iscoms).* Biotechnol. Appl. Biochem., 1988. **10**(2): p. 161-172.

110. Schnurr, M., et al., *Tumor antigen processing and presentation depend critically on dendritic cell type and the mode of antigen delivery.* Blood, 2005. **105**(6): p. 2465-2472.

111. Duewell, P., et al., *ISCOMATRIX Adjuvant Combines Immune Activation with Antigen Delivery to Dendritic Cells In Vivo Leading to Effective Cross-Priming of CD8+ T Cells.* J. Immunol., 2011. **vol. 187 no. 1 55-63**.

112. Glauert, A.M., J.T. Dingle, and J.A. Lucy, *Action of Saponin on Biological Cell Membranes.* Nature, 1962. **196**(4858): p. 953-955.

113. Skene, C.D. and P. Sutton, *Saponin-adjuvanted particulate vaccines for clinical use.* Methods, 2006. **40**(1): p. 53-59.

114. Sjölander, A., et al., *Immune responses to ISCOM® formulations in animal and primate models.* Vaccine, 2001. **19**(17-19): p. 2661-2665.

115. Hu, K.F., K. Lövgren-Bengtsson, and B. Morein, *Immunostimulating complexes (ISCOMs) for nasal vaccination.* Adv. Drug Delivery Rev., 2001. **51**(1-3): p. 149-159.

116. Waite, D.C., et al., *Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21.* Vaccine, 2001. **19**(28-29): p. 3957-3967.

117. Scifinder®. *Chemical Abstracts Service: Columbus, OH, 2011 RN 58-08-2 (accessed October 10, 2011).* 2011 10 October 2011 [cited 2011 October];

Available from: https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf.

118. Birrenbach, G. and P.P. Speiser, *Polymerized micelles and their use as adjuvants in immunology.* J. Pharm. Sci., 1976. **65**(12): p. 1763-1766.

119. Kenley, R.A., et al., *Poly(lactide-co-glycolide) decomposition kinetics in vivo and in vitro.* Macromolecules, 1987. **20**(10): p. 2398-2403.

120. Wise, D.L., et al., *Opportunities and challenges in the design of implantable biodegradable polymeric systems for the delivery of antimicrobial agents and vaccines.* Adv. Drug Delivery Rev., 1987. **1**(1): p. 19-40.

121. Aguado, M.T. and P.H. Lambert, *Controlled-Release Vaccines-Biodegradable Polylactide/Polyglycolide (PL/PG) Microspheres as Antigen Vehicles.* Immunobiology, 1992. **184**(2â€“3): p. 113-125.

122. Gupta, R.K., et al., *In vivo distribution of radioactivity in mice after injection of biodegradable polymer microspheres containing 14C-labeled tetanus toxoid.* Vaccine, 1996. **14**(15): p. 1412-1416.

123. Alonso, M.J., et al., *Biodegradable microspheres as controlled-release tetanus toxoid delivery systems.* Vaccine, 1994. **12**(4): p. 299-306.

124. Alonso, M.J., et al., *Determinants of Release Rate of Tetanus Vaccine from Polyester Microspheres.* Pharmaceutical Research, 1993. **10**(7): p. 945-953.

125. Men, Y., et al., *A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide.* Vaccine, 1995. **13**(7): p. 683-689.

126. Sanchez, A., et al., *Pulsed controlled-release system for potential use in vaccine delivery.* J. Pharm. Sci., 1996. **85**(6): p. 547-552.

127. Tobío, M., et al., *A Novel System Based on a Poloxamer/ PLGA Blend as a Tetanus Toxoid Delivery Vehicle.* Pharm. Res., 1999. **16**(5): p. 682-688.

128. Ramya, R., et al., *Poly(lactide-co-glycolide) microspheres: A potent oral delivery system to elicit systemic immune response against inactivated rabies virus.* Vaccine, 2009. **27**(15): p. 2138-2143.

129. Murillo, M., et al., *Polyester Microparticles as a Vaccine Delivery System for Brucellosis: Influence of the Polymer on Release, Phagocytosis and Toxicity.* J. Drug Targeting, 2002. **10**(3): p. 211-219.

130. Aparecida, d.S.S., et al., *A subunit vaccine based on biodegradable microspheres carrying rHsp65 protein and KLK protects BALB/c mice against tuberculosis infection.* Hum. Vaccines, 2010. **6**(12): p. 1047-1053.

131. Carcaboso, A.M., et al., *Immune response after oral administration of the encapsulated malaria synthetic peptide SPf66.* Int. J. Pharm., 2003. **260**(2): p. 273-282.

132. Saini, V., et al., *Comparison of humoral and cell-mediated immune responses to cationic PLGA microspheres containing recombinant hepatitis B antigen.* Int.J.Pharm., 2011. **408**(1-2): p. 50-57.

133. Fischer, S., et al., *One-step preparation of polyelectrolyte-coated PLGA microparticles and their functionalization with model ligands.* J. Controlled Release, 2006. **111**(1-2): p. 135-144.

134. Gomez, J.M.M., et al., *Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis.* J. Controlled Release, 2008. **130**(2): p. 161-167.

135. Sharp, F.A., et al., *Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome.* Proc. Natl. Acad. Sci. U. S. A., 2009. **106**(3): p. 870-875.

136. Fischer, S., et al., *Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response.* J. Drug Targeting, 2009. **17**(8): p. 652-661.

137. Kazzaz, J., et al., *Encapsulation of the immune potentiators MPL and RC529 in PLG microparticles enhances their potency.* J. Controlled Release, 2006. **110**(3): p. 566-573.

138. Vila, A., et al., *Design of biodegradable particles for protein delivery.* J. Controlled Release, 2002. **78**(1-3): p. 15-24.

139. Brooking, J., S.S. Davis, and L. Illum, *Transport of Nanoparticles Across the Rat Nasal Mucosa.* J. Drug. Target., 2001. **9**(4): p. 267-279.

140. Hillery, A.M. and A.T. Florence, *The effect of adsorbed poloxamer 188 and 407 surfactants on the intestinal uptake of 60-nm polystyrene particles after oral administration in the rat.* Int. J. Pharma., 1996. **132**(1-2): p. 123-130.

141. Csaba, N., et al., *PLGA:Poloxamer and PLGA:Poloxamine blend nanoparticles: new carriers for gene delivery.* Biomacromolecules, 2005. **6**(1): p. 271-278.

142. Csaba, N., et al., *Design and characterisation of new nanoparticulate polymer blends for drug delivery.* J Biomater Sci Polym Ed, 2004. **15**(9): p. 1137-51.

143. Csaba, N., A. Sanchez, and M.J. Alonso, *PLGA: Poloxamer and PLGA: Poloxamine blend nanostructures as carriers for nasal gene delivery.* J. Controlled Release, 2006. **113**(2): p. 164-172.

144. Paolicelli, P., et al., *Surface-modified PLGA-based nanoparticles that can efficiently associate and deliver virus-like particles.* Nanomedicine, 2010. **5**(6): p. 843-853.

145. Garinot, M., et al., *PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination.* J. Controlled Release, 2007. **120**(3): p. 195-204.

146. Gupta, P.N., et al., *M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B.* J. Drug Targeting, 2007. **15**(10): p. 701-713.

147. Ataman-Onal, Y., et al., *Surfactant-free anionic PLA nanoparticles coated with HIV-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models.* J. of Controlled Release, 2006. **112**(2): p. 175-185.

148. Paolicelli, P., et al., *Surface-modified PLGA-based nanoparticles that can efficiently associate and deliver virus-like particles.* Nanomedicine, 2010. **5**(6): p. 843-53.

149. Moon, J.J., et al., *Antigen-Displaying Lipid-Enveloped PLGA Nanoparticles as Delivery Agents for a Plasmodium vivax Malaria Vaccine.* PLoS ONE, 2012. **7**(2): p. e31472.

150. Arbós, P., et al., *Quantification of the bioadhesive properties of protein-coated PVM/MA nanoparticles.* Int.J.Pharm., 2002. **242**(1-2): p. 129-136.

151. Tamayo, I., et al., *Poly(Anhydride) Nanoparticles Act as Active Th1 Adjuvants through Toll-Like Receptor Exploitation.* Clin. Vaccine Immunol., 2010. **17**(9): p. 1356-1362.

152. Gómez, S., et al., *Development of a Novel Vaccine Delivery System Based on Gantrez Nanoparticles.* J. Nanosci. Nanotechnol., 2006. **6**(9-10): p. 3283-3289.

153. Layton, R.C., et al., *Delta inulin polysaccharide adjuvant enhances the ability of split-virion H5N1 vaccine to protect against lethal challenge in ferrets.* Vaccine, 2011. **29**(37): p. 6242-6251.

154. Mata, E., et al., *Enhancing immunogenicity to PLGA microparticulate systems by incorporation of alginate and RGD-modified alginate.* Eur. J. Pharm. Sci., 2011. **44**(1-2): p. 32-40.

155. Verheul, R.J., et al., *Covalently stabilized trimethyl chitosan-hyaluronic acid nanoparticles for nasal and intradermal vaccination.* J. Controlled Release, 2011. **156**(1): p. 46-52.

156. Nishimura, K., et al., *Adjuvant activity of chitin derivatives in mice and guinea-pigs.* Vaccine, 1985. **3**(5): p. 379-384.

157. Marchand, C., et al., *C3, C5, and factor B bind to chitosan without complement activation.* J. Biomed. Mater. Res., Part A, 2009. **93A**(4): p. 1429-1441.

158. Gorzelanny, C., et al., *Human macrophage activation triggered by chitotriosidase-mediated chitin and chitosan degradation.* Biomaterials, 2010. **31**(33): p. 8556-8563.

159. Calvo, P., et al., *Chitosan and Chitosan/Ethylene Oxide-Propylene Oxide Block Copolymer Nanoparticles as Novel Carriers for Proteins and Vaccines.* Pharm. Res., 1997. **14**(10): p. 1431-1436.

160. Vila, A., et al., *Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice.* Eur. J. Pharm. Sci., 2004. **57**(1): p. 123-131.

161. Vila, A., et al., *Design of biodegradable particles for protein delivery.* Journal of Controlled Release, 2002. **78**(1–3): p. 15-24.

162. Rezaei, M.A. and M.J. Alonso, *Preparation and evaluation of chitosan nanoparticles containing diphtheria toxoid as new carriers for nasal vaccine delivery in mice.* Arch. Razi Inst., 2006. **61**(1): p. 13-25.

163. Amidi, M., et al., *N-Trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model.* Vaccine, 2007. **25**(1): p. 144-153.

164. Sluetter, B., et al., *Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination.* J. Controlled Release, 2009. **138**(2): p. 113-121.

165. Prego, C., et al., *Chitosan-based nanoparticles for improving immunization against hepatitis B infection.* Vaccine, 2010. **28**(14): p. 2607-2614.

166. Vicente, S., et al., *Polysaccharide-based nanocapsules as vehicles for nasal immunization against hepatitis B*, in *2nd Pharm. Sci. Fair*2009: Nice, France.

167. Vicente, S., et al., *Adjuvant formulations based on polysaccharidic nanocapsules as potential single-dose vaccines*, 2010: Seattle.

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ANTECEDENTES, HIPÓTESIS Y OBJETIVOS

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ANTECEDENTES

1. La nanotecnología se presenta como una estrategia prometedora en el desarrollo de nuevas vacunas, denominadas nanovacunas, destinadas a prevenir enfermedades para las cuales aun no existe una vacuna efectiva o para mejorar aquéllas que ya se encuentran en el mercado. Estas nanovacunas pueden ofrecer interesantes ventajas, tales como: i) proteger el antígeno de factores de degradación externos; ii) incrementar la interacción del antígeno con las células del sistema inmune; iii) permitir la interacción simultánea del antígeno e inmunoestimulantes con las células inmunes a fin de potenciar la respuesta inmune; iv) mejorar el transporte del antígeno a través de barreras mucosas, permitiendo así la administración sin agujas [1].

2. Existen determinados biopolímeros tales como los poli(aminoácidos) (protamina y poliarginina) y los polisacáridos (quitosano y sulfato de dextrano) que ofrecen un especial atractivo en el diseño de sistemas de liberación de vacunas debido a una serie de propiedades, y en particular, por su capacidad para i) organizarse formando diferentes nanoestructuras; ii) promover la translocación de estas estructuras a través de membranas [2, 3] y iii) estimular el sistema inmune [2, 4, 5].

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[1] **Correia-Pinto, J.F. et al.** *Vaccine delivery carriers: insights and future perspectives.* Int J Pharm, 2013. **440**(1): p. 27-38.

[2] **Martínez Gómez, J.M. et al.** *Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis.* Journal of Controlled Release, 2008. 130 (2): p. 161-167.

[3] **Lozano, M.V. et al.** *Polyarginine nanocapsules: a new platform for intracellular drug delivery.* Journal of Nanoparticle Research, 2013. **15**(3): p. 1-14.

[4] **Kerkmann, M., et al.** *Immunostimulatory properties of CpG-oligonucleotides are enhanced by the use of protamine nanoparticles*. Oligonucleotides, 2006. 16(4): p. 313-22.

[5] **Mattner, F., et al.** *Vaccination with Poly-l-Arginine As Immunostimulant for Peptide Vaccines: Induction of Potent and Long-Lasting T-Cell Responses against Cancer Antigens*. Cancer Research, 2002. 62(5): p. 1477-1480.

3. Existen determinados aceites y materiales lipídicos de interés en la formulación de vacunas debido a su demostrada capacidad adyuvante, como es el escualeno, aceite utilizado en nanoemulsiones adyuvantes presentes en el mercado [6], el ácido linoléico [7, 8] y la lisofosfatidilcolina.

4. La amplia variedad de nanotecnologías disponibles hacen factible la co-asociación de diversos biomateriales de naturaleza polimérica y oleosa, junto con uno o varios antígenos y moléculas immunomoduladoras.

[10] **Fox, C.B. et al.** *Squalene Emulsions for Parenteral Vaccine and Drug Delivery.* Molecules, 2009. 14 (9): p. 3286-3312.

[11] **Mena, J. et al.** *Linoleic acid increases adhesion, chemotaxis, granule release, intracellular calcium mobilisation, MAPK phosphorylation and gene expression in bovine neutrophils.* Veterinary Immunology and Immunopathology, 2013. **151**(3–4): p. 275-284.

[12] **Perrin-Cocon, L. et al.** *Lysophosphatidylcholine is a natural adjuvant that initiates cellular immune responses.* Vaccine, 2006. **24**(9): p. 1254-63

HIPÓTESIS

1. El recubrimiento de los antígenos proteicos particulados mediante polímeros con propiedades bioadhesivas y/o inmunoadyuvantes, tales como la protamina, poliarginina, sulfato de dextrano, alginato y el poly (I:C), pueden mejorar el transporte del antígeno a través de barreras mucosas, favorecer la interacción con las células inmunes y potenciar la respuesta de estas células contra el antígeno asociado.

2. La asociación de antígenos peptídicos, poco inmunogénicos, junto con moléculas inmunoestimulantes tales como el poly (I:C), a nanopartículas basadas en el biopolímero quitosano pueden permitir la eficiente co-liberación de ambos en las células inmunes, siendo así capaz de inducir respuestas inmunes sinérgicas y efectivas.

3. La asociación de un conjunto biomateriales adyuvantes, tales como la lisofosfatidilcolina, el ácido linoléico, el escualeno y el quitosano, en la forma de estructuras nanométricas tipo nanoemulsión y nanocápsulas, permite optimizar y combinar las propiedades inmunoadyuvantes de estas moléculas a la vez que aportar una adecuada protección y presentación del antígeno asociado a las mismas.

OBJECTIVOS

Teniendo en cuenta los antecedentes expuestos y las hipótesis planteadas, el objetivo global de la presente tesis doctoral ha sido el diseño y desarrollo de diversos sistemas nanométricos destinados a actuar como adyuvantes de vacunas. Estos nanosistemas están constituidos a partir de biomateriales con propiedades adyuvantes intrínsecas, a fin de potenciar y modular una respuesta inmune especifica frente a antígenos particulados y peptídicos tras su administración por vía parenteral y/o nasal. Para lograr este objetivo global se han planteado los siguientes objetivos experimentales:

Diseño y desarrollo de nanovacunas basadas en el recubrimiento de proteínas virales particuladas:

Partiendo del antígeno de la hepatitis, el objetivo ha sido el de recubrirlo con diferentes biopolímeros con propiedades bioadhesivas e inmunoadyuvantes tales como la protamina, poliarginina, sulfato de dextrano, alginato y el poly (I:C), formando una o múltiples capas a fin de dotarlo de diferentes propiedades. Los sistemas resultantes han sido caracterizados en cuanto a sus propiedades fisicoquímicas, estabilidad y actividad biológica por medio de ensayos *in vitro* e *in vivo*. Estos resultados aparecen recogidos en el capítulo 3 de la presente memoria.

Diseño y desarrollo de sistemas nanométricos como vehículos transportadores de antígenos peptídicos

En este apartado se han seguido dos estrategias:

1. Diseño y desarrollo de nanoestructuras a base de quitosano capaces de co-asociar uno o varios antígenos peptídicos de baja capacidad inmunogénica junto con otros agentes inmunoestimulantes tales como el poly (I:C). Los sistemas resultantes han sido caracterizados en cuanto a sus propiedades fisicoquímicas, estabilidad y actividad biológica por medio de ensayos *in vitro* e *in vivo*. Estos resultados aparecen recogidos en el capítulo 4 de la presente memoria.

2. Diseño y desarrollo de nanoemulsiones y nanocápsulas basadas en lípidos con propiedades inmunoadyuvantes (lisofosfatidilcolina, ácido linoléico y escualeno) y

quitosano capaces de incrementar la interacción de antígenos peptídicos con las células inmunes y provocar la activación de las mismas. Los sistemas resultantes han sido caracterizados en cuanto a sus propiedades fisicoquímicas, estabilidad y actividad biológica mediante ensayos *in vitro* e *in vivo*. Estos resultados aparecen recogidos en el capítulo 5 de la presente memoria.

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CAPÍTULO 3

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CAPÍTULO 3

MULTI-ENVELOPING OF PARTICULATED ANTIGENS WITH BIOPOLYMERS AND IMMUNOSTIMULANT POLYNUCLEOTIDES

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

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ABSTRACT

The main objective of this work was to study the possibility of enveloping particulated antigens using biomaterials and polynucleotides with immunostimulant properties, and to assess the value of this potential adjuvant strategy. The nanoparticulated structure (22 nm) of the recombinant Hepatitis B surface antigen (rHBsAg) virus-like particle (VLP) has inspired us to use it as a substrate in the design of multi-enveloped nanoparticles. Our approach has relied on the adsorption of polymers and immunostimulants by electrostatic interactions. For the first layer, and taking into account the negative charge of the rHBsAg, we studied its interaction with the positively charged polymers protamine (PR) and polyarginine (PARG). These polymer-coated antigen nanostructures were further enveloped using negatively charged polymers such as dextran sulfate (DS) and alginate (ALG), as well as the immunostimulant polynucleotide poly (I:C) (pIC). The combination of these materials, allowed us to design four different prototypes of multi-enveloped nanoparticles (HB:PR; HB:PR:DS; HB:PARG:ALG; HB:PARG:pIC) with very small sizes (< 50 nm). The inversion of the surface charge observed upon the sequential adsorption of the macromolecular layers, confirmed the multilayer structure of the nanoparticle. We evaluated selected multi-enveloped antigen nanostructures, i.e. HB:PR:DS and HB:PARG:ALG, with respect to their ability to be internalized by macrophages and also for their capacity to generate humoral immune responses (IgG titers), following either intranasal or intramuscular administration to mice. The results showed that although these novel formulations generated modest IgG titers, the IgG1/IgG2a ratio was more balanced that the one observed for the alum-adsorbed antigen. Overall, here we show how to envelop particulate antigens with immunoadjuvant layers and the impact of this enveloping process for the antigen presentation to the immune system. Beyond the variables investigated in this work, the results highlight the possibility to protect and modify antigens presentation using a simple layer-by-layer approach.

1. INTRODUCTION

Vaccination is the most cost-effective preventive intervention against disease to date [1], and while the majority of the present vaccines are based on attenuated and inactivated whole-cell vaccines, the current tendency is to design vaccines based on subunit antigens. These subunit vaccines are based on specific components of pathogen, and so are less prone to provoke adverse reactions [2]. Among the subunit antigens, virus-like particles (VLP), formed by self-assembled viral proteins, are particularly interesting due to their superior immunogenicity [3, 4]. Notable examples of VLP based vaccines are the present hepatitis B (HB) and the human papillomavirus (HPV) vaccines, which are based on the recombinant hepatitis B surface antigen (rHBsAg) [5] and on the HPV L1 protein, respectively [6]. In addition, other VLP based vaccines, such as RTS,S for malaria and ACAM-FLU-A for Influenza A, are currently in the clinical development stage [7]. Despite their immunogenicity, these particulated antigens still need to be associated to a suitable vaccine adjuvant in order to generate an effective immune response. Most commonly, subunit antigens are adsorbed onto alum salts as adjuvant, due to their long record of safe use and effectiveness [8]. However, these adjuvants present several limitations. The use of alum requires the tight control of the storage conditions, as freezing provokes aggregation and loss of effectiveness [9]. This is a particularly important problem because the maintenance of the global cold chain has significant limitations [10] and can lead to a decreased immunization coverage worldwide [11]. Additionally, the use of alum is limited to parenteral administration, which requires specific training to perform and can cause blood-borne pathogen contamination by the re-use of syringes [12]. Finally, alum salts generate a T_h2 biased immune response and they lack the capacity to stimulate cell-mediated immunity [13].

The use of nanotechnology-based approaches is known to offer opportunities for improving the formulation of vaccines. Specific advantages of the nanoformulations described in the literature include: i) their improved thermostability, as compared to the alum-adjuvanted vaccines [14, 15]; ii) their capacity to facilitate the transport of antigens across mucosal barriers and generate humoral and mucosal responses [15, 16]; iii) their ability to enhance and prolong the immune responses (single-dose vaccination) [17]; iv) their potential to modulate T_h1/T_h2 immune responses [16, 17].

It has been hypothesized that nanocarriers may facilitate antigen presentation to T cells due to their resemblance with virus in terms of size and surface characteristics [18, 19]. These antigen-loaded nanocarriers are captured in the region of administration by local antigen presenting cells (APC), particularly dendritic cells (DCs) [20]. Then, DCs process the antigen while migrating to the lymph nodes through the lymphatic vessels [18]. In the lymph nodes, once the antigen is processed, the DC present individual peptides associated to the major histocompatibility complex (MHC) class II molecules to specific T lymphocytes, triggering the adaptive immune response, with the generation of memory T and B cells [21]. In addition to this peripheral uptake of antigen-loaded nanocarriers by DCs, it has been recently proposed that small nanoparticles (< 100 nm) could target antigens directly to the DCs in the lymph nodes [18, 22, 23]. As the immune cell population in these locations is much more concentrated that in peripheral tissues [24], such strategy could potentially provide a better and higher antigen presentation to immune cells, increasing the immune response and decreasing local reactions to the vaccine [23, 25]. An additional advantage of these ultra-small nanocarriers, as compared to larger particles or alum- based vaccines, relies on the fact that they could be sterilized by filtration.

Despite this information, most of the antigen delivery carriers described until now presents a size larger than 100 nm [26]. Therefore, taking into account this technological gap, our main goal was to rationally design enveloped virus-like particle antigens with a final size less than 50 nm. For this purpose, our approach has been to use the antigen itself as a substrate for the adsorption of polymers and polynucleotides with adjuvant properties. As a model antigen we have chosen the recombinant hepatitis B surface antigen (rHBsAg) in its VLP form [5]. This antigen is formed by the self-assembly of rHBsAg proteins with associated lipids, and presents an average particle diameter of 22 nm with surface negative charge [27]. Because of its negative surface charge, we have selected for the first enveloping layer two different cationic polymers: polyarginine (PARG) and protamine (PR). The second enveloping layer consisted of anionic polysaccharides, such as dextran sulfate (DS) or alginate (ALG), or a synthetic double stranded based RNA, poly (I:C) (pIC).

The rational for the selection of the polymers was as follows. Polyarginine is a cationic polyamino acid, which has been shown to favor the uptake of antigens by antigen presenting cells [28] and, as a consequence, to enhance the T cell responses *in vivo*

against associated antigens [29]. These interesting adjuvant properties have led to its inclusion in a hepatitis C vaccine (HCV), which is currently in clinical trials [30]. On the other hand, Protamine is a mixture of arginine-rich polypeptides, with membrane translocating properties [31], which has also shown the capacity to increase the uptake of both microparticles [32] and nanoparticles by phagocytic cells [31]. With regard to the selection of the negatively charged polysaccharides, dextran sulfate and alginate are both linear anionic polymers exhibiting immunoadjuvant properties. Alginate has been found to activate macrophages [33] inducing the production of pro-inflammatory cytokines [34], possibly through the interaction with Toll like receptors (TLR) [35]. There is also evidence of the capacity of dextran sulfate to increase cell-mediated immunity [36] and antibody responses [37] in different animal models. Finally, the polynucleotide poly (I:C) is an agonist of the Toll like receptor 3 (TLR-3) that is present in dendritic cells. TLR-3 recognizes double strand RNA (dsRNA) associated to viral infection, leading to the cell activation and secretion of interferon alpha/beta, playing an important role in the defense against viruses. Through these mechanisms poly (I:C) can promote cell activation and maturation [38], potentiating both humoral and cellular immune responses [39, 40]. The adsorption of the poly (I:C) onto the enveloped antigen was thought to be particularly important in order to achieve simultaneous delivery of the antigen and the immunostimulant to the immune cells.

In brief, this work describes the development of small nanovaccines (<50 nm) through a multi-enveloping method using HB VLP as model antigen, and their characterization regarding physicochemical properties, uptake by immune cells and *in vivo* capacity to generate an effective immune response to the HB antigen after intramuscular (i.m.) and intranasal (i.n.) administration.

2. MATERIAL AND METHODS

2.1. Materials

Poly-L-arginine hydrochloride with molecular weight of 5 – 15 kDa (P4663), dextran sulfate sodium salt with molecular weight of 9 – 20 kDa (D6924) and Phosphate Buffer saline (PBS) were obtained from Sigma–Aldrich (Madrid, Spain). Alginate with molecular weight of 4kDa was obtained from Danisco (Copenhagen, Denmark). Protamine sulfate was kindly donated by LEO Pharma (Denmark). Polyinosinic-

polycytidylic acid of low molecular weight (0.2 – 1 kB) (Poly(I:C)-LMW) was purchased from Invivogen (San Diego, USA).

The recombinant hepatitis B surface antigen (rHBsAg) was kindly donated by Shantha Biotechnics Ltd. (Hyderabad, India) as an aqueous suspension in PBS with a protein concentration of 1.13 mg/mL.

The 5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA, SE) *single isomer* (Catalogue # C2211) and the Slide-A-Lyzer Dialysis Cassettes, 2K MWCO were purchased from ThermoFisher Scientific (Massachusetts, USA).

The Amicon Ultra-4 mL Centrifugal Filters with 100kDa pore and low-binding regenerated cellulose membrane were purchased to Merck Millipore (Massachusetts, USA).

DMEM (Dulbecco's Modified Eagle Medium) was purchased from Life Technologies Corporation (CA, USA).

2.2. Preparation of multi-enveloped particles using hepatitis B antigen as substrate

First, an aliquot of the antigen suspension diluted with ultrapure water was introduced in an Amicon-4 100 kDa filtration tube and centrifugated at 5,000 rpm for 5 minutes at 15 ºC in a Universal 32 centrifuge (Hettich, Tuttlingen, Germany). After repeating this procedure three times, the antigen was recovered from the filter and diluted to the final concentration of 0.1 mg/ml. This final solution was then used as template for the adsorption of the first polymer layer.

2.2.1. Preparation of HB:PR and HB:PARG – First layer adsorption

A volume of 0.8 ml of protamine or polyarginine solution (0.1 mg/ml) was mixed in a glass tube with 0.8 ml of HB solution (0.1 mg/ml) under magnetic stirring (200 rpm). The magnetic stirring was maintained for 5 minutes and, then, the resulting suspension was incubated for 20 minutes at room temperature in order to allow the complex formation. Afterwards, the formulation was filtered using an Amicon-4 100 kDa and diluted to a final volume of 4 ml with ultrapure water. This ultrafiltration procedure was repeated 3 times and subsequently the concentrated dispersion of the complexes HB:PR or HB:PARG was recovered and diluted to a final HB concentration of 0.1 mg/ml).

2.2.2. Preparation of HB:PR:DS, HB:PARG:ALG and HB:PARG:pIC – Second layer adsorption

A volume of 0.785 ml of dextran sulfate (0.4 mg/ml), alginate (0.4 mg/ml) or poly (I:C) (0.1 mg/ml) solution was mixed in a glass tube with 0.785 ml of HB:PR or HB:PARG formulation (HB at 0.1 mg/ml) under magnetic stirring (200 rpm) to constitute the HB:PR:DS, HB:PARG:ALG or the HB:PARG:pIC formulation, respectively. The formulations were kept under stirring for 5 minutes and then they were incubated at room temperature for 20 minutes. Likewise the first layer, the formulations were filtered with an Amicon-4 100 kDa and diluted to a final volume of 4 ml. This ultrafiltration procedure was repeated three times and subsequently the concentrated dispersion of the complexes HB:PR:DS, HB:PARG:ALG and HB:PARG:pIC was recovered and diluted to a final HB concentration of 0.1 mg/ml. The final theoretical mass ratio for HB:PR:DS, HB:PARG:ALG and HB:PARG:pIC was 1:1:4, 1:1:4 and 1:1:1, respectively.

2.3. Physicochemical characterization

The particle size and polydispersity index (PdI) were evaluated by Dynamic Light Scattering (DLS) and its surface charge (zeta potential) by Electrophoretic Light Scattering (ELS) using a Zetasizer Nano ZS90 (Malvern Instruments, UK). The measurements were performed at 25ºC with a detection angle of 173º, in ultrapure water.

2.4. Determination of protamine and polyarginine adsorption and antigen enveloping efficiency

The protamine and polyarginine adsorption to the HB particles was evaluated by an indirect measurement based on the free polyamino acid in solution (micro BCA (mBCA) protein assay from Pierce (Thermo Fisher Scientific, MA, USA) after incubation with the antigen. The efficiency of adsorption (E.A.%) was calculated based on the difference between the theoretical concentration of the polyamino acid (0.1 mg/ml), and the concentration determined by the mBCA.

The amount of antigen coated by the polymers was determined indirectly using a microtiter plate-based sandwich ELISA kit (Murex HBsAg Version 3, Murex Biotech Ltd.; Dartford, UK). The HB enveloping efficiency (E.E.%) was calculated by the difference between the theoretical amount of antigen and the amount of non-enveloped antigen determined in suspending medium. It was assumed that the enveloped antigen particles with polymers, when incubated in the microwells, would not be recognized by the anti-HB antibodies of ELISA assay.

2.5. Stability of the multi-enveloped particles in aqueous suspension

2.5.1. Stability during storage

The multi-enveloped nanoparticles,HB:PR1:1, HB:PR:DS1:1:4, HB:PARG:ALG1:1:4 and HB:PARG:pIC1:1:1, at HB concentration of 0.1 mg/ml, were stored at 4 ºC for up to a 1 month and then their size and PdI were determined as described in the section 2.3.

2.5.2. Stability upon dispersion in simulated biological medium

The stability of the multi-enveloped nanoparticles HB:PR 1:1, HB:PR:DS 1:1:4. HB:PARG:ALG 1:1:4 and HB:PARG:pIC 1:1:1, at HB concentration of 0.05 mg/ml, in PBS at 37 ºC, was tested for up to 4 hours.

2.6. Preparation of fluorescently labeled HB:PR:DS and HB:PARG:ALG multi-enveloped particles

In order to perform *in vitro* cell uptake studies, HB:PR:DS and HB:PARG:ALG particles were prepared with fluorescently labeled protamine (PRF) and polyarginine (PARGF), respectively. The dye chosen was the 5-carboxy tetramethylrhodamine succinimidyl ester (TAMRA) and the labeling was performed based on the manufacturer's instructions. Briefly, protamine and polyarginine were dissolved in 0.1 M sodium bicarbonate buffer, pH 8.0, at 10 mg/ml concentration. In parallel, TAMRA was dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml. An aliquot of the TAMRA solution (0.1 ml) was poured slowly over 1 ml of the polyamino acid solution under magnetic stirring and left for 1 hour at room temperature. After incubation, the labeled polyamino acid solution was dialyzed in Slide-A-Lyzer Dialysis Cassete 2K MWCO for

48 h, to remove free TAMRA. After dialysis, the labeled protamine (PRF) or polyarginine (PARGF) was adsorbed onto the HB antigen and then the polysaccharide dextran sulfate or alginate was added over the PRF or PARGF layers to form the fluorescently labeled HB:PRF:DS or HB:PARGF:ALG particles, respectively. The labeled formulations were, then, characterized for their size, PdI and surface charge as described in 2.3.

2.7. *In vitro* cell studies

2.7.1. Cells and culture

The adherent RAW 264.7 cells, a murine macrophage cell line (ATCC, Manassas, VA), were cultured in RPMI supplemented with 10% (v/v) fetal bovine serum (FBS) (PAA; Pasching, Austria), 2 mM glutamine and 100 U/ml of penicillin/streptomycin, in a humidified atmosphere at 37 °C in 5% carbon dioxide atmosphere. Cells were split every other day to maintain 70–80% of confluent cultures.

2.7.2. Internalization studies in macrophages

The uptake of HB:PR:DS and HB:PARG:ALGby Raw 264.7 cells was examined using a confocal microscope (Leica SP5, Germany). Briefly, cells (3 x 10^5) were seeded on a glass cover-slip (Menzel-Gläser, Braunschweig, Germany) in a 24-well plate (Falcon 3047, BD Biosciences, USA) and left overnight at 37 ºC to permit cell adhesion. The following day, cells were incubated with TAMRA-labeled HB:PRF:DS and HB:PARGF:ALG formulation (in a mass equivalent to 10 µg of HB), in RPMI with 10% FBS for 30 min at 37 ºC, in the dark. After several washes with PBS to remove non-internalized particles, the cells were fixed with 4% formaldehyde for 10 min and then incubated with Alexa Fluor 488-phalloidin (Invitrogen, Thermo Fisher Scientific, USA) for 20 min to stain the cellular cytoskeleton. Finally, the cover-slips containing the attached cells were mounted on slides in the presence of ProLong® Gold Antifade mounting medium (Invitrogen, Thermo Fisher Scientific, USA), containing DAPI as nucleus stain, and analyzed in a confocal microscope (Confocal Laser Scanning Microscopy (Leica SP5)).

2.8. *In vivo* studies

2.8.1. Animals

Female BALB/c mice (4–5 weeks) were housed in filter-top cages in a 12 h light/12 h dark cycle at constant temperature (22 °C) and provided with a standard diet and water *ad libitum*. The animals were kept conscious during the immunizations and bleeding. All protocols were in accordance with the guidelines of the Spanish regulations (Royal Decree 1201/2005) regarding the use of animals in scientific research and under the approval of the Ethical Committee of the University of Vigo.

2.8.2. Preparation of the control vaccine using alum as adjuvant.

The HB antigen was used either soluble (suspended in PBS) for intranasal (i.n.) administration or adsorbed in aluminum hydroxide (HB-Alum) for intramuscular (i.m.) administration as positive control. Briefly, Alum (Alhydrogel ®, Sigma-Aldrich, St. Louis, MO) was incubated with the HB antigen in a 3:1 (v:v) ratio for 30 minutes at 4 ºC under gentle agitation. Afterwards, the suspension was centrifuged (10,000 x g, 10 minutes, 4ºC) and the pellet was resuspended in an isotonic saline solution. The dose of antigen administered (10 μg) was the same in the control vaccines, as in the rest of formulations.

2.8.3. Immunization and bleeding schedule

In total, three *in vivo* trials were performed in mice, in order to evaluate the capacity of 1) the HB:PR, 2) the HB:PR:DS and HB:PARG:ALG and 3) the HB:PARG:pIC systems, to stimulate an immune response against the HB antigen by i.n. and i.m. routes.

In all cases, the initial pool of mice was randomly distributed in groups of ten animals for each formulation and administration route (i.m. or i.n.). The antigen dose was 10 μg, either for test formulations or controls. In the i.m. mice test groups, the test formulations and control were administered at day 0 and 28 (0 and 4 weeks) in the hind leg of the mice, in a volume of 0.06 ml (HB at 0.167 mg/ml). In the i.n. mice test groups, the formulations and control were administered at day 0, 28 and 112 (0, 4 and 16 weeks), in a volume of 0.02 ml (HB at 0.5 mg/ml) by instillation of 0.01 ml in each nostril.

The blood samples were collected in all cases from the mouse maxillary vein at day 42, 126 and 183 (corresponding to weeks 6, 18 and 26).

2.8.4. Determination of anti-HB IgG in the serum of immunized mice by ELISA

Specific antibody levels against HB in mice sera were evaluated by ELISA at different time points. For this purpose, MaxiSorp ® 96-well plates (Nunc, Denmark) were coated with a HB solution at 5 µg/ml, in carbonate buffer (pH 9.6), and incubated overnight at 4 ºC. Plates were then blocked with BSA 1% in PBS for 1 hour at 37ºC. In order to quantify specific anti HB-IgG, both serum samples and a mouse monoclonal anti-HB IgG antibody (Acris Antibodies GmbH; Hiddenhausen, Germany), were serially diluted and incubated for 2 hours at 37 ºC. Afterwards, secondary antibodies (goat anti-mouse IgG from Southern Biotech, AL, USA) were added to the wells and incubated for 1 hour at 37 ºC. Bound antibodies were finally revealed with 2,2´-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and the absorbance read at 405 nm. To transform antibody levels in µg/mL to international units (mIU/mL), a similar protocol was carried out in parallel using a control rabbit anti-HB IgG (Biokit, Barcelona, Spain) of known concentration (mIU/ml).

The IgG1/IgG2a ratio was also evaluated in a pool of sera from mice immunized with HB:PARG:pIC and HB-Alum by i.m. route. In this case, the same protocol was followed but specific anti-mouse IgG1 and IgG2a secondary antibodies were used. The ratio IgG1/IgG2a was then calculated based on the absorbance levels detected. All serum samples were tested at least twice and in duplicate.

3. RESULTS AND DISCUSSION

Virus-like particles (VLP) antigens are having an increasing presence in the immunization protocols. Despite their good safety profile and immunogenicity, these new vaccines need to be administered in association with adjuvants in order to generate robust immune responses. Inspired by the nanoparticulated nature of the VLP, we have designed, a strategy intended to increase the immune response against these antigens. This strategy relies on the use of a particulated antigen as a substrate for the adsorption of polymers with immune adjuvant properties. The antigen chosen was the hepatitis B VLP antigen (HB), due to its nanometric size (22 nm) and negative surface charge. On the other hand, the biomaterials selected for the enveloping process were: protamine (Pr), polyarginine (PARG), dextran sulfate (DS), alginate (ALG). Finally, the immunomodulator poly (I:C) (pIC), was also associated to the nanostructures in order to evaluate its potential for enhancing the immune response against the enveloped antigen. With this strategy, four multi-enveloped systems (HB:PR; HB:PR:DS; HB:PARG:Alg; HB:PARG:pIC) (**Figure 1**) were developed and characterized. Finally, their behavior upon contact with dendritic cells and upon *in vivo* administration was assessed.

3.1. Preparation and characterization of multi-enveloped nanoparticles

The HB antigen in a PBS stock solution was washed with ultrapure water (by ultrafiltration) in order to decrease the salt content before its enveloping with the polymers. As the adsorption of the polymers was based on electrostatic interactions, it was found important to reduce the presence of salts that could shield the electrostatic attraction between molecules of opposite charge [41]. This operation lead to a decrease of the antigen sample number-distribution, as before ultrafiltration it presented a size of 53 nm while after this process the size was of 22 nm (data not shown). After incubation of the antigen with the polymers, the formulation was also ultrafiltered to separate the multi-enveloped nanosystem from free polymer and allow the concentration of the formulation. This was considered an important step, particularly after the first coating (protamine and polyarginine), in order to eliminate the free polymer molecules that could complex with the negatively charged polymers (dextran sulfate, alginate and poly (I:C)) and interfere with the antigen enveloping process.

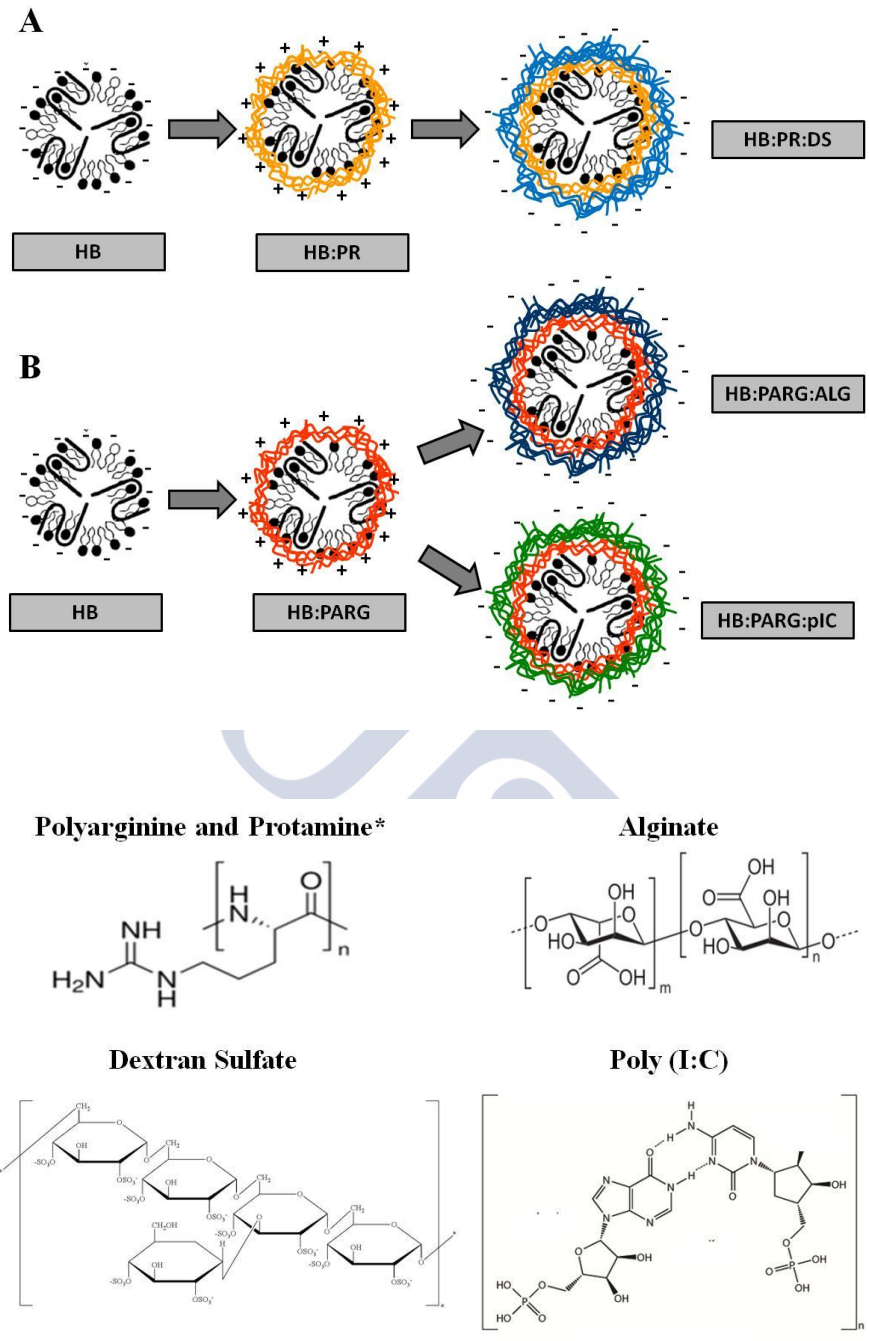


Figure 1. *Illustration of the sequential Protamine (PR) and Dextran Sulfate (DS) adsorption to the antigen HB to originate the HB:PR and HB:PR:DS compositions (**A**) and of the polyarginine (PARG) and alginate (ALG) or poly (I:C) (pIC) to form the HB:PARG and HB:PARG:ALG or HB:PARG:pIC compositions, respectively (**B**) and of the polymers involved in the multi-enveloping process. * The protamine is a mixture of polyamino acids molecules composed majorly by Arginines.*

The DLS and ELS techniques were considered the most practical techniques to characterize the antigen and to monitor its progressive coating by the polymers. In order to have a reliable signal with these techniques, the minimal HB concentration needed was 0.05 mg/ml and this concentration was taken as reference for characterization of the multi-enveloped systems. The initial DLS intensity-distribution size analysis of the antigen alone indicate a size greater than 100 nm (**Annex 1A**), which is in contradiction with previously published work [27, 42]. However, when the DLS signal was evaluated through a volume-distribution (**Annex 1B**) and number-distribution size (**Annex 1C**), we observed that the majority of the HB particles in solution had in fact a size close to 22 nm (22 ± 1) and that most part of antigen volume was from particles with this size. Such difference between intensity and number evaluation can be due to the presence of a small number of big particles resultant from HB particles agglomeration. In DLS, the intensity signal is directly proportional to the size of the particles, so even if the quantity of small particles is greater than that of the large ones, the intensity distribution profile will predominantly reflect the presence of large particles [43]. As the number-distribution size matched perfectly with the referenced size of the HB particles, this parameter was considered to be the most adequate for the characterization of antigen and to follow its progressive coating through the multi-enveloping process. The presence of the refereed HB particles aggregates could explain the high polydispersity (PdI 0.3). As this nanostructure was used as a template for the polymer adsorption, all the multi-enveloped structures developed presented also a significant high PdI (**Figure 2**). The ELS analysis of the antigen alone confirmed its negative surface charge potential. After the addition of the protamine or polyarginine, this surface charge became positive, suggesting that the antigen surface was coated by the positive polyamino acids. Finally, when the dextran sulfate, alginate or poly (I:C) were added to the formulations, the zeta potential detected was again negative, thus indicating that the antigen particles had been coated by the negative polymer. The adsorption of subsequent polymer layers led to consecutive negative-positive-negative surface charge inversions and also to a subtle increase of the size from the original 22 nm to about 40 nm, upon formation of the multilayer structure.

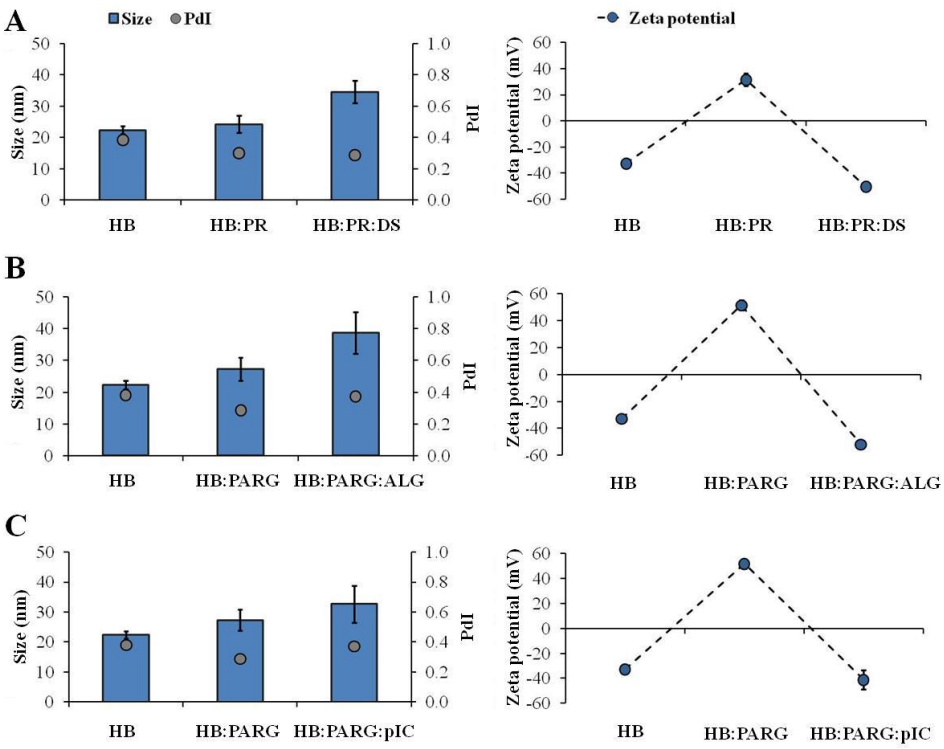


Figure 2. *Evolution on the size (Size), polydispersity index (PdI) and surface charge (zeta potential) of the multi-enveloped systems HB:PR and HB:PR:DS (**A**) upon the adsorption of protamine (PR) and dextran sulfate (DS) onto the hepatitis B antigen (HB), of the systems HB:PARG and HB:PARG:ALG (**B**) upon the adsorption of polyarginine (PARG) and alginate (ALG) and of the system HB:PARG:pIC (**C**) upon the adsorption of polyarginine and poly (I:C) (pIC), respectively.*

3.2. Determination of protamine and polyarginine adsorption and antigen enveloping efficiency

To evaluate the percentage of protamine and polyarginine adsorbed onto the antigen particles, the concentration of the non-associated polymers was determined by mBCA. Under the selected experimental conditions, the percentage of polyarginine and protamine adsorbed was 23% and 13%, respectively (**Table 1**). This indicates that the amount of polymers used exceeded the amount required for the enveloping process. These positively charged polymers are hydrophilic and the mechanism driving the interaction with the antigen is based on the ionic interaction with the negatively charge antigen HB, as illustrated in **Figure 1**.

Table 1. Summary of the adsorption efficiency (E.A.) of protamine and polyarginine on the HB antigen and percentage of enveloped antigen (HB Enveloped).

Formulation	E.A. (%)	HB Enveloped (%)
HB:Protamine	13 ± 5	67 ± 2
HB:Polyarginine	23 ± 0.4	65 ± 3

On the other hand, the amount of HB antigen coated by the polymers was determined by ELISA. Interestingly, although the amounts of protamine and polyarginine adsorbed to HB were quite different, the HB enveloping efficiency was relatively high (> 60%) for both polyamino acids (**Table 1**). A possible explanation for the higher amount of polyarginine adsorbed could be found in the greater density of positive charges in this polymer. In addition, the higher molecular weight of polyarginine (5-15kDa) vs. protamine (5 kDa) could have also influenced the amount of polymer adsorbed. The slightly larger size and higher positive surface charge identified for HB:PARG compared with the HB:PR are also supporting this hypothesis (**Figure 2**).

3.3. Stability of multi-enveloped particles in aqueous suspension

A critical aspect of the colloidal carriers is the preservation of their stability upon storage and also upon incubation in simulated biological fluids. Indeed, very small nanoparticles, especially those formed by ionic complexation, often have a tendency to destabilize themselves. Interestingly, irrespective of the nature of the coating polymer, the nano-enveloped particles remained stable in terms of particle size distribution, upon storage for at least one month at 4 ºC (**Annex 2**). On the other hand, the behavior of the different nanostructures upon incubation in PBS at 37 ºC, was slightly different. The HB:PR formulation showed a slight and rapid size increase (from 40 to 60 nm size) (**Figure 3A**) upon incubation in PBS. However, following this initial increase, the size and also the PdI remained stable for the rest of the assay. On the other hand, the HB:PR:DS prototype exhibited an adequate stability in PBS (**Figure 3B**) while the HB:PARG:ALG system, aggregated rapidly when dispersed in this media. Finally, the HB:PARG:pIC preserved its size and PdI for up to 4 hours in PBS (**Figure 3C**).

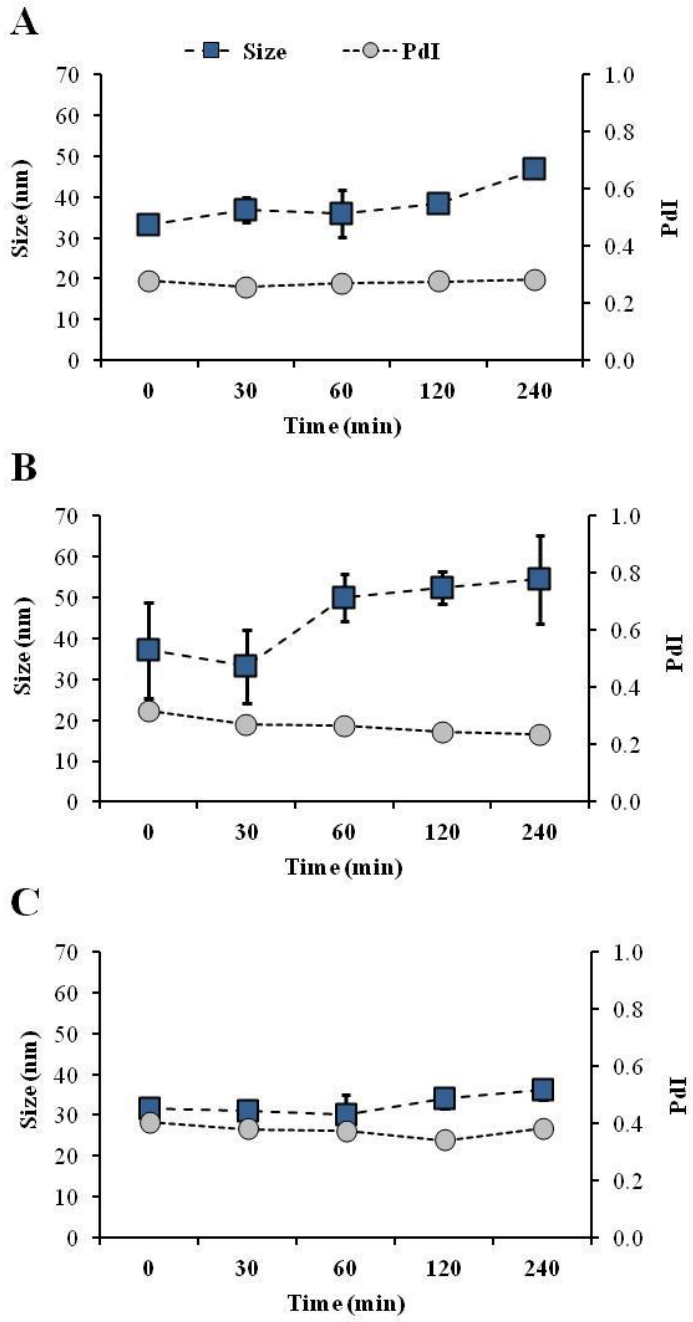


Figure 3. *Evaluation of size number distribution (Size) and polydispersity index (PdI) after 4 hours at 37 ºC in PBS of the formulations HB:PR (**A**), HB:PR:DS (**B**) and HB:PARG:pIC (**C**).*

The different behavior of HB:PARG:ALG and HB:PARG:pIC systems (which share the same HB:PARG core) in PBS, could be related to the different ionic forces between the first and second enveloping layers. The great negative charge density of poly (I:C) might be responsible for the firm association between the two layers.

Overall, the conclusion is that the stability of the multiple enveloping of antigen nanoparticles is dependent on the balance between the attractive forces of the polymer-polymer and polymer-antigen layers and between the polymer layer and the components of the incubation medium.

3.4. *In vitro* internalization studies of fluorescently labeled multi-enveloped particles

As indicated in the introduction, in order to facilitate the antigen presentation to the immune system, nanoparticles should have the capacity to enter the APCs. To verify the possibilities for this internalization process to occur, the HB:PR:DS and HB:PARG:ALG nanosystems were prepared using TAMRA-labeled polymers and incubated with macrophages (RAW 264.7). The dye was covalently linked to the polyamino acids prior to the enveloping process. The DLS and ELS analysis of the labeled formulations confirmed that all the physicochemical parameters of the multilayer systems remained constant, with only a small decrease in the zeta potential values of the HB:PARG:ALG formulation (**Annex 3**).

The internalization process of the nanosytems into the macrophages was visualized by confocal microscopy (**Figure 4**).

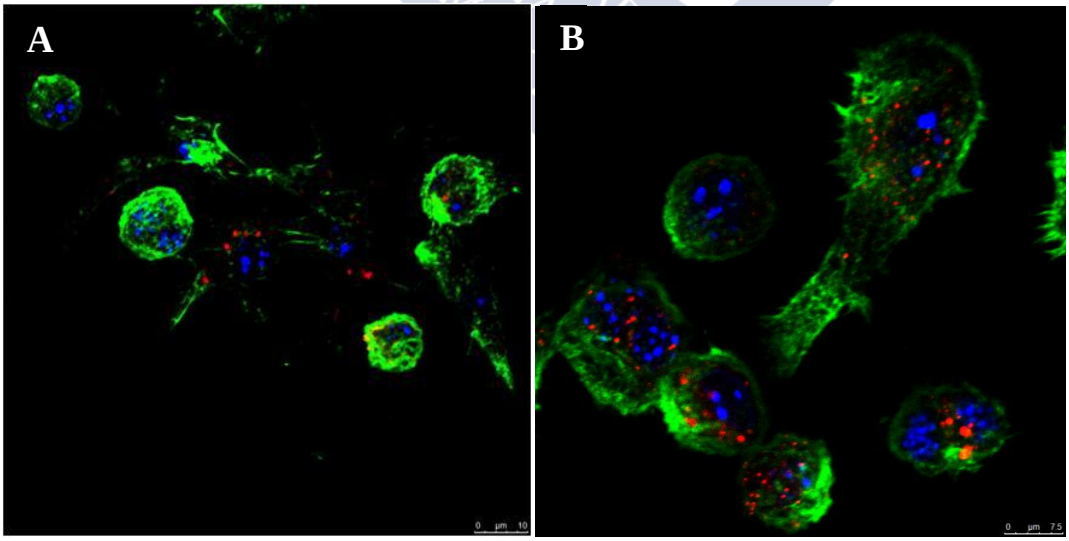


Figure 4. *Evaluation of the uptake of fluorescent HB:PR:DS (**A**) and HB:PARG:ALG particles (**B**) by RAW 264.7 cells by confocal microscopy (red channel: TAMRA (labeled particles); green channel: Alexa Fluor 488-phalloidin (cytoskeleton); blue channel: DAPI (nucleus)).*

The images obtained suggest a more pronounced presence of the HB:PR:DS nanoparticles, with respect to the HB:PARG:ALG ones, into the cells although more detailed studies should be realized to confirm this data.

3.5. *In vivo* studies

The ability of the HB:PR, HB:PR:DS and HB:PARG:ALG or HB:PARG:pIC formulations to promote a systemic humoral immune response by i.n. or i.m. routes against the hepatitis B antigen, was assessed in three different *in vivo* trials with BALB/c mice. The antigen dose was 10 μg for the formulations and for the alum-adsorbed antigen used as positive control (same mass ratio as in licensed vaccines) [44]. The specific anti-HB IgG concentration (μg/ml) in mouse sera was determined by ELISA and converted to international units (mIU/ml) in order to be able to compare with the protective anti-HB IgG levels observed in humans (10 mIU/ml) [45].

Following intramuscular immunization, it was found that the prototypes investigated, led to a steady anti-HB IgG profile close to 100 mIU/ml (values between 179 and 62 mIU/ml) during the whole experiment (**Figure 5**). Although these levels were lower than those observed for the alum-adjuvanted vaccine, it is important to mention that they were always superior to 10 mIU/ml, a concentration known to be protective in humans. On the other hand, the effect of the presence of poly (I:C) in the formulation was only shown at early times.

When testing the formulations by intranasal route and in order to better understand the adjuvant effect of enveloping the antigen, the antigen alone was also administered by the i.n. route. Despite being a highly immunogenic antigen, the HB alone generated almost undetectable IgG titers (2 mIU/ml). Overall, the responses were similar for the different prototypes and were low at short times (below 10 mIU/ml), however, they increased over time reaching IgG levels close to 60 mIU/ml (**Figure 6**). These values were also significantly higher than those attained for the free antigen.

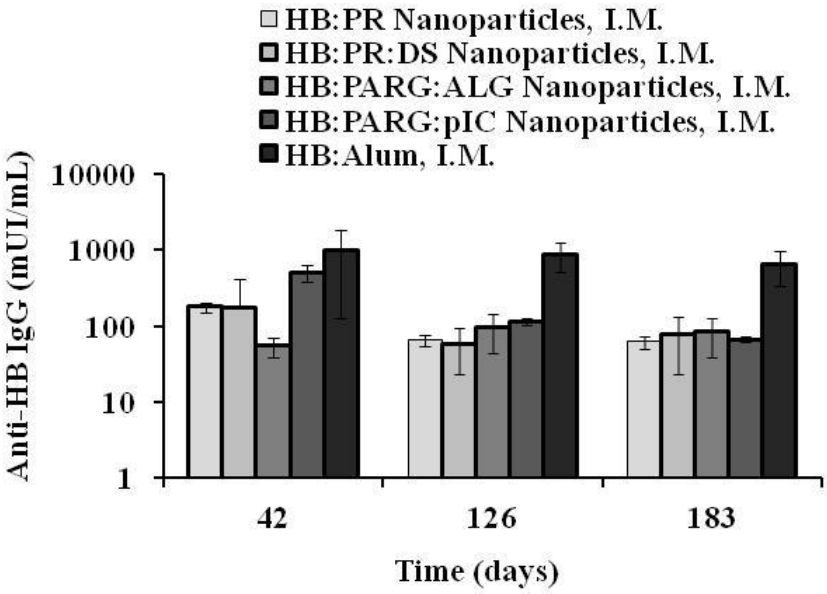


Figure 5. *Systemic humoral (IgG) immune response observed in mice upon intramuscular (0 and 4 weeks) immunization with Hepatitis B antigen coated with Protamine (HB:PR), Protamine and Dextran Sulfate (HB:PR:DS), Polyarginine and Alginate (HB:PARG:ALG) or Polyarginine and poly (I:C) (HB:PARG:pIC). Hepatitis B antigen adsorbed to Alum (HB:Alum) was used as positive control. The plot represents the average of anti-HB IgG quantified at different time points after primary immunization (mean ± SEM, n = 10).*

The modest humoral response observed for all the multi-enveloped systems, suggests that there might be an intrinsic limitation of the multi-enveloping strategy for the hepatitis B antigen. This limitation might be related to the recent theory that underlines the importance of the exposure of a repetitive epitope array in the generation of an effective immune response [3]. Such theory has been used to justify the superiority of VLP antigens as compared to soluble antigens [6, 7]. However, despite of this, it is clear that rHBsAg antigen needs to be associated with Alum in order to activate the immune cells and generate an effective immune response. On the other hand, the improved behavior of the multi-enveloped formulations as compared to the VLP antigen alone, following intranasal administration, suggests that the enveloping polymers exert a certain role. This role could be simply related to the capacity of polyarginine to facilitate the transport of proteins across the nasal mucosa [46] or it could also be related to the adjuvant effect of the biomaterials [29, 31, 33, 37, 39].

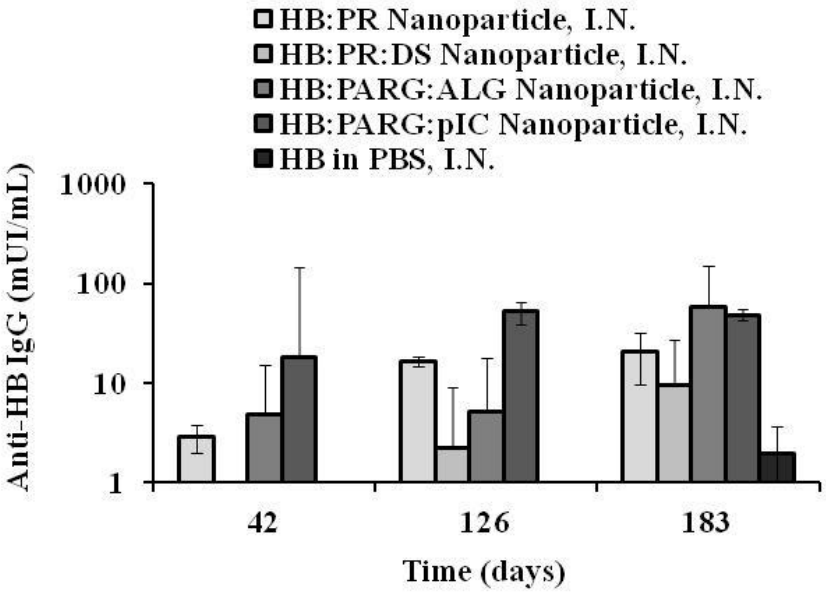


Figure 6. *Systemic humoral (IgG) immune response observed in mice upon intranasal (0, 4 and 16 weeks) immunization with Hepatitis B antigen coated with Protamine (HB:PR), Protamine and Dextran Sulfate (HB:PR:DS), Polyarginine and Alginate (HB:PARG:ALG) or Polyarginine and poly (I:C) (HB:PARG:pIC). Hepatitis B antigen dispersed in PBS (HB in PBS) was used as positive control. The plot represents the average of anti-HB IgG quantified at different time points after primary immunization (mean ± SEM, n = 10).*

The theory about the limited exposure of the antigen epitopes in the enveloped systems would agree with the positive results previously obtained by our group for a chitosan nanocapsules prototype, which have VLP rHBsAg antigen exposed on their surface [16, 17]. However, this theory would be in disagreement with the results of previous works that have shown that the coating of rHBsAg antigen [47] or its entrapment within polymers such as chitosan [14], may also lead to significant responses following either i.m. or i.n. administration. Therefore, given the different nature of the biomaterials and the different sizes of the vaccine nanostructures it is difficult to draw a conclusion at this point. Another explanation for the limited response obtained for the enveloped nanosystems could be related to the small size of the nanostructures. Even though, we have postulated that small size could facilitate the direct access of the antigen to the lymph nodes, the possibility that these ultra-small particles would be less recognizable by the antigen presenting cells, cannot be discarded.

Finally, based on previous works [39, 40], it was expected that the association of poly (I:C) to the nanostructure would lead to an enhanced response. Keeping in mind that this immunostimulant would mainly have an impact on the cell responses (associated to Th1 responses) [48], the IgG1/IgG2a ratio of the mice immunized with the HB:PARG:pIC formulation were compared with those observed for the alum-absorbed antigen (**Figure 7**).

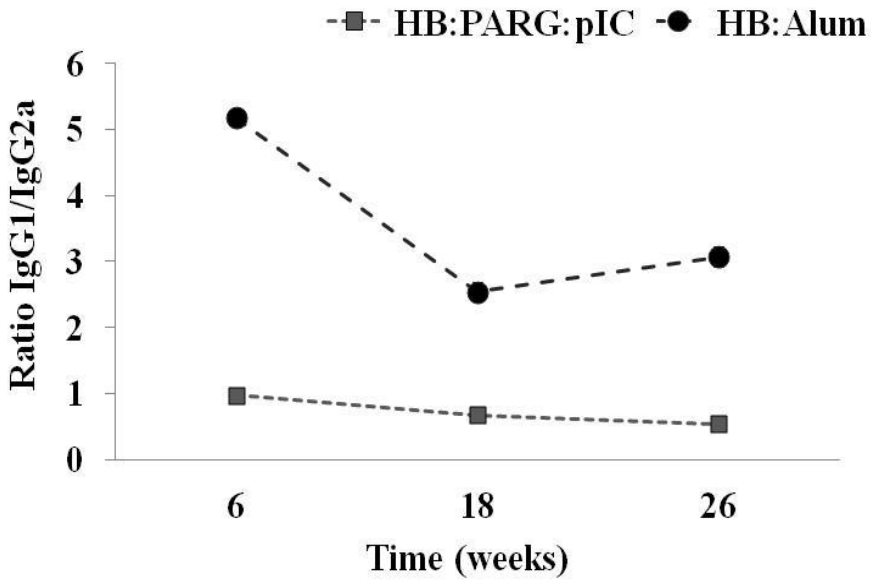


Figure 7. *Ratio between the IgG1 and IgG2a subtypes in the serum of mice immunized with the multi-enveloped system composed by the Hepatitis B antigen coated with polyarginine and poly (I:C) (HB:PARG:pIC) (grey squares) and with the same antigen adsorbed to alum (HB:Alum) (black circle).*

As the IgG1 and IgG2a are characteristic of a T_h2 and T_h1 biased-response, respectively [49], we assumed that a IgG1a/IgG2 ratio superior to 1 was indication of a T_h2 polarized response while a IgG1/IgG2a ratio inferior to 1 was the indication of a T_h1 polarized response [50]. The activation of lymphocytes T_h2 has been associated to a more preponderant humoral response while the activation of T_h1, to an enhanced cellular based immunity [51]. The IgG1/IgG2a ratio indicates that the alum markedly shifted the lymphocytes towards a T_h2 profile, which is a well known characteristic of this adjuvant [13]. On the contrary, in the case of the HB:PARG:pIC formulation, it was found that the initially balanced T_h1/T_h2 response (IgG1a/IgG2 = 0.97) shifted, at the end of the assay, towards a clear polarization of a T_h1 response (IgG1a/IgG2 = 0.54). The increased activation of T_h1 cells by the HB:PARG:pIC formulation can be an

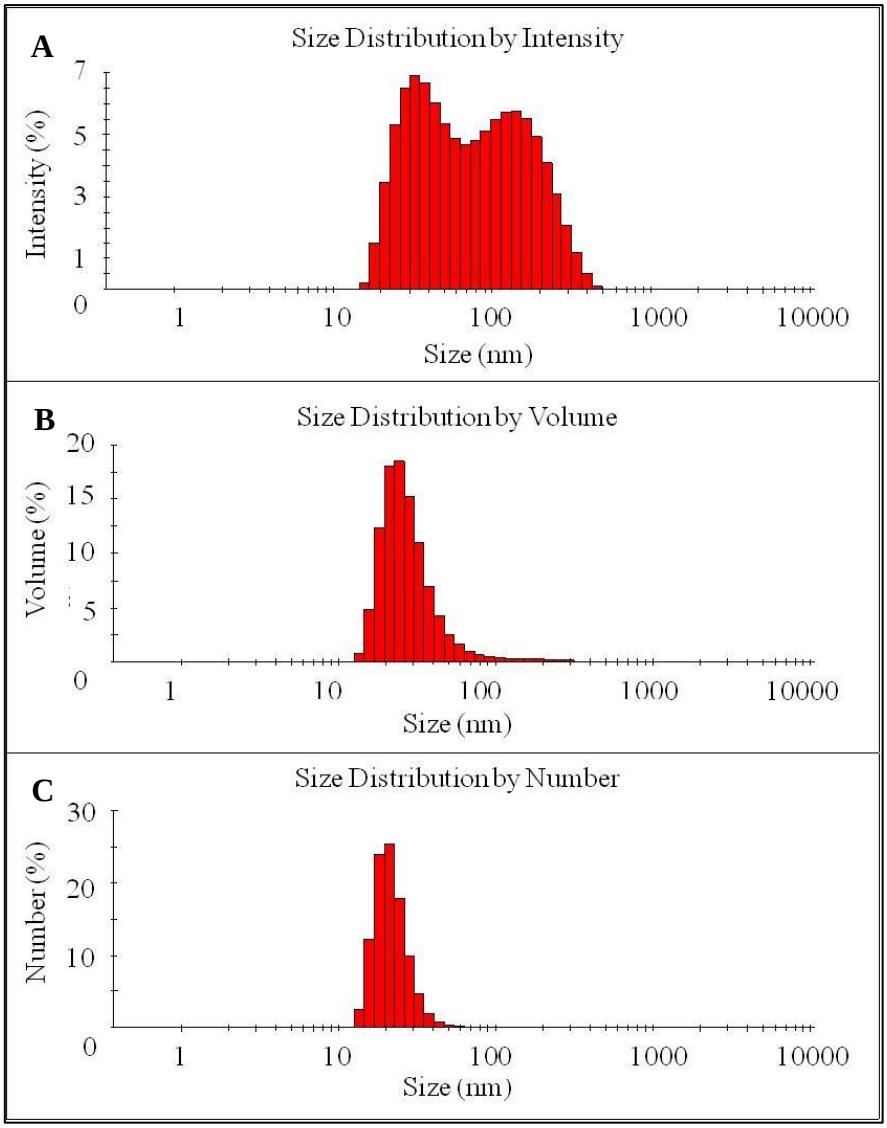
interesting advantage of this system, as these cells stimulate the development of cellular based immune responses capable of eliminating virus infected cells [52].

CONCLUSION

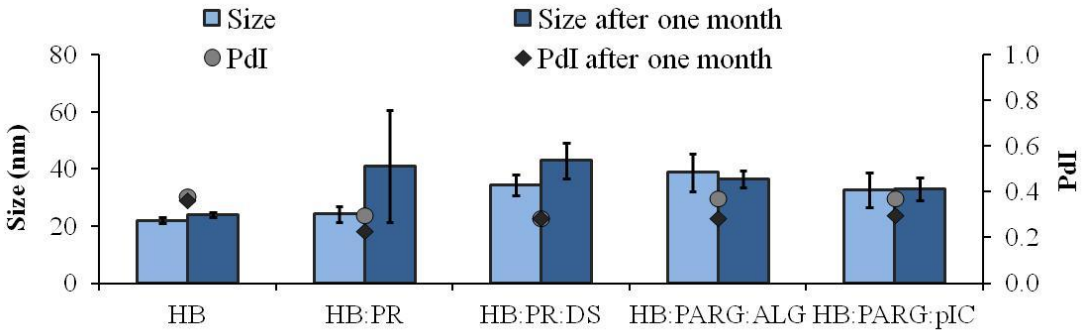
Here we present the design and evaluation of a new strategy for the immunomodulation of immune responses associated to particulated antigens. The strategy involves using the HB particulated antigen as a model template, and its enveloping with a number of biomaterials and immunostimulators. The HB multi-enveloped systems were capable of generating protective IgG titers (>10 mIU/ml) but the IgG titers achieved were lower than those of the alum-adsorbed antigen. There were no important differences between the activity of the HB:PR, HB:PR:DS and HB:PARG:ALG systems despite their diverse composition and structure, which suggests an intrinsic limitation of this multi-enveloping strategy. Nevertheless, the association of the immunostimulant poly (I:C) to the enveloped antigen led to a more balanced T_h1/T_h2 response, as compared to the one observed for the alum-absorbed antigen. This could be taken as an indication of the improved cellular responses, which would be of importance for the complete elimination of viral infections. Overall, this work provides new insight on the properties that are determinant of the behavior of the antigen delivery nanocarriers and underlines the fact that a very small size might not be a good option for enhancing the immune response against particulated antigens.

SUPPORTING INFORMATION

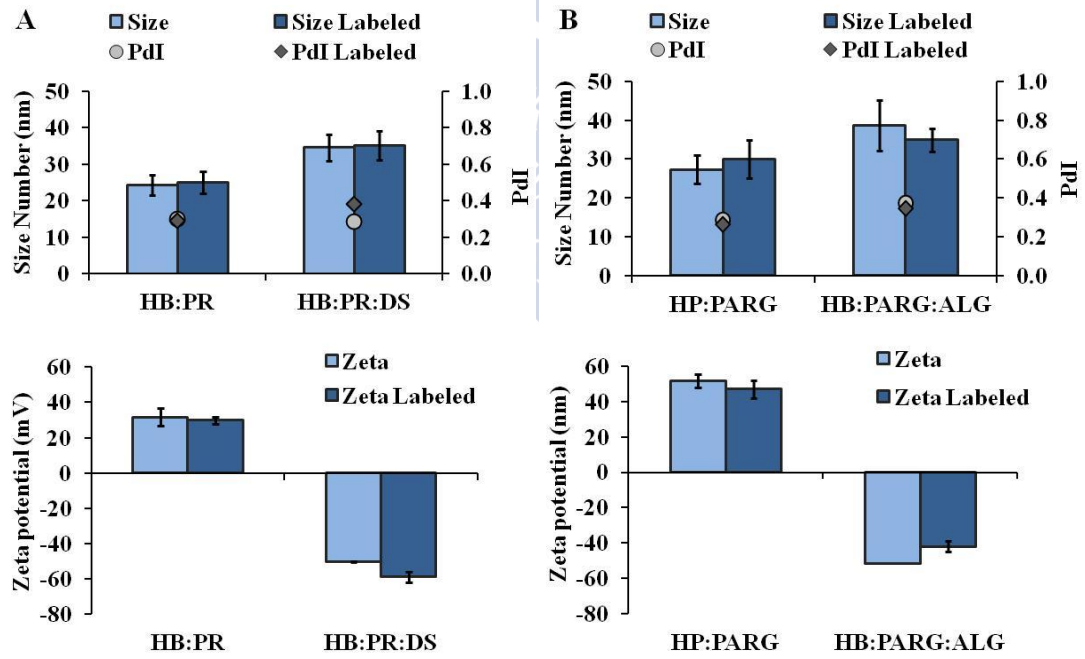
Annex 1. Comparison of the DLS signal analysis of the HB antigen alone through three different size distributions: Intensity (**A**), Volume (**B**) and Size (**C**).



Annex 2. Evolution of size (Size number) and polydispersity index (PdI) after one month at 4 ºC of the HB coated with protamine (HB:PR), with protamine and dextran sulfate (HB:PR:DS), with polyarginine and alginate (HB:PARG:ALG) or with polyarginine and poly (I:C) (HB:PARG:pIC).



Annex 3. Comparison of the size number distribution (Size), polydispersity index (PdI) and surface charge (Zeta potential) of non labeled and labeled HB:PR formulations (**A**) and of non labeled and labeled HB:PARG and HB:PARG:ALG formulations (**B**).



REFERENCES

1. Zepp, F., *Principles of vaccine design—Lessons from nature.* Vaccine, 2010. **28, Supplement 3**(0): p. C14-C24.

2. Huang, D.B., J.J. Wu, and S.K. Tyring, *A review of licensed viral vaccines, some of their safety concerns, and the advances in the development of investigational viral vaccines.* Journal of Infection, 2004. **49**(3): p. 179-209.

3. Jegerlehner, A., et al., *Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation.* Eur J Immunol, 2002. **32**(11): p. 3305-14.

4. Grgacic, E.V.L. and D.A. Anderson, *Virus-like particles: Passport to immune recognition.* Methods, 2006. **40**(1): p. 60-65.

5. Keating, G.M. and S. Noble, *Recombinant hepatitis B vaccine (Engerix-B): a review of its immunogenicity and protective efficacy against hepatitis B.* Drugs, 2003. **63**(10): p. 1021-51.

6. Schiller, J.T. and D.R. Lowy, *Raising Expectations For Subunit Vaccine.* J Infect Dis, 2014. **24**.

7. Kushnir, N., S.J. Streatfield, and V. Yusibov, *Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development.* Vaccine, 2012. **31**(1): p. 58-83.

8. Clements, C.J. and E. Griffiths, *The global impact of vaccines containing aluminium adjuvants.* Vaccine, 2002. **20, Supplement 3**(0): p. S24-S33.

9. Chen, D., et al., *Characterization of the freeze sensitivity of a hepatitis B vaccine.* Hum Vaccin, 2009. **5**(1): p. 26-32.

10. Matthias, D.M., et al., *Freezing temperatures in the vaccine cold chain: A systematic literature review.* Vaccine, 2007. **25**(20): p. 3980-3986.

11. Davaalkham, D., et al., *Administration of hepatitis B vaccine in winter as a significant predictor of the poor effectiveness of vaccination in rural Mongolia: evidence from a nationwide survey.* J Epidemiol Community Health, 2007. **61**(7): p. 578-84.

12. Drucker, E., P.G. Alcabes, and P.A. Marx, *The injection century: massive unsterile injections and the emergence of human pathogens.* The Lancet. **358**(9297): p. 1989-1992.

13. Gupta, R.K., *Aluminum compounds as vaccine adjuvants.* Adv Drug Deliv Rev, 1998. **32**(3): p. 155-172.

14. Prego, C., et al., *Chitosan-based nanoparticles for improving immunization against hepatitis B infection.* Vaccine, 2010. **28**(14): p. 2607-14.

15. Vila, A., et al., *Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice.* Eur J Pharm Biopharm, 2004. **57**(1): p. 123-31.

16. Vicente, S., et al., *Co-delivery of viral proteins and a TLR7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy.* Journal of Controlled Release, 2013. **172**(3): p. 773-781.

17. Vicente, S., et al., *A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach.* PLoS ONE, 2013. **8**(4): p. e62500.

18. Bachmann, M.F. and G.T. Jennings, *Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns.* Nat Rev Immunol, 2010. **10**(11): p. 787-796.

19. Scheerlinck, J.P. and D.L. Greenwood, *Virus-sized vaccine delivery systems.* Drug Discov Today, 2008. **13**(19-20): p. 882-7.

20. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.

21. Pozzi, L.A., J.W. Maciaszek, and K.L. Rock, *Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop effector function, and differentiate into memory cells.* J Immunol, 2005. **175**(4): p. 2071-81.

22. Manolova, V., et al., *Nanoparticles target distinct dendritic cell populations according to their size.* Eur J Immunol, 2008. **38**(5): p. 1404-13.

23. Reddy, S.T., et al., *In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles.* Journal of Controlled Release, 2006. **112**(1): p. 26-34.

24. Reddy, S.T., et al., *Exploiting lymphatic transport and complement activation in nanoparticle vaccines.* Nat Biotech, 2007. **25**(10): p. 1159-1164.

25. Junt, T., et al., *Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells.* Nature, 2007. **450**(7166): p. 110-114.

26. Correia-Pinto, J.F., N. Csaba, and M.J. Alonso, *Vaccine delivery carriers: insights and future perspectives.* Int J Pharm, 2013. **440**(1): p. 27-38.

27. Zhao, Q., et al., *Maturation of Recombinant Hepatitis B Virus Surface Antigen Particles.* Human Vaccines, 2006. **2**(4): p. 174-180.

28. Buschle, M., et al., *Transloading of tumor antigen-derived peptides into antigen-presenting cells.* Proceedings of the National Academy of Sciences, 1997. **94**(7): p. 3256-3261.

29. Mattner, F., et al., *Vaccination with Poly-l-Arginine As Immunostimulant for Peptide Vaccines: Induction of Potent and Long-Lasting T-Cell Responses against Cancer Antigens.* Cancer Research, 2002. **62**(5): p. 1477-1480.

30. Valneva Austria GmbH, *Phase II Study of Immunization With a Hepatitis C Virus (HCV) Antigen Peptide Vaccine*, 2008: In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2015 March 17] Available from: http://clinicaltrials.gov/show/NCT00602784 NLM Identifier: NCT00602784.

31. Reynolds, F., R. Weissleder, and L. Josephson, *Protamine as an efficient membrane-translocating peptide.* Bioconjugate Chemistry, 2005. **16**(5): p. 1240-1245.

32. Martínez Gómez, J.M., et al., *Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis.* Journal of Controlled Release, 2008. **130**(2): p. 161-167.

33. Yang, D. and K.S. Jones, *Effect of alginate on innate immune activation of macrophages.* J Biomed Mater Res A, 2009. **90**(2): p. 411-8.

34. Kurachi, M., et al., *Comparison of the activities of various alginates to induce TNF-alpha secretion in RAW264.7 cells.* Journal of Infection and Chemotherapy, 2005. **11**(4): p. 199-203.

35. Flo, T.H., et al., *Involvement of toll-like receptor (TLR) 2 and TLR4 in cell activation by mannuronic acid polymers.* J Biol Chem, 2002. **277**(38): p. 35489-95.

36. McCarthy, R.E., L.W. Arnold, and G.F. Babcock, *Dextran sulphate: an adjuvant for cell-mediated immune responses.* Immunology, 1977. **32**(6): p. 963-974.

37. Kerlin, R.L. and D.L. Watson, *Effect of dextran sulphate on IgG subclass of antibody in efferent popliteal lymph of sheep.* Immunol Cell Biol, 1987. **5**: p. 411-7.

38. Lundberg, A.M., et al., *Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems*. Vol. 110. 2007. 3245-3252.

39. Durand, V., et al., *Shaping of adaptive immune responses to soluble proteins by TLR agonists: a role for IFN-alpha/beta.* Immunol Cell Biol, 2004. **82**(6): p. 596-602.

40. Sloat, B. and Z. Cui, *Nasal Immunization with Anthrax Protective Antigen Protein Adjuvanted with Polyriboinosinic–Polyribocytidylic Acid Induced Strong Mucosal and Systemic Immunities.* Pharmaceutical Research, 2006. **23**(6): p. 1217-1226.

41. Van de Steeg, H.G.M., et al., *Polyelectrolyte adsorption: a subtle balance of forces*. Langmuir, 1992. **8**(10): p. 2538-2546.

42. Diminsky, D., et al., *Comparison between hepatitis B surface antigen (HBsAg) particles derived from mammalian cells (CHO) and yeast cells (Hansenula*

polymorpha): composition, structure and immunogenicity. Vaccine, 1997. **15**(6-7): p. 637-47.

43. Instruments, M., *Dynamic light scattering: an introduction in 30 minutes.* Technical Note Malvern, MRK656-01, 2012: p. 1-8.

44. Rustgi, V.K., C.J. Schleupner, and D.S. Krause, *Comparative study of the immunogenicity and safety of Engerix-B administered at 0, 1, 2 and 12 months and Recombivax HB administered at 0, 1, and 6 months in healthy adults.* Vaccine, 1995. **13**(17): p. 1665-1668.

45. West, D.J. and G.B. Calandra, *Vaccine induced immunologic memory for hepatitis B surface antigen: implications for policy on booster vaccination.* Vaccine, 1996. **14**(11): p. 1019-1027.

46. Miyamoto, M., et al., *Effect of poly-l-arginine on the nasal absorption of FITC-dextran of different molecular weights and recombinant human granulocyte colony-stimulating factor (rhG-CSF) in rats.* International Journal of Pharmaceutics, 2001. **226**(1–2): p. 127-138.

47. Tafaghodi, M., et al., *Hepatitis B surface antigen nanoparticles coated with chitosan and trimethyl chitosan: impact of formulation on physicochemical and immunological characteristics.* Vaccine, 2012. **30**(36): p. 5341-5348.

48. Hafner, A.M., B. Corthesy, and H.P. Merkle, *Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant.* Adv Drug Deliv Rev, 2013. **65**(10): p. 1386-99.

49. Finkelman, F.D., et al., *Lymphokine control of in vivo immunoglobulin isotype selection.* Annual review of immunology, 1990. **8**(1): p. 303-333.

50. Cribbs, D.H., et al., *Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with β-amyloid.* International Immunology, 2003. **15**(4): p. 505-514.

51. Paul, W.E. and R.A. Seder, *Lymphocyte responses and cytokines.* Cell, 1994. **76**(2): p. 241-251.

52. Graham, M.B., V.L. Braciale, and T.J. Braciale, *Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection.* J Exp Med, 1994. **180**(4): p. 1273-82.

CAPÍTULO 4

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CAPÍTULO 4

CHITOSAN-POLY (I:C)-PADRE BASED NANOPARTICLES AS DELIVERY VEHICLES FOR SYNTHETIC PEPTIDE VACCINES

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ABSTRACT

The favorable safety profile of peptide antigens has prompted the search of adjuvants capable of increasing the immunization efficiency of these intrinsically poor immunogenic antigens. The integration of both immunostimulants and peptide antigens within nanometric delivery systems for their co-delivery to the immune cells is currently considered a promising peptide-based vaccination strategy. With this idea in mind, the potential synergistic effect of the immunostimulant poly (I:C) and chitosan, integrated into a nanostructure, was explored. In addition, a universal T-Helper peptide (PADRE) was incorporated into the nanoparticles in order to promote a strong T helper response. The value of this nanostructured combination of materials was assessed for a peptide antigen, 1338aa, which contained the amino acid sequence of a specific epitope (17-36aa) from the HPV-16 L2 protein. Nanoparticles consisting of chitosan (CS) and poly (I:C) (pIC) could be obtained by the ionic gelation technique by adjusting the amount of both components to promote their adequate interaction. These nanoparticles exhibited a nanometric size (<300 nm), a high positive surface charge (> +40 mV) and a very high pIC association efficiency (>96%). They also showed capacity for the association of both the 1338aa and PADRE peptides. The influence of the presence of pIC and PADRE in the nanocomposition, as well as that of the peptide presentation form (encapsulated versus surface adsorbed) on the antibody induction was evaluated in a preliminary *in vivo* study. The data obtained highlights the possibility to engineer nanoparticles through the rational combination of a number of adjuvant molecules together with the antigen.

1. INTRODUCTION

The advent of synthetic peptide-based antigens has brought new opportunities and also new challenges to the development of effective vaccines. These synthetic and chemically well-defined molecules, designed from known epitopes of tumor cells and pathogens, present no risk of mutation or reversion associated to whole pathogen-based vaccines. From the technological point of view, they are easy to produce under appropriate quality control practices [1] and present little risk of contamination by pathogenic or toxic substances [2]. However, due to their purity, these entities lack of "danger signals" [3] and consequently are incapable of activating immune cells. Furthermore, the peptide-based antigens present in general limited MHC recognition, which contributes to their poor intrinsic immunogenicity [4]. Therefore, there is a need to design adjuvant systems specifically adapted to facilitate the efficient presentation of peptide-based antigens without compromising their favorable safety profile. Within this frame, nanoparticles made of biodegradable polymers are receiving increasing attention. Among the different nanotechnology-based adjuvant systems, nanoparticles made of chitosan (CS), a biodegradable polysaccharide [5], are particularly attractive since these nanoparticles have already proven to be efficient adjuvant systems for a variety of protein antigens [6, 7]. In addition to their capacity for the association of peptides and proteins, CS nanoparticles are well known for their ability to efficiently complex polynucleotides, protect them from enzyme degradation, and deliver them to cells [8]. Therefore, in principle, such delivery system could offer the possibility of associating simultaneously antigens and polynucleotide-based immunostimulants and delivering them to the target site in a controlled manner.

As a model peptide-based antigen that would potentially benefit from the chitosan-based nanotechnology, we have chosen the peptide (1338aa), which comprises a specific amino sequence (13-38aa) from the Human Papilloma Virus 16 (HPV-16) L2 capsid protein. Currently available HPV vaccines are based on the L1 protein from the HPV capsid, which self assembles into virus-like particles (VLPs) [9]. These vaccines induce high levels of type-specific neutralizing antibodies, however these antibodies have a limited ability to protect against all oncogenic HPV types [10, 11]. On the other hand, the L2 minor capsid protein does not assemble into VLPs and is less immunogenic but it contains conserved domains that elicit broad cross-type neutralizing antibodies [12], which makes L2 a potential target for a novel HPV vaccine. The amino

acid sequence 17-36aa of this protein has been identified as a highly conserved neutralizing epitope among the HPV strains [13] and presents a great potential as antigen for a cross-protective HPV vaccine.

In order to enhance the adjuvant properties of chitosan nanoparticles, we have incorporated into the nanostructure the immunostimulant poly (I:C) (pIC), a synthetic analog of the viral double-stranded RNA (dsRNA). This compound mimics a molecular pattern associated with viral infections and it is an agonist of the Toll-like receptor 3 (TLR3), present in the endosomes of antigen presenting cells (APC). The activation of TLR3 promotes dendritic cell maturation and the production of T cell chemokines [14], thereby potentiating both humoral and cellular immune responses [15, 16]. Nevertheless, the systemic delivery of pIC can also provoke adverse effects, such as autoimmunity [17] and chronic inflammatory diseases [18], as observed in rodent models. In the context of this work it has been assumed that the incorporation of this immunostimulant into CS nanoparticles could protect it from serum nucleases and facilitate its uptake by the APCs, consequently leading to a reduction of the effective dose and potential adverse effects [19, 20].

Finally, in addition to the HPV L2 antigenic peptide and the pIC immunostimulant, we associated a T-Helper Pan HLA-DR epitope (PADRE) peptide to the composition. These peptides present specific amino acid sequences that can bind to the majority of MHC class II alleles [21] and have already shown to be capable of increasing the immune response of associated peptide-based antigens [22, 23]. Accordingly, the inclusion of the PADRE peptide in the nanoparticle is expected to provide a greater MHC recognition of the 1338aa peptide and consequently, an increased immune response against the antigen.

To summarize, the objective of this work was to design and develop a CS based nanoparticle with the capacity to co-deliver a peptide-based antigen with the immunostimulants pIC (as danger signal) and the PADRE peptide (to increase T helper responses). The ultimate goal has been the identification of potential synergistic effects among the three major components with regard to their ability to generate an effective antibody response. The expected low immunogenicity of the L2 peptide 1338aa allied with its great potential as antigen for an improved HPV vaccine makes it an excellent antigen model for the study of novel particle-based vaccines with intrinsic adjuvant

activities. Even though the synergistic effect of the combination of the T helper peptides and pIC for immunization purposes has already been observed for a peptide-based antigen from HPV [23] this is, to the best of our knowledge, the first time that both molecules are combined in nanoparticles as an adjuvant system to solve the specific limitations of peptide-based antigens.

2. MATERIALS AND METHODS

2.1. Materials

Peptide 1338aa and peptide PADRE were chemically synthesized (95% and 99% purity, respectively) by Genemed Synthesis (San Antonio, USA). Amino acid sequence and physicochemical characteristics are described in **Table 1**. Polyinosinic-polycytidylic acid of low molecular weight (0.2 – 1 kB) (Poly (I:C)-LMW) was purchased from Invivogen (San Diego, USA). Ultrapure chitosan hydrochloride salt (Protasan UP Cl 113, Mw: 50,000-150,000 g/mol) was purchased from Novamatrix (Norway). Degree of deacetylation was confirmed by elemental analysis to be 75 ± 2%. Pentasodium tripolyphosphate (TPP), trehalose, TFA (Trifluoroacetic acid ≥99%), ammonium hydroxide and heparin were obtained from Sigma-Aldrich (Madrid, Spain). Chitosanase 10U purified from Streptomyces griseus (C4163-01) was purchased from United States Biological (MA, USA). SYBR Gold® and Nunc Maxisorp™ MicroWell™ flat-bottom 96-well plates were purchased from Thermo Fisher Scientific (Waltham, USA). HPLC-grade acetonitrile and water were purchased from Scharlab (Spain) and Fisher Chemical (Thermo Fisher Scientific, USA).

Human serum adsorbed and peroxidase labeled anti-mouse IgG (H+L) goat antibody was purchased from KPL (MD, USA). The secondary anti-Rabbit IgG horseradish peroxidase linked whole antibody from donkey was purchased from Amersham (GE Healthcare Life Sciences, UK). ABTS was acquired from Roche (Basel, Switzerland). Imject® Alum was obtained from Pierce Biotechnology (USA).

2.2. Design and preparation of CS and pIC based nanoparticles

In order to obtain a monodisperse population of nanometric particles with efficient pIC incorporation, different TPP and pIC concentrations were tested. The TPP concentrations were 0, 0.25, 0.625 mg/ml while the pIC were 0.25, 0.625 and 1.25

mg/ml, with a constant volume of 0.2 ml. The CS concentration and volume was fixed at 1 mg/ml and 0.5 ml, respectively.

The formulation was produced upon addition of the TPP and/or pIC phase over the CS solution under magnetic stirring during 5 minutes. The resulting nanoparticles were let to stay for 30 minutes and, then, they were isolated by ultracentrifugation at 20,000 RCF, 4 ºC for 2 hours (Centrifuge 5430R, Eppendorf AG, Germany) on a glycerol bed. The supernatant was removed and the pellet (0.1 ml) was resuspended in ultrapure water with a pipette. The resulting particles were evaluated in terms of hydrodynamic diameter (size), polydispersity (PdI), derived count rate (DCR) and surface charge (zeta-potential). The positive-to-negative charge (P/N) ratio was defined as the ratio between the maximum number of protonable primary amines in CS (considering the 75% deacetylation degree determined by elemental analysis) and the sum of negative phosphate groups from TPP and pIC.

2.3. Physicochemical characterization

Particle size, PdI and DCR were evaluated by Dynamic Light Scattering (DLS) and zeta-potential by Electrophoretic Light Scattering (ELS) using a Zetasizer Nano ZS90 (Malvern Instruments, UK). The measurements were performed at 25 ºC with a detection angle of 173º, in distilled water. The morphology of the nanoparticles was examined by transmission electron microscopy (CM 12 Philips, Eindhoven, The Netherlands). The nanoparticles were placed on copper grids with Formvar films and stained with 2% (w/v) phosphotungstic acid solution. The grids were left overnight in an oven at 60 ºC to dry and then observed with TEM. The solutions and formulation pH was determined with a Sartorius Docu-pH Benchtop Meters (ThermoFisher Scientific, USA).

2.4. Evaluation of pIC loading in nanoparticles

The encapsulation efficiency of pIC in the nanoparticles was calculated from the amount of free pIC detected in the supernatant collected upon ultracentrifugation. The free pIC was determined by absorbance at 260 nm (Abs 260 nm) with a NanoDrop 2000 (Thermo Fisher Scientific) and quantified by interpolation in a linear standard curve (R^2

= 0.9988) produced with solutions of pIC solubilized in the nanoparticles supernatant in a concentration range from 2.5 to 80 μg/ml.

The indirect evaluation of the encapsulation of pIC was confirmed by electrophoresis in a 1% agarose gel using a Sub-Cell GT 96/192 electrophoresis system (Bio-Rad Laboratories Ltd., England). Briefly, the isolated nanoparticles were stained with SYBR Gold® and then 0.025 ml of formulation was added (equivalent to 8.4 μg pIC) to the agarose gel wells. After applying 90 V for 45 minutes, the gels were evaluated with an UV transilluminator (Molecular Imager® Gel Doc™ XR, Bio-Rad, CA, USA) and analyzed with Image Lab™ Software (Bio-Rad, CA, USA).

The stability of the pIC encapsulation was tested by incubating the nanoparticles with anionic heparin, based on the method by Csaba et al [24], and verifying its release in an agarose gel. In summary, fresh isolated nanoparticles and freeze dried nanoparticles were incubated in a 1.2 mg/ml heparin solution for 2 hours at 37 °C in order to have an excess of heparin in relation to the pIC (mass ratio pIC/heparin 1/3) and induce the competitive displacement of the pIC by the strong anionic polymer.

In order to verify that the pIC remained intact after encapsulation, the nanoparticles were incubated with chitosanase (an enzyme capable of hydrolyzing the CS into oligosaccharides, releasing the associated pIC) [7, 24]. In summary, 0.1 ml of fresh isolated nanoparticles (0.4608 mg), were incubated with 1.1 U of chitosanase in a 50 mM acetate buffer pH 5.5, for 4 hours at 37 ºC under agitation. After incubation, an aliquot (0.019 ml) was taken and tested in an agarose gel 1%. This same protocol was also applied to freeze dried particles (described below) to verify pIC stability in the nanoparticles after the freeze drying process.

2.5. Peptide Association to Nanoparticles

Firstly, the 1338aa peptide was solubilized in sterile water at the concentration of 10 mg/ml while the PADRE peptide, due to its higher hydrophobicity, was solubilized in a 0.07 M ammonium hydroxide solution to the final concentration of 5 mg/ml. The association of the peptides to the CS/TPP/pIC nanoparticles was achieved either by incorporation of the peptides into the TPP/pIC containing phase (**Protocol A**), during the nanoparticles formation process (section 2.2.), or by their incubation with the preformed nanoparticles (**Protocol B**), as illustrated in **Figure 1**. In the first case, the peptides were added to the aqueous phase containing TPP and pIC, at the concentration

of 0.125 mg/ml (pH 8.8). The whole mixture of components was added to the CS phase (1 mg/ml, pH 4.80) under magnetic stirring, and then left for 1 hour at room temperature to produce the formulation NP E2. In the second case, a 0.1 ml of the solution of 1338aa alone (pH 6.4) or of the solution of 1338aa and PADRE (pH 9.6) at the concentration of 0.25 mg/ml, was poured over the nanoparticle suspension (pH 5) under magnetic stirring to generate the formulation NP A1 and NP A2, respectively. The stirring was maintained for 5 minutes, and then the formulation was left for 1 hour at room temperature. Finally, a loaded nanoparticle formulated with the same method as NP A2, but without pIC (NP C), was also produced as a control. The theoretical loading of each peptide with respect to the CS mass was 5% in all cases. After the incubation period, the formulations were isolated by ultracentrifugation at 20,000 RCF, 4 ºC for 2 hours on a glycerol bed (Centrifuge 5430R, Eppendorf AG, Germany).

2.6. Evaluation of the 1338aa and PADRE association

To evaluate the peptide association efficiency, the loaded nanoparticles were first isolated by ultracentrifugation, as previously described. The supernatant was eliminated and the nanoparticles pellet was recovered and digested with chitosanase in the same conditions as reported above (section 2.4). The peptide released after nanoparticle digestion was determined by an ultra performance LC (UPLC) coupled with UV detection system (ACQUITY UPLC H-Class, Maryland, USA) using an a reverse phase Acquity UPLC BEH C18 column (1.7 um 130 Å 2.1 x 100 mm) and a gradient elution. The mobile phase A consisted of TFA 0.1% (v/v) aqueous solution and the mobile phase B of TFA 0.1% (v/v) in acetonitrile. The rest of UPLC conditions are described in the **Annex 1**. For the standard curve, the peptides were diluted in the digested matrix of nanoparticles (NP B) at concentrations between 3.125 and 100 µg/ml.

2.7. Freeze-drying of nanoparticles

Lyophilization was conducted in a VirTis Genesis 25L lyophilizer (Model SQ EL-85, SP Scientific, Pennsylvania, USA). The samples were frozen overnight at -20 °C and then transferred to the lyophilizer. They underwent an initial drying step for 24 h at -35 °C at 2-10 millitorr (mtorr) followed by a secondary drying for another 24 h at 0 °C and finally a third step of 16 hours at 20 °C at the same pressure. Trehalose was used as a cryoprotectant at different concentrations (0, 2.5%, 5% and 10% w/v), while the

nanoparticle concentration was evaluated at 42% and 25% (v/v). After freeze drying, loaded nanoparticles were reconstituted with ultrapure water (0.1 ml) in order to analyze their particle size and PdI. The same freeze drying cycle was used to dehydrate the isolated formulations and determine the formulation process yield and the nanoparticle concentration by gravimetric analysis.

2.8. Immunization studies

The ability of nanoparticles to induce an antibody response against the 1338aa peptide was assessed in 6- to 8-week-old female BALB/c mice obtained from the National Cancer Institute (NCI), housed and handled under specific pathogen–free conditions in the animal care facilities at the NCI, Bethesda. Groups of 5 mice were randomly assigned and immunized intramuscularly (i.m) in the anterior thigh, with a first and second boost given 21 and 42 days after, respectively. The immunization dose of 1338aa was 5 μg (0.05 ml).

The control, alum-adsorbed antigen, was prepared according to the manufacturers specifications. Briefly, the Imject® Alum was agitated until complete homogenization of the dispersion and then it was added dropwise over a 1338aa solution in PBS, producing a final dispersion of alum salt and 1338aa at the concentration of 0.1 mg/ml. This dispersion was left under agitation for 1 hour at room temperature before administration. As an additional control, the same formulation without pIC (NP C) was used.

The blood samples (0.2 ml) were collected by retro-orbital bleeding at 0, 3, 6 and 8 weeks after the first vaccination. The animals were anesthetized (isofluorane 5% in oxygen vapor) before each immunization and blood sample collection. The blood samples were ultracentrifugated for 10 minutes at 14,926 RCF and then 0.05 ml of serum was collected. Serum IgG levels for 1338aa were determined for each sample by ELISA. For this purpose, Maxisorp® 96-wells were coated with 0.1 ml of the 1338aa peptide at 5 μg/ml in PBS and incubated overnight at 4 ºC. After washing with PBS, the wells were blocked with 0.2 ml of 5% bovine serum albumin in carbonate-bicarbonate buffer for 2 hours at 37 ºC. After washing again with PBS, 0.1 ml of the respective serum, diluted 1:50 in PBS with 2% FBS, was added and incubated 1 hour at 37 ºC. After new wash with PBS and 0.05% Tween 20, 0.1 ml of secondary antibody (goat anti-Mouse IgG (H+L) labeled with HPR) diluted 1:1000 in PBS with 1% FBS was

added to the wells, and left incubating for 1 hour at room temperature. Finally, the wells were washed again with PBS, and then the ABST substrate was added to the wells. After 40 minutes incubation, the absorbance was determined in a microplate reader (405 nm, POLARstar OPTIMA, BMG-Labtech Gmbh, Germany). All the washing and blocking steps were done with an automated microplate washer (ELx405 Select Deep Well Microplate Washer, Biotek, USA). All serum samples were tested in duplicate.
As positive control for the 1338aa peptide coating of the wells, HPV16 L2 peptide 17-36aa rabbit antiserum was used in a dilution of 1:1000 [13], and as secondary antibody, an anti-rabbit IgG was used in a dilution of 1:2000.

3. RESULTS AND DISCUSSION

The objective of the present work was to design a nanocarrier, specifically adapted for the co-association of antigens and different kinds of immunostimulants. The final goal was to explore the synergistic effect of different adjuvant molecules assembled together in a nanostructure. With this idea in mind we chose three main components for the formation of the nanostructures: chitosan, known for its capacity to facilitate antigen presentation to the immune system when presented in a nanometric structure [6, 7], poly (I:C), a potent immunostimulant and a T-Helper peptide (PADRE). To assess the value of this nanocomposition, we selected a challenging HPV related peptide-based antigen (1338aa) (**Figure 1**). This peptide was either adsorbed onto the nanoparticles surface, or incorporated within the nanoparticle structure. Here we present the key aspects of the development and characterization of these new nanostructures and the results of an exploratory *in vivo* efficacy assay.

3.1. Design and characterization of Chitosan-Poly (I:C) based nanoparticles

The development process of the multicomponent nanocomposition consisted of several subsequent steps. The first step involved the encapsulation of poly (I:C) in CS based nanoparticles. Based on previous work from our group [26], we selected CS/TPP mass ratios of 4/1 and 8/1 for the formation of the nanoparticles. The TPP is an ionic cross-linking agent that promotes the gelation of chitosan and facilitates the formation of well defined spherical nanoparticles. The CS/pIC mass ratio tested was 4/2, 4/1 and 4/0.4, which is equivalent to a pIC theoretical loading of 10, 25 and 50%, respectively, in relation to the total amount of CS used for particle preparation (**Table 2**). Simple

complexes of CS and pIC, without TPP, were also produced as previously described [24, 27]. These nanoparticles and nanocomplexes were expected to protect poly (I:C) from degradation [24, 28], and facilitate its delivery in the immune cells, where its receptor (TLR3) is located.

Table 1. Physicochemical characteristics of the peptides

Peptide	Amino acid sequence	MW	Purity	pI	Charge at pH			
					5.7	6.4	8.8	9.6
1338aa	ASAWQLYKTCKQAGTCPPDIIP KVEGDDDDDD	3411	95%	3.94	-4	-9	-6	-7
PADRE	(D-Ala)K(Cha)VAAWTLKA(D-Ala) DDDDDD	1973	99%	3.85	-4	-4	-4	-5

*MW: Molecular weight as provided by manufacturer; (Cha): Cyclohexylalanine; (D-Ala): D-Alanine; pI: Theoretical pI predicted by online software [25]; Charge at pH: Charge calculated according to the method described in **Annex 2**).*

The nanoparticles were formed spontaneously upon the addition of TPP and pIC to the CS phase. As shown in the **Table 2**, the CS/TPP/pIC mass ratio 4/1/1 and 4/1/2 (25% and 50% pIC loading, respectively) led either to the formation of aggregates or to highly polydisperse particles, as the P/N ratio was close to neutrality (1/1.13 and 1/0.95). The aggregation of the colloidal system at these mass ratios could result from the lack of inter-particular repulsive force due to the saturation of the CS amino groups. Accordingly, the CS/TPP/pIC 4/1/0.4 (correspondent to 10% pIC loading), presented a higher P/N ratio (1/0.85) and formed nanometric population of particles with low polydispersity.

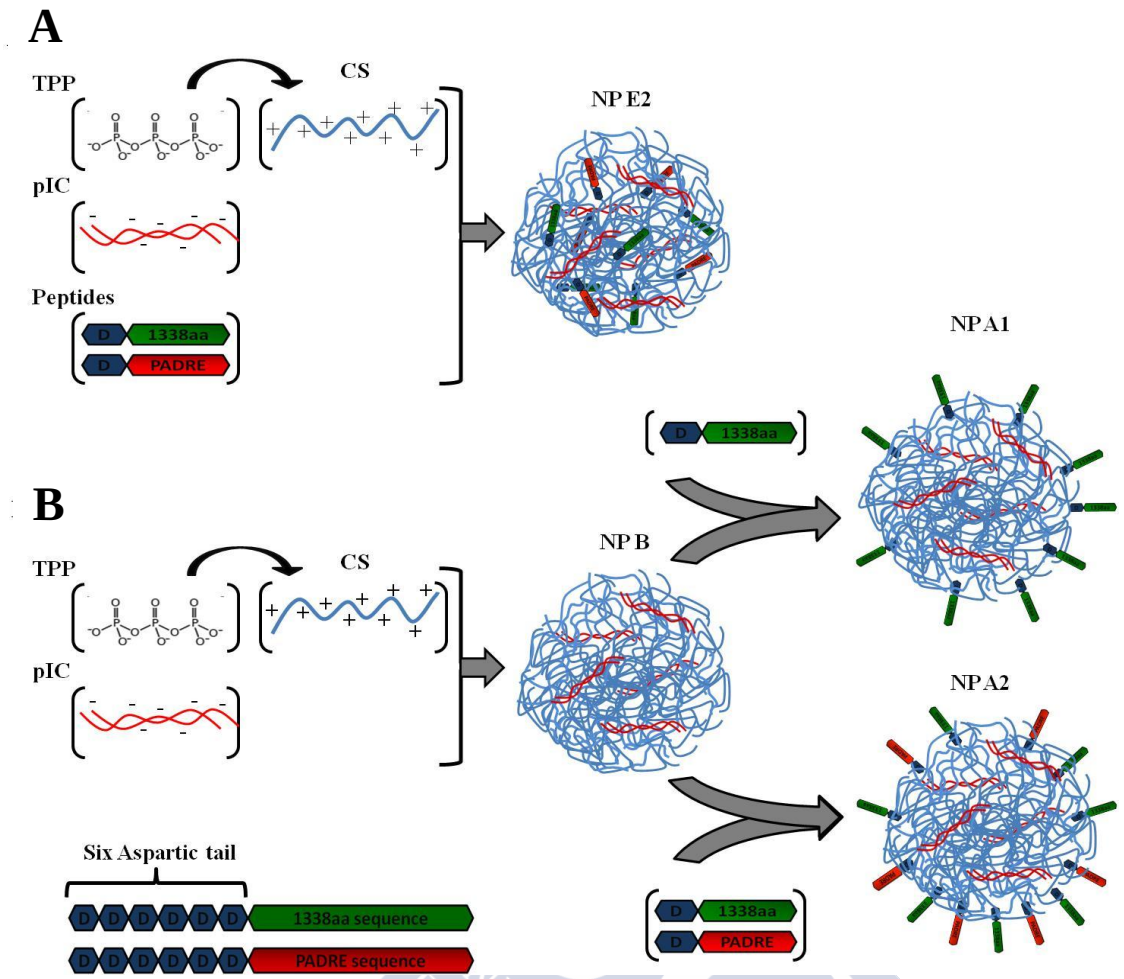


Figure 1. *Illustration of peptide-loading into nanoparticles either by encapsulation of both peptides to generate the formulation NP E2 (**A**), by adsorption of 1338aa or of 1338aa and PADRE to generate the formulation NP A1 and NP A2, respectively (**B**). Representation of both peptides, divided in their active part (1338aa sequence and PADRE sequence) and its six aspartic acid segment designed to promote their interaction with the CS amino groups.*

At CS/TPP 8/1 mass ratio it was possible to obtain nanometric structures with greater proportion of pIC (higher pIC loading), possibly due to the greater number of available amino groups for interaction with the pIC and to avoid colloidal aggregation. Accordingly, an increase in the surface charge of the resulting particles was also observed with increasing P/N ratios.

The complexes formed by ionic interaction of CS and pIC without TPP, presented a high PdI as compared with nanoparticles produced by ionic gelation of CS with TPP [24].

Table 2. Physicochemical characterization of Chitosan-Poly (I:C) nanoparticles at different mass ratios

CS/TPP/pIC	P/N	pIC (%)	Size (nm)	PdI	ζ (mV)	Derived Count Rate
4/1/2	1/1.13	50	Aggr.	-	-	-
4/1/1	1/0.95	25	369 ± 27	0.5	+32 ± 3	108264
4/1/0.4	1/0.85	10	284 ± 15	0.2	+41 ± 1	46537
8/1/4	1/0.74	50	363 ± 14	0.3	+42 ± 2	64724
8/1/2	1/0.56	25	290 ± 25	0.2	+43 ± 5	24955
8/1/0.8	1/0.46	10	302 ± 23	0.2	+55 ± 3	10495
8/0/4	1/0.35	50	350 ± 24	0.3	+49 ± 1	49503
8/0/2	1/0.17	25	243 ± 8	0.3	+56 ± 1	19691
8/0/0.8	1/0.07	10	172 ± 5	0.2	+56 ± 1	6064

CS/TPP/pIC mass ratio: Mass ratio between chitosan, TPP and poly (I:C); P/N charge ratio: Charge ratio between the amine groups of CS and the sum of the phosphate groups of TPP and pIC; pIC (%): Loading of poly (I:C) in particles in relation to the chitosan mass; PdI: Polydispersity Index; ζ: Zeta potential. (Means ± S.D., n=4).

The entanglement of the pIC in the gelled matrix of CS and TPP may provide a better entrapment of the polynucleotides in comparison to the simple electrostatic interaction between CS and pIC, as observed previously for pDNA [24] and dsRNA [28]. Thus, the gelled nanostructure could be more adequate for preventing the premature release of pIC. This is a critical issue given the potential toxicity associated to the systemic release of pIC [29].

Among the CS/TPP/pIC mass ratios tested, those consisting of 4/1/0.4 and 8/1/2 led to the formation of nanoparticles with an acceptable size (<300 nm) and PdI (<0.3). On the other hand, the yield of the nanoparticles formation process was much higher for the ratio 4/1/0.4 than for 8/1/2 (yield of 77 and 47%, respectively). Therefore, the CS/TPP/pIC mass ratio 4/1/04 (from now on named NP B) was selected for further

experiments. These nanoparticles, exhibited a spherical shape (**Figure 2**) and a high positive surface charge (+41 mV). This positive charge, which indicates that pIC was conveniently entrapped within the chitosan matrix, is an important feature for the subsequent adsorption of the anionic peptides 1338aa and PADRE and also to facilitate nanoparticle uptake by antigen presenting cells [30].

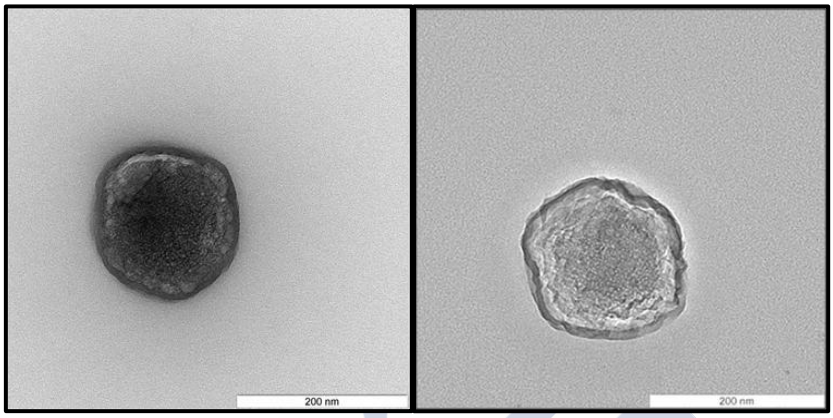


Figure 2. *Morphology of Chitosan/TPP/Poly (I:C) nanoparticles (NP B) visualized by transmission electron microscopy. The scale bars correspond to 200 nm.*

3.2. Association and release of pIC from CS/TPP/pIC nanoparticles

As determined by spectrophotometry, the association efficiency of pIC in the selected mass ratio composition CS/TPP/pIC 4/1/0.4 was close to 100%. This high pIC association efficiency was further confirmed by agarose gel electrophoresis. The migration patterns presented in **Figure 3**, show the absence of bands corresponding to free pIC in the nanoparticles suspending medium (obtained upon centrifugation of the suspension) (well 7) or in the nanoparticle suspension (well 8). This high efficiency is in agreement with previous work showing the high capacity of CS/TPP nanoparticles for the association of DNA and dsRNA [24, 28]. To verify if the pIC was firmly associated to the nanoparticles, the system was incubated with heparin, a strong polyanion that might compete for the CS amino groups and displace loosely attached polynucleotide material [27]. The results showed that after incubation with an excess of heparin (1.2 mg/ml), only a small amount of pIC migrated (well 9), which illustrates the high affinity of pIC towards CS/TPP nanoparticles. This is an important result as it is known that the cell extracellular matrix contains polyanions similar to heparin that might favor the disassociation of polynucleotides [31]. In order to assess the stability of the pIC, the

nanoparticles were digested with chitosanase to facilitate the release of pIC, which was then evaluated by agarose gel electrophoresis. The results showed that the migration pattern of the released pIC molecules was the same as that of the control pIC. Consequently, it can be concluded that pIC was firmly associated to the nanoparticles and could be released in an appropriate manner as a result of the polymer degradation. These results are in agreement with those previously reported showing the capacity of chitosan/TPP nanoparticles to deliver DNA [24] and dsRNA to different cells [28], including macrophages [32]. However, from our knowledge, this is the first report disclosing the potential of chitosan nanoparticles for the association of pIC as a strategy for enhancing the immunogenic response to the associated antigens.

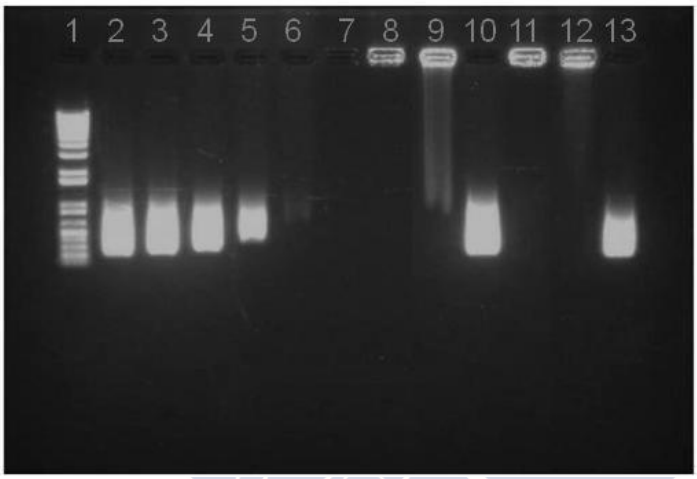


Figure 3. *Agarose gel assay on pIC release from NP B (Chitosan/TPP/Poly (I:C) without peptides): 1) DNA Ladder; 2) 8 μg pIC; 3) 4 μg pIC; 4) 2 μg pIC; 5) 1 μg pIC; 6) 0.5 μg pIC in solution; 7) NP B Supernatant; 8) NP B Pellet; 9) NP B Pellet incubated with heparin; 10) NP B Pellet digested with chitosanase; 11) NP B Pellet freeze dried; 12) NP B Pellet freeze dried and incubated with heparin; 13) NP B Pellet Freeze dried incubated with chitosanase.*

3.3. Evaluation of the 1338aa and PADRE peptide association to CS/TPP/pIC nanoparticles

The peptides 1338aa and PADRE were designed to incorporate HPV16 L2 neutralizing and T-helper amino acid sequences, respectively. In addition to these active sequences, a segment of six aspartic acid molecules was also incorporated at the C-terminus region of both peptides (**Table 1**). This segment was intended to work as an electrostatic

anchor for the positively charged chitosan molecules, thus leaving the active region of the peptide available for interaction with its specific receptors (**Figure 1**). As shown in **Table 1**, at the formulation pH (5.74) the peptides are expected to present a high negative charge, which would enable the electrostatic interaction with the positively charged nanoparticles (**Table 2**).

Two different protocols were used for the association of the peptides to the nanoparticles in order to promote their localization, either inside (**Protocol A**) or on the surface of the nanoparticles (**Protocol B**) (**Figure 1**). The nanoparticles obtained, following both protocols, presented a nanometric size, a low pDI and a positive zeta-potential (**Table 3**).

Table 3. Physicochemical characteristics and loading efficiency of Chitosan/TPP/Poly (I:C) nanoparticles (Chitosan/pIC) loaded with 1338a and PADRE peptides

Formulation	Code	Size (nm)	PdI	ζ (mV)	1338aa (%)	PADRE (%)
Chitosan/pIC without peptide (Blank)	**NP B**	284 ± 15	0.2	+41 ± 1	N/A	N/A
Chitosan/pIC-1338aa Adsorbed	**NP A1**	299 ± 23	0.2	+49 ± 5	39 ± 1	N/A
Chitosan/pIC/PADRE-1338aa Adsorbed	**NP A2**	274 ± 9	0.2	+47 ± 5	50 ± 5	75 ± 13
Chitosan/pIC/PADRE-1338aa Entrapped	**NP E2**	290 ± 28	0.2	+48 ± 4	67 ± 8	71 ± 3

Chitosan/pIC-1338aa Adsorbed: Chitosan/TPP/Poly (I:C) nanoparticle with 1338aa adsorbed at surface; Chitosan/pIC/PADRE-1338aa Adsorbed: Chitosan/TPP/Poly (I:C) nanoparticle with PADRE and 1338aa adsorbed at surface; Chitosan/pIC/PADRE-1338aa Entrapped: Chitosan/TPP/Poly (I:C) nanoparticle with PADRE and 1338aa encapsulated; Size: Particle size distribution; PdI: Polydispersity Index; ζ: Zeta potential; 1338aa (%): Percentage of peptide 1338aa associated to the nanoparticle; PADRE (%): Percentage of peptide PADRE associated to the nanoparticle. Loading values were obtained from the direct determination of the peptide released peptide after the digestion of the nanoparticles with chitosanase. (Means ± S.D., n=3).

In both methods, the resulting peptide-loaded nanoparticles presented a positive surface charge, which in the case of the particles produced by the **Protocol B** indicates that their surface was not totally covered by the adsorbed peptide molecules. In **Protocol A**, the peptides were dissolved together with TPP and pIC and added to the CS phase to facilitate simultaneous 1338aa and PADRE entrapment within the nanoparticle matrix (loading efficiency of 67 and 71%, respectively) (**Table 3**). The basic pH of the TPP/pIC/peptides phase (pH 8.8) possibly increased the anionic character of the peptides (**Table 1**), thereby improving their interaction with CS. In **Protocol B**, the peptides were simply adsorbed onto the nanoparticle surface and, as expected, the antigen association efficiency was lower (39 – 50%) [33] although the PADRE association remained almost unaltered (75%). Interestingly, when the peptide 1338aa was adsorbed onto the nanoparticles in combination with the PADRE peptide, it had higher association than when added alone (50% and 39%, respectively) (**Table 3**).

A possible cause for this could be that the combined solution of 1338aa and PADRE had a higher pH (9.6) than that of the 1338aa peptide alone (6.4). This could have increased the negative charge of 1338aa (**Table 1**) and, consequently, provide a more important interaction with the positively charged CS. The higher charge density of PADRE compared with 1338aa peptide (1 charge/3 amino acids and 1 charge/3.6 amino acids, respectively) could explain its overall higher association (loading efficiency of 70-75% vs 50%-67%). By digesting the nanoparticles and analyzing the released peptides by UPLC, it was also possible to verify that both peptides maintained their stability upon association to the nanoparticles (**Annex 3**). The mild formulation conditions probably contributed to the stability observed both for the pIC and peptides associated to the nanoparticles. In addition, all the formulations maintained their physicochemical characteristics for at least one year at 4 ºC (**Figure 4**).

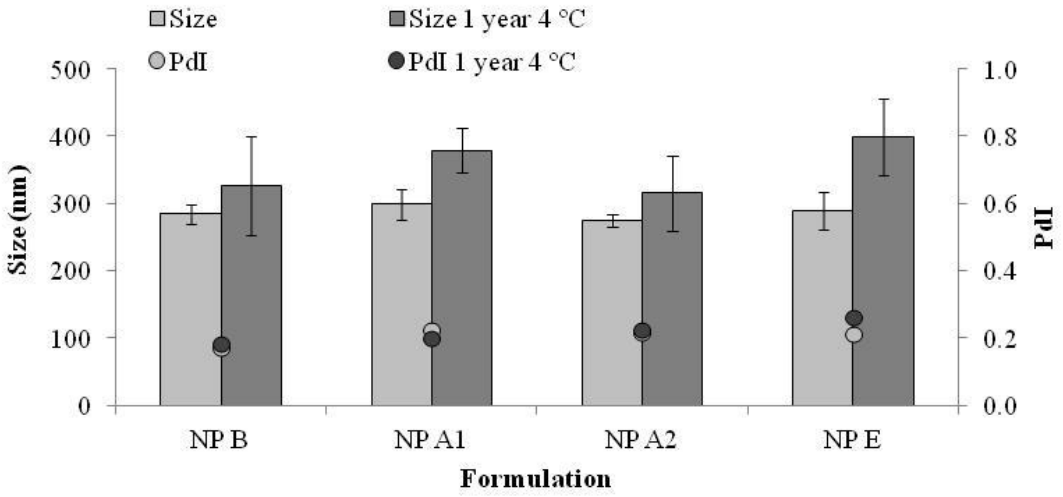


Figure 4. *Particle size distribution (Size) and polydispersity index (PdI) after 1 year storage at 4 ºC in suspension of nanoparticles without peptides (NP B), nanoparticles with 1338a adsorbed at surface (NP A1), with 1338aa and PADRE adsorbed at surface (NP A2) and both peptides entrapped in the matrix (NP E).*

3.4. Freeze-drying of CS/TPP/pIC nanoparticles

In general, the dehydration of the nanoparticle formulations helps preventing particle aggregation and degradation [34]. For this reason, the feasibility of freeze-drying the CS/TPP/pIC nanoparticles was explored. For this, trehalose was chosen as a cryoprotectant [34] and the influence of the formulation concentration (42 and 25% (v/v)) and the trehalose concentration (10, 5 and 1% (w/v)) in the resuspension of the dried formulation was studied. The resulting powder cakes were resuspended with 0.1 ml of ultrapure water to provide for a final antigen concentration of 0.1 mg/ml. A 42% (v/v) nanoparticle concentration at 5% trehalose (w/v) generated an adequate cake, which could be easily resuspended. The resuspended particles presented only a modest variation in size, PdI and zeta-potential (between 10-15%), as shown in **Annex 4**. An additional positive effect of the developed freeze-drying method is that due to the final trehalose concentration (12%), the resuspended solution was close to isotonic.

The stability of the pIC associated to the freeze-dried nanoparticles upon one week storage at room temperature was analyzed by agarose gel electrophoresis (**Figure 3**). Similar to the fresh particles, the absence of the free pIC band in the gel (well 12) indicates that the pIC remains firmly attached to the particle after the freeze-drying (well 11) even after incubation with heparin at 37 ºC (well 12). Furthermore, upon

digestion of the nanoparticle with chitosanase, the pIC band became visible with a migration pattern and intensity (well 13) similar to those of the control (well 2). This indicates that these nanoparticles can effectively protect the pIC from degradation during freeze-drying and upon storage in a dry powder form. After confirming the suitability of the freeze drying procedure for the pIC-containing nanoparticles, the peptide-loaded CS/TPP/pIC formulations were freeze-dried in the same conditions. The results showed that these formulations also preserved their physicochemical characteristics after resuspension, with only minor variation in size and zeta-potential (**Figure 5**).

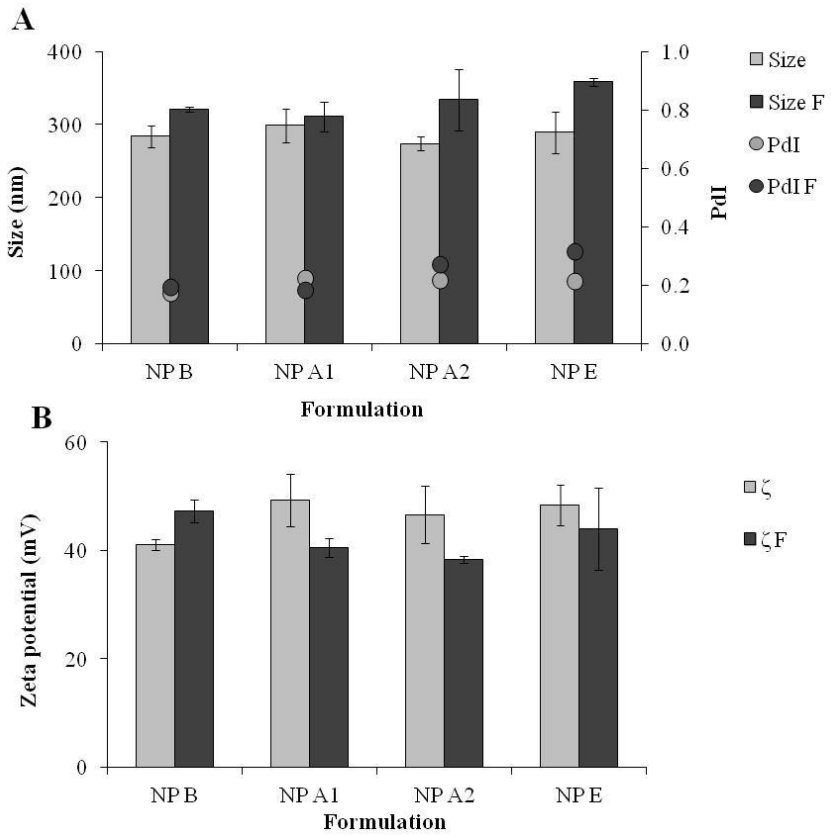


Figure 5. *Physicochemical evaluation of nanoparticles without peptides (NP B), nanoparticles with 1338a adsorbed at surface (NP A1), with 1338aa and PADRE adsorbed at surface (NP A2) and both peptides entrapped in the matrix (NP E) after freeze drying and resuspension operation. Size: Size of the formulation before Freeze-Drying; Size F: Size after Freeze-Drying; PdI: Polydispersity of formulation before Freeze-Drying; PdI F: PdI after Freeze-Drying; ζ: Zeta-potential of formulation before Freeze-Drying; ζ F: Zeta-potential after Freeze-Drying.*

3.5. Immunization studies

A preliminary vaccination study was conducted to evaluate the influence of the different components and their organization on the ability of the nanoparticle to generate a humoral immune response. The formulations selected, presenting different 1338aa and PADRE arrangements, were: nanoparticles with the peptide 1338aa alone adsorbed at the surface (NP A1), with the peptide 1338aa and PADRE both adsorbed at the surface (NP A2) and the nanoparticles with both peptides encapsulated (NP E2). These formulations and also three controls, the alum-adsorbed antigen, nanoparticles without peptides (NP B) and nanoparticles with peptides but without pIC (NP C), were administered to mice by the intramuscular route and the IgG response was monitored for up to 8 weeks (**Figure 6**).

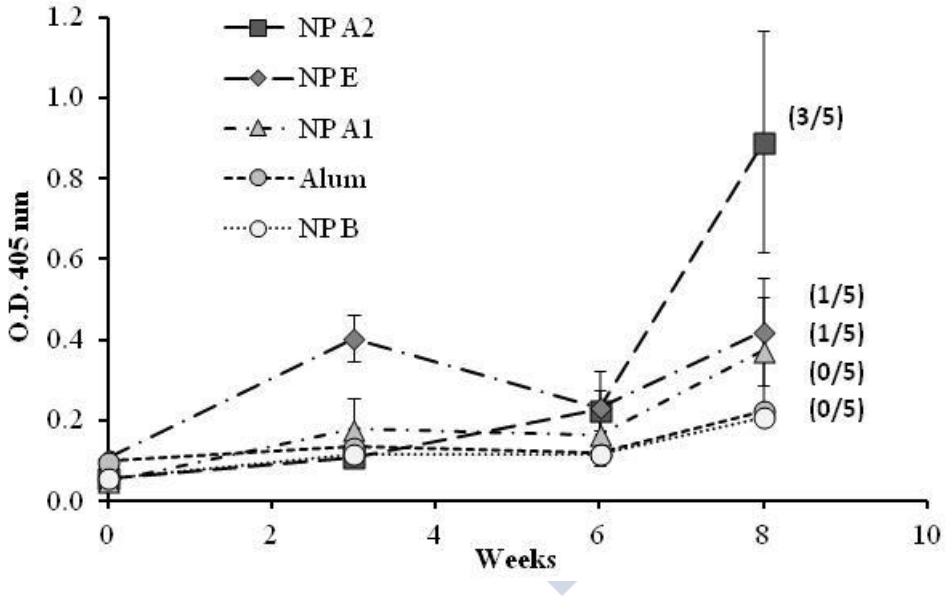


Figure 6. *1338aa peptide-specific IgG responses in diluted serum (1/50) of mice immunized with different formulations by intramuscular route. NP B: Chitosan/TPP/Poly (I:C) nanoparticles without peptides; NP C: Chitosan/TPP nanoparticles with 1338aa and PADRE adsorbed on their surface; NP A1: Chitosan/TPP/Poly (I:C) nanoparticles with 1338aa adsorbed on their surface; NP A2: Chitosan/TPP/Poly (I:C) nanoparticles with 1338aa and PADRE adsorbed on their surface; NP E: Chitosan/TPP/Poly (I:C) nanoparticles with 1338aa and PADRE encapsulated. (Number / 5): responsive mice in the total 5 mice per group. O.D. 4 times higher than those of the control NP B (0.2) was considered a positive response.*

The O.D. values for IgG shown in **Figure 6** indicate that alum was unable to enhance the immune response against the 1338aa peptide. On the other hand, peptide-loaded CS nanoparticles without pIC, known for their capacity to increase IgG responses against various protein antigens [6, 7, 35-37], failed to generate peptide-specific IgG responses. These results underline the difficulty in generating an immune response against poorly immunogenic peptides. With regard to the behavior of the formulation adjuvanted with either pIC, PADRE or both, the results were variable, exhibiting from 1 to 3 responding animals out of 5 immunized mice. Among the formulations tested, the composition containing both pIC and PADRE was capable of generating significant IgG levels in 3 out of 5 mice. These results suggest that both T helper and immunostimulant signals are required in order to achieve satisfactory antibody responses. In addition, the display of the peptide on the nanoparticle surface also appears to be critical. Indeed, the number of responding mice was greater when both components, pIC and PADRE, were adsorbed rather than entrapped.

The HPV L2 epitope 17-36aa [13], present in the peptide 1338aa, has already been tested in mice [38] although at an higher antigen dose (40 μg). In this study it was observed that the Freund`s adjuvant was unable to stimulate an effective immune response against this peptide, thus confirming the low immunogenicity of this antigen. However, the conjugation of this peptide to other T Helper peptide (P25) and to the immunostimulant Pam_2Cys (a TLR2 agonist), resulted very effective in terms of IgG responses. From the comparison of these previous data with those obtained in the current study, we could formulate different hypotheses. First, there is the possibility that either the antigen (17-36aa) or the immunostimulants (P25 and Pam2Cys), or their combination were more appropriate for enhancing the immune response. In this sense, it is important to keep in mind that, although sharing the same epitope, the peptide 1338aa presents a longer amino acid chain and an additional six aspartic segment in the C-terminus region, which could potentially lead to a different processing and presentation by the immune cells [3]. A second hypothesis could be that the conjugation to the T helper peptide to the peptide antigen facilitates its presentation to the immune system.

The poor immunogenicity of peptide-based antigens has been associated, among other factors, to their inability to establish a highly repetitive epitope pattern, which results in a limited recognition by the APCs [39]. Based on this, the adsorption of the peptide 1338aa onto the surface of the nanoparticles was supposed to adequately display the

required epitope patterns repeatedly in a nanometric structure, resembling a virus. This hypothesis was partially confirmed by the higher response elicited by the prototypes with adsorbed antigen (NP A2), as compared to the prototypes entrapping the same (NP E2). However, the possibility to further improve the presentation of the peptide epitopes cannot be discarded. As the epitope region of the peptide was connected directly with the six aspartic segment used for adsorption to the nanoparticle, it is possible that part of the active region got embedded in the nanoparticle matrix hindering its recognition by immune cells. The addition of a generic peptide spacer between the particle binding motif and the target peptide could avoid this possibility and provide for a more efficient presentation of the target peptide to B cells. Furthermore, while CS/TPP nanoparticles have demonstrated capacity for sustained release of proteins [7, 33], the release profile with these peptides has not been studied and should be explored in detail in further studies. On the other hand, according to the data presented, and in agreement with previous studies [24, 40], the pIC is firmly associated to the nanoparticles and its release may take longer than expected. As indicated previously, the introduction of pIC into the nanoparticles was expected to potentiate DC maturation [14] under the assumption that it would be released in endocytic vesicles along with the bound peptides. It has already been observed that for an optimal immune response with pIC, it is important that its delivery occurs simultaneously with the antigen of interest [41].

Therefore, this study provides an overall illustration of the potential of peptide-based antigens in conjunction with immunostimulatory molecules and it also provides insights into the complexity of the engineering process. Probably, a large array of different peptide and immunostimulant combinations should be performed according to their structural organization and size in order to assess the value of nanotechnology in the design of novel peptide-based nanovaccines.

4. CONCLUSIONS

Here we report for the first time, a chitosan nanostructure designed to co-deliver a TLR3 agonist (Poly (I:C)) and a T-helper peptide peptide (PADRE) and function as adjuvant system for a HPV-derived peptide antigen (1338aa). These nanoparticles are produced by ionic gelation, a mild and gentle method which permits, by a subtle modification of the formulation procedure, either the encapsulation or the adsorption of the target peptides. The capacity of combining different materials (polysaccharides,

polynucleotides and polyamino acids) in the form of nanoparticles, and the possibility to freeze-dry them highlights the versatility of this system and opens the possibility of integrating other immune stimulants. The capacity of inducing an immune response towards this particularly poor immunogenic antigen 1338aa is in contrast to the low efficacy observed in the same study for alum salts. In conclusion, the preliminary data presented here shows the potential of these new prototypes as vaccine adjuvants for the delivery of peptide-based antigens.

Annex 1. UPLC conditions and peptide retention times

Gradient			Additional features		Retention time (min)	
Time (min)	Phase A	Phase B	Flux (ml/min)	0.2	1338aa in dig.	4.674
0	80	20	Wavelength Abs (nm)	280	PADRE in dig.	4.927
1.50	80	20	Column Temp. (ºC)	35 ºC		
1.51	55	45	Filter time Constant	Normal		
3.00	80	20	Volume injected (ul)	5		

1338aa in dig.: 1338aa dissolved in Chitosan/TPP/Poly (I:C) nanoparticle digested matrix; PADRE in dig.: PADRE dissolved in Chitosan/TPP/Poly (I:C) nanoparticle digested matrix.

Annex 2. Calculation of the peptides charge at specific pH

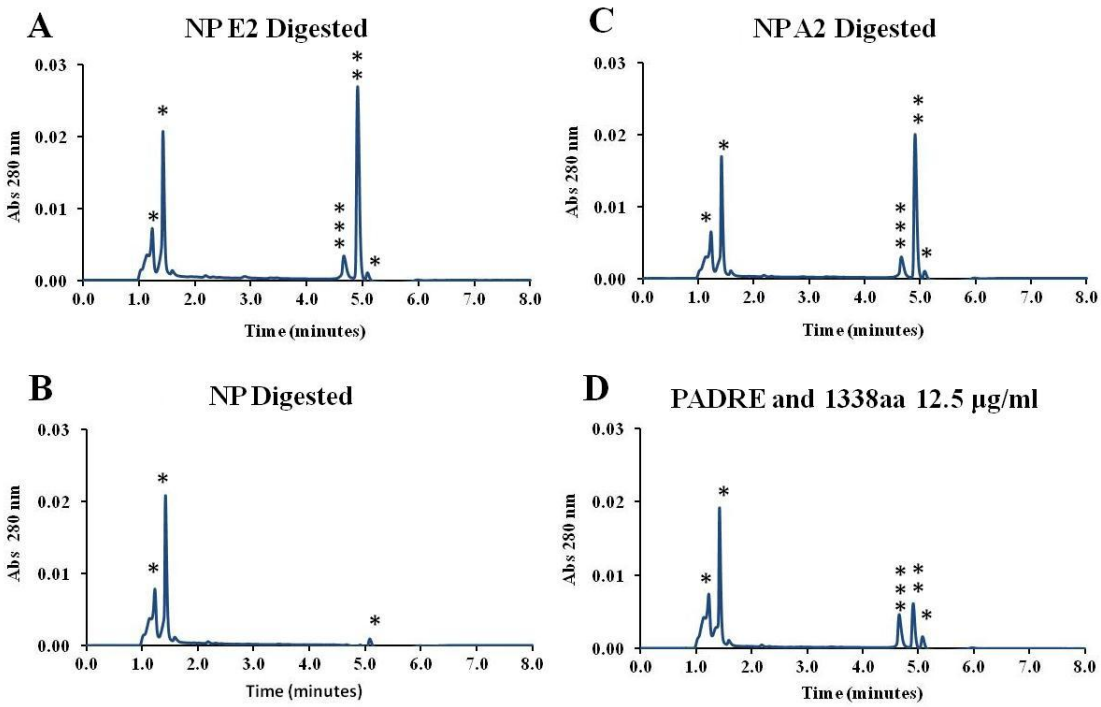
Calculation was realized with the following equation:

$$Z = \sum_i Ni \frac{10^{pKai}}{10^{pH} + 10^{pKai}} - \sum_j Nj \frac{10^{pH}}{10^{pH} + 10^{pKaj}}$$

In which Z represents the Net charge of the peptide sequence, Ni the number of arginine, lysine and histidine residues and the N-terminus, and pKai, their pKa. The Nj represents the number of aspartic acid, glutamic acid, cysteine and tyrosine residues and C-terminus, and the pKaj, their pKa.

Note: In both predictions, the non-standard residue ciclohexylalanine had to be ignored and the D-Alanine was replaced by the L-Alanine for calculation purposes which increases the error. However both amino acids are neutral so the influence in the pI and charge according pH should be negligible. In the other hand the aspartic tail should always be quite dominant in terms of peptide charge properties.

Annex 3. Comparison between UPLC peaks from digested blank nanoparticles and peptide-loaded nanoparticles, and the free PADRE and 1338aa peptides in digested nanoparticle matrix.



*Comparison between UPLC peaks of digested peptide-loaded nanoparticles (A: encapsulated B: surface adsorbed) with blank nanoparticles (C) and with free PADRE and 1338aa peptides in digested nanoparticle matrix as controls (D); *: Peaks from residues of digested nanoparticles; **: Peak from PADRE peptide; ***: Peak from 1338aa peptide.*

Annex 4. Optimization of freeze drying conditions with CS/TPP/pIC nanoparticles

NP B Vol. (ml)	[NP B] (v/v)%	[Tre] (w/v)%	Size (nm)	PdI
0.1	25	5	294 ± 8	0.2
		2.5	332 ± 19	0.3
		1	437 ± 47	0.5
	42	10	320 ± 20	0.2
		5	322 ± 4	0.2
		2.5	363 ± 53	0.3

NP B Vol.: Initial volume of CS/TPP/pIC nanoparticle in the freeze-drying assay; [NP B] (v/v) %: Nanoparticle concentration; [Tre] (v/v) %: Initial trehalose concentration (Means ± S.D., n=3).

REFERENCES

1. Yang, D., et al., *Peptide Vaccines.* New Vaccine Technologies, 2001: p. 214.

2. WHO, *Annex 1 - Guidelines for the production and quality control of synthetic peptide vaccies*. World Health Organ Technical Report Series. Vol. 889. 1999: WHO Expert Committee on Biological Standardization.

3. Claire-Anne Siegrist, *Vaccine immunology*, in *Vaccines (Sixth Edition)*, Offit Stanley A., Plotkin Walter A., and Orenstein Paul A., Editors. 2013, W.B. Saunders: London. p. 14-32.

4. Lanzavecchia, A., *Identifying strategies for immune intervention.* Science, 1993. 260(5110): p. 937-44.

5. Halim, A.S., et al., *Biocompatibility and Biodegradation of Chitosan and Derivatives*, in *Chitosan-Based Systems for Biopharmaceuticals*2012, John Wiley & Sons, Ltd. p. 57-73.

6. Vila, A., et al., *Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice.* Eur J Pharm Biopharm, 2004. 57(1): p. 123-31.

7. Prego, C., et al., *Chitosan-based nanoparticles for improving immunization against hepatitis B infection.* Vaccine, 2010. 28(14): p. 2607-14.

8. Mao, S., W. Sun, and T. Kissel, *Chitosan-based formulations for delivery of DNA and siRNA.* Adv Drug Deliv Rev, 2010. 62(1): p. 12-27.

9. Lowy, D.R. and J.T. Schiller, *Prophylactic human papillomavirus vaccines.* The Journal of Clinical Investigation, 2006. 116(5): p. 1167-1173.

10. Giroglou, T., et al., *Immunological analyses of human papillomavirus capsids.* Vaccine, 2001. 19(13-14): p. 1783-93.

11. Bosch, F.X. and S. de Sanjose, *Chapter 1: Human papillomavirus and cervical cancer--burden and assessment of causality.* J Natl Cancer Inst Monogr, 2003. 31: p. 3-13.

12. Roden, R.B., et al., *Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes.* Virology, 2000. 270(2): p. 254-7.

13. Gambhira, R., et al., *A protective and broadly cross-neutralizing epitope of human papillomavirus L2.* J Virol, 2007. 81(24): p. 13927-31.

14. Lundberg, A.M., et al., *Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems*. Vol. 110. 2007. 3245-3252.

15. Durand, V., et al., *Shaping of adaptive immune responses to soluble proteins by TLR agonists: a role for IFN-alpha/beta.* Immunol Cell Biol, 2004. 82(6): p. 596-602.

16. Sloat, B. and Z. Cui, *Nasal Immunization with Anthrax Protective Antigen Protein Adjuvanted with Polyriboinosinic–Polyribocytidylic Acid Induced Strong Mucosal and Systemic Immunities.* Pharmaceutical Research, 2006. 23(6): p. 1217-1226.

17. Sobel, D.O., et al., *Poly I:C Induces Development of Diabetes Mellitus in BB Rat.* Diabetes, 1992. 41(4): p. 515-520.

18. Cunningham, C., et al., *The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C).* Brain, Behavior, and Immunity, 2007. 21(4): p. 490-502.

19. Jennings, G.T. and M.F. Bachmann, *Designing recombinant vaccines with viral properties: a rational approach to more effective vaccines.* Curr Mol Med, 2007. 7(2): p. 143-55.

20. Hafner, A.M., B. Corthésy, and H.P. Merkle, *Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant.* Advanced Drug Delivery Reviews, 2013. 65(10): p. 1386-1399.

21. Alexander, J., et al., *Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides.* Immunity, 1994. 1(9): p. 751-761.

22. Alexander, J., et al., *Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce specific high titer IgG antibody responses.* J Immunol, 2000. 164(3): p. 1625-33.

23. Wu, C.Y., et al., *Improving therapeutic HPV peptide-based vaccine potency by enhancing CD4+ T help and dendritic cell activation.* J Biomed Sci, 2010. 17(88): p. 1423-0127.

24. Csaba, N., M. Koping-Hoggard, and M.J. Alonso, *Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery.* Int J Pharm, 2009. 382(1-2): p. 205-14.

25. Bioinformatics, S.S.I.o. *SIB ExPASy Bioinformatics Resource Portal*. 2011 08/01/2014]; Available from: http://web.expasy.org/protparam/.

26. Calvo, P., et al., *Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers.* Journal of Applied Polymer Science, 1997. 63(1): p. 125-132.

27. Koping-Hoggard, M., et al., *Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers.* Gene Ther, 2004. 11(19): p. 1441-52.

28. Katas, H. and H.O. Alpar, *Development and characterisation of chitosan nanoparticles for siRNA delivery.* Journal of Controlled Release, 2006. 115(2): p. 216-225.

29. Hafner, A.M., B. Corthesy, and H.P. Merkle, *Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant.* Adv Drug Deliv Rev, 2013. 65(10): p. 1386-99.

30. Harush-Frenkel, O., et al., *Targeting of nanoparticles to the clathrin-mediated endocytic pathway.* Biochemical and Biophysical Research Communications, 2007. 353(1): p. 26-32.

31. Ruponen, M., et al., *Extracellular and intracellular barriers in non-viral gene delivery.* J Control Release, 2003. 93(2): p. 213-7.

32. Howard, K.A., et al., *RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system.* Mol Ther, 2006. 14(4): p. 476-84.

33. Calvo, P., et al., *Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines.* Pharmaceutical Research, 1997. 14(10): p. 1431-6.

34. Abdelwahed, W., et al., *Freeze-drying of nanoparticles: Formulation, process and storage considerations.* Advanced Drug Delivery Reviews, 2006. 58(15): p. 1688-1713.

35. Vila, A., et al., *Design of biodegradable particles for protein delivery.* Journal of Controlled Release, 2002. 78(1–3): p. 15-24.

36. Lugade, A.A., et al., *Single low-dose un-adjuvanted HBsAg nanoparticle vaccine elicits robust, durable immunity.* Nanomedicine, 2013. 9(7): p. 923-34.

37. Garcia-Fuentes, M. and M.J. Alonso, *Chitosan-based drug nanocarriers: where do we stand?* J Control Release, 2012. 161(2): p. 496-504.

38. Alphs, H.H., et al., *Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2.* Proc Natl Acad Sci U S A, 2008. 105(15): p. 5850-5.

39. Schiller, J.T. and D.R. Lowy, *Raising Expectations For Subunit Vaccine.* J Infect Dis, 2014. 24.

40. Koping-Hoggard, M., et al., *Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo.* Gene Ther, 2001. 8(14): p. 1108-21.

41. Schlosser, E., et al., *TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses.* Vaccine, 2008. 26(13): p. 1626-37.

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CAPÍTULO 5

LYSOPHOSPHATIDYLCHOLINE AND LINOLEIC ACID BASED NANOSYSTEMS FOR VACCINATION

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

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ABSTRACT

The clinical use of safe and effective nanoemulsions (AS03 and MF59®), as vaccine adjuvants has shown the potential of lipid materials in the formulation of adjuvant systems. Based on this evidence, the objective of this work has been to engineer nanostructures combining a number of lipid materials presenting immunostimulant properties, with the polysaccharide chitosan, and to explore their potential vaccine adjuvanticity. Thus, using Lysophosphatidylcholine (LS), Linoleic Acid (LA) and Squalene (SQ), we designed two nanoemulsions (LA:LS and SQ:LA:LS) and by combining with Chitosan (CS), two nanocapsules (LA:LS:CS and SQ:LA:LS:CS). The prototypes were produced by the solvent displacement technique and exhibited a nanometric size (< 200 nm) and, either a positive (between +39 and +37 mV) or a negative (between -28 and -36 mV) surface charge, depending on the presence of CS in the formulation. The SQ-containing formulations showed the best stability profile upon storage (stable for 3 months at 4 ºC and 1 month at room temperature) and also upon incubation in physiological simulated media (stable for 24 hours in DMEM and RPMI with FBS). The formulations were evaluated for complement activation (all active it except LA:LS), cytotoxicity (IC50 between 16 and 47 μg/ml), cytokines production (IL-8 increased release) and Reactive Oxygen Species (ROS) production (increased ROS levels). Due to their higher stability and IC50, the SQ-containing formulations were chosen for a preliminary association assay with three different peptide antigen models (association between 7 - 45%). The formulations were found to have a low IC50 and low antigen association and need to be further optimized in order to form an adequate vaccine adjuvant.

1. INTRODUCTION

Vaccination is considered, nowadays, the most cost-effectiveness public health intervention [1]. The progressive refinement of antigens, regarding their precision and purity, has raised the need of innovative adjuvants that may compensate for their lack of danger signals [2]. In particular, the recent development of synthetic peptide-based antigens, aimed at targeting specific epitopes of the pathogens [3], has prompted the research oriented to the design of novel vaccine adjuvants.

Recently, the nanoemulsion-based adjuvants named as AS03 and MF59®, have proven to be superior than the widely established alum salts [4, 5] for some specific antigens such as influenza (Pandemrix® and Focetria®) [6]. These formulations are known to provoke the recruitment of leukocytes to the adjuvant region and their activation [7, 8], a fact that could be associated to the emission of danger signals by specific components of the formulation, but also to their nanometric size [9, 10].

Based on this knowledge, we have assumed that it is possible to engineer nano-emulsions with robust adjuvant properties and an acceptable toxicity profile. With this idea in mind, we have selected lysophosphatidylcholine (LS) as a surfactant [11] for the formulation of nanoemulsions containing oils, such as linoleic acid (LA) and squalene (SQ), all of them known for their adjuvant properties.

Indeed, LS is an endogenous lysophospholipid produced by the hydrolysis of the phosphatidylcholine of the cell membranes (**Annex 1**) and exhibits a specific immunostimulant behavior [12]. It is recognized as an endogenous danger signal released by cells during infection or trauma [13]. It is also known to work as a chemoattractant for T cells [14, 15], monocytes [16, 17] and professional phagocytes [12, 17, 18]. These chemotactic properties have been already confirmed following intracutaneous administration in humans [19]. Furthermore, LS has been shown to activate lymphocytes [20-22] and monocytes [23] promoting their differentiation in dendritic cells [24] and their subsequent maturation [25], as well as stimulate the antibody production by B cells [21]. Some authors have already proposed to take advantage of LS immunostimulant properties and used it as a simple mixture with the antigen model hen egg lysozyme (HEL). The results, after subcutaneous administration to mice, indicated that LS has adjuvant properties comparable to those of alum salts in

terms of humoral response (IgG) [13]. Other authors have also reported the absorption enhancing capacity of LS following its co-administration with proteins by the nasal route [26].

With regard to the oils selected to formulate the nanoemulsions, linoleic acid (LA) is an unsaturated lipid (18:2(n-6)) that it is present in emulsions for parenteral nutrition [27, 28]. It has been reported to act as a chemoattractant [29, 30], a promoter of neutrophiles activation [28, 30, 31] and an activator of the Nuclear factor-kappa B (NF-kB) [32], which is involved in the stimulation of both the innate and adaptive immune response [33]. On the other hand, squalene is a triterpene oil already used in MF59 and AS03 [34]. Due to its highly hydrophobic nature, squalene was selected to act as a reservoir for the slow release of lysophosphatidylcholine and linoleic acid, thus potentially reducing their toxicity and improving their presentation to the immune system [35, 36].

The above indicated nanoemulsions were also coated with Chitosan (CS), with the final purpose of increasing the nanoemulsion intranasal adjuvant capability. Indeed, CS is a positively charged and biodegradable polysaccharide [37] that exhibits mucoadhesive properties [38]. In fact, in previous works from our group, we have found the possibility to enhance the immune response of the recombinant hepatitis B virus surface antigen (rHBsAg) administered intranasally upon its association to CS nanocapsules [39].

Taking this into account, the objective of this work could be summarized as the rational design of nanometric adjuvants consisting of oils, surfactants and chitosan. Two nanoemulsions (containing LA and LS and with or without SQ) and two CS nanocapsules formulations (containing LA and LS and with or without SQ) were developed and evaluated in terms of: (i) stability in simulated physiological mediums and storage conditions; (ii) *in vitro* toxicity; (iii) capacity to stimulate the production of proinflammatory cytokines and reactive oxygen species (ROS) by immune cells; (iv) ability to activate the complement in plasma; (v) capability to associate peptide-based antigens.

2. MATERIALS AND METHODS

2.1. Materials

Ultrapure CS hydrochloride salt, with a molecular weight of 50 - 150 kDa and a deacetylation degree of 75 - 90% (Protasan UP Cl 113), was purchased from Novamatrix (Norway). The deacetylation degree of the CS used was confirmed by elemental analysis to be of 75 ± 2%.

Synthetic Lysophosphatidylcholine (LS) 18:0 (1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine) (Purity >99%; MW: 523.7; CMC: 4 μM) was obtained from Avanti Polar Lipids (Alabama, USA).

Linoleic acid (LA) (L1376), Trehalose (D-(+)-Trehalose dehydrate) and TFA (Trifluoroacetic acid ≥99%), were obtained from Sigma–Aldrich (Madrid, Spain).

Squalene (SQ) was purchased from Merck KGaA (Darmstadt, Germany).

DMEM (Dulbecco's Modified Eagle Medium) and RPMI (Roswell Park Memorial Institute medium) and FBS (Fetal Bovine Serum) were purchased to Life Technologies Corporation (CA, USA). Acetonitrile (Supragradient HPLC) and Ethanol was purchased from Scharlab (Barcelona, Spain). Water HPLC Gradient was purchased from Fisher Chemical (Thermo Fisher Scientific, USA).

2.2. Preparation of nanoemulsions and nanocapsules

Two nanoemulsions (LA:LS and SQ:LA:LS) and two nanocapsule formulations (LA:LS:CS and SQ:LA:LS:CS) were prepared by solvent displacement technique. For the LA:LS and LA:LS:CS, an organic phase composed of LA and LS (10 and 2 mg/ml, respectively) in 2 ml of ethanol was poured over 10 ml of ultrapure water containing CS (0.2 mg/ml) or not, under magnetic stirring. Formulations composed of SQ:LA:LS and SQ:LA:LS:CS, were prepared using the same procedure but with a organic phase composed of SQ, LA and LS (5, 2 and 2 mg/ml, respectively) in 2 ml of ethanol and the aqueous phase was 10 ml of ultrapure water or CS aqueous solution (0.1 mg/ml), respectively. The stirring was maintained for 5 minutes and then the ethanol was removed by evaporation under vacuum (Büchi Labortechnik AG, Flawil, Switzerland) to a final volume of 5 ml. Finally, the nanoemulsions and nanocapsules were isolated by ultracentrifugation at 61.740 g for 1 h, at 15 ºC in an Ultracentrifuge Optima L90 K with rotor Beckman Type 70.1 Ti (Beckman Coulter, CA, USA). The lipid phase recovered (1 ml) was then diluted in ultrapure water and characterized.

To determine the nanoparticles concentration, 0.5 ml of the lipid phase obtained after ultracentrifugation, was dehydrated in a lyophilizer VirTis Genesis 25L (Model SQ EL-85, SP Scientific, Pennsylvania, USA) using a freeze drying cycle. In resume, the samples were frozen overnight in a -20 °C freezer and then transferred to the lyophilizer. In here, it were submitted to an initial drying step for 24 h at -35 °C at 2-10 millitorr (mtorr) followed by a secondary drying for another 24 h at 0 °C and finally a third step of 16 hours at 20 °C at the same pressure. After the dehydration, the formulation was weighted and the concentration calculated.

2.3. Physicochemical characterization

The particle size distribution and polydispersity index (PdI) of the formulations were evaluated by Dynamic Light Scattering (DLS) and its surface charge (zeta potential) by Electrophoretic Light Scattering (ELS) using a Zetasizer Nano ZS90 (Malvern Instruments, UK). Each analysis was performed in triplicate at 25 ºC with a detection angle of 173º, after a ten-fold dilution of the isolated formulation in distilled water.

The morphological characterization of the particles was performed with transmission electronic microscopy (TEM) (Philips CM 12, Eindhoven, Netherlands). Briefly, the samples were placed on copper grids with Formvar films and then stained with 2% (w/v) phosphotungstic acid solution. Afterwards, the grids were left overnight in an oven at 60 ºC to dry and then they were observed with TEM.
The pH was determined with a Sartorius Docu-pH Benchtop Meters (ThermoFisher Scientific, USA).

2.4. Nanoemulsions and nanocapsules stability

2.4.1. Stability during storage as an aqueous suspension

The blank nanoemulsions and nanocapsules were diluted a ten-fold in ultrapure water, stored at 4 ºC and then the size and surface charge were evaluated after 1, 3 and 6 months. These formulations were also stored at room temperature (RT) at the same dilution for up to one month, after which, the size and surface charge were evaluated.

2.4.2. Stability upon dispersion in simulated biological medium

The blank nanoemulsions and nanocapsules were diluted ten-fold in two different simulated physiological mediums, namely DMEM and RPMI supplemented with 10% FBS, and their size was monitored for up to 24 hours at 37 ºC.

2.5. Freeze drying studies

The capacity of the formulations to be freeze-dried and recover its initial characteristics after reconstitution, was assessed using trehalose as cryoprotectant at different (w/v) concentrations (5%, 2.5% and 0%) and using the same freeze drying cycle protocol described in 2.2. The nanoemulsions and nanocapsules suspensions were diluted a ten-fold in the trehalose solution at a final volume of 0.8 ml. After freeze drying, the nanoparticles were reconstituted with ultrapure water (0.8 ml) and the particle size and PdI were analyzed.

2. 6. Analysis of complement activation

The formulations ability to activate the complement cascade was studied through the evaluation of the hydrolysis of the C3 complement factor by Western-blot. A pool of human plasma from healthy donors was incubated with three different concentrations (10, 100 and 1000 μg/ml) of the formulations in the presence of a veronal buffer (pH 7.4). Equal volumes of plasma, buffer and formulation (50 μl each) were mixed together and incubated at 37 °C for 1 h. Cobra venom factor (CVF) (Quidel Corporation; San Diego, CA) and PBS were used as positive and negative controls, respectively. After incubation, the mixture was centrifuged at 16,000×g for 30 min. Supernatants containing complement proteins were loaded (2 μl) onto a 10% SDS-PAGE gel and then transferred to a PVDF membrane (Immun-Blot, Bio-Rad; Hercules, CA) using the Transblot Semidry Transfer Equipment (Bio-Rad; Hercules, CA). PVDF membranes were blocked overnight at 4 °C with 5% non-fat dry milk in TBST. Then the membrane was washed and incubated for 90 min at RT with a mouse mAb against human C3 dilute 1:1000. After intensive washes, membranes were incubated with secondary polyclonal goat anti-mouse IgG Abs conjugated with alkaline phosphatase diluted 1:2000 at RT for 1 h. The membrane was finally revealed with BCIP.

2.7. *In vitro* studies

2.7.1. Cells and culture

The adherent RAW 264.7 murine macrophage and the human promyeloblast cell line HL60 were purchased from ATCC (Manassas, VA). The cells were cultured in RPMI supplemented with 10% (v/v) heated-inactivated fetal bovine serum (FBS) (PAA; Pasching, Austria), 2 mM glutamine and 100 U/ml of penicillin/streptomycin (hereafter called complete medium), at 37 °C in 5% CO_2 atmosphere. Cells were split every day to maintain 70–80% confluent cultures.

The human peripheral blood mononuclear cells (PBMCs) were obtained from three healthy donors (the project was approved by the Clinical Research Ethical Committee of Galicia 2013/272, and written informed consent for participation in the study was obtained from the donors). Briefly, 15 ml of heparinized blood were diluted with equal volume of PBS and centrifuged through a Ficoll-Hypaque gradient in a relation 7:3 (Blood diluted:ficoll) at 1600 rpm for 30 minutes at 20 ºC, in order to separate mononuclear leukocyte, granulocyte and eritrocyte. The mononuclear cells in the interface between Ficoll and plasma were collected with a Pasteur pipette and wash twice with complete medium by centrifugation at 1200 rpm for 5 minutes at 20 ºC.

2.7.2. Citotoxicity evaluation: xCELLigence® system

The cytotoxicity of the formulations was evaluated by the xCELLingence® RTCA DP (Roche Diagnostics; Penzberg, Germany), following the manufacturer's instructions. This instrument measures the variations of impedance caused by cell growth when cells are incubated on plates containing electrodes. The xCELLigence® device was placed inside the incubator (37 °C and 5% CO_2) and 1.5×10^4 RAW 264.7 cells per well were incubated with 200 µl RPMI 10% FBS for 20-23 h until the exponential phase was reached. At that point, nanocapsules and nanoemulsions were added at six different concentrations (250, 125, 62.5, 31.25, 15.63 and 7.81 µg/ml) and incubated for 48 h at 37 ºC. The culture medium and the formulations alone (without cells) were used as negative controls. Continuous monitoring of impedance (that is correlated with cell viability) was performed during the whole procedure with intervals of 15 min between each impedance measurement.

2.7.3. Cytokine production: FlowCytomix™ system

The cytokine production was evaluated by flow cytometry (FC500, Beckman-Coulter, FL, USA), with a Human Th1/Th2 11plex FlowCytomix™ Multiplex Kit (eBioscience, Vienna, Austria), following manufacturer instructions.

The formulations were incubated at the concentration of 10 and 100 μg/ml, in 1×10^5 of PBMC, for 24 hours at 37 ºC. As positive control it was used the phytohaemagglutinin (PHA) plus the lipopolysaccharide (LPS) (InvivoGen, San Diego, CA) to activate both B cells and T cells, respectively. As negative control it was used the culture medium. Two independent experiments, each one in duplicate, were performed for each of the samples tested.

2.7.4. Reactive Oxygen Species (ROS) production

The formulations effect in the intracellular ROS production was evaluated by measuring the oxidation of 2',7-dichlorofluorescein diacetate (DCFH-DA). This marker can be oxidized by ROS and converted to the fluorescent compound. In resume, the formulations were incubated at 10 and 100 μg/ml concentration, in $1x10^5$ HL60 cells in 96 well plates, during 1 and 12 hours at 37 ºC. After the incubation, cells were collected and centrifuged at 100 g for 5 minutes, resuspended in PBS containing 5 μM of DCFH-DA and then incubated at 37 ºC during 30 minutes. Finally, cells were washed twice with PBS and analysed by flow cytometry. The Mean Fluorescence Intensity (MFI) was normalized to negative control. As positive control it was used 10 μM of phorbol 12-myristate 13-acetate (PMA). As negative control it was used complete medium.

2.8. Peptide-based antigen loading

To evaluate the capacity of the nanoemulsion SQ:LA:LS and nanocapsule SQ:LA:LS:CS to associate peptide-based antigens, three model peptide-based antigens from the simian immunodeficiency virus (SIV) (68-4, 69-2 and 69-4), with different physicochemical properties (**Table 1**), were associated to the formulations. In brief, an aliquot (0.005 ml) of the peptide solution (10 mg/ml) was poured over an aliquot (0.095 ml) of the isolated formulation to obtain a final concentration of 0.5 mg/ml antigen (with a theoretical loading of 3.5% and 3.3% for SQ:LA:LS and SQ:LA:LS:CS, respectively). The resulting dispersions were incubated for up to two hours under agitation at 300 rpm in a plate agitator at room temperature (RT). Afterwards, the

physicochemical properties of the loaded formulations were evaluated and the peptide association efficiency was determined. A preliminary stability test at room temperature for one week was also realized with the loaded formulations.

2.9. Determination of peptide association efficiency (PA)

The peptide association (PA) was evaluated through the determination of the free peptide in solution (indirect measurement) (IPA) and the released peptide after particle disruption (direct measurement) (DPA). The loaded formulation nanoparticles were isolated using Amicon Ultra-0.5 mL Centrifugal Filters 30 kDa (Merck Millipore, USA) by ultracentrifugation at 10,000 g for 10 minutes at 15 ºC in a Universal 32 centrifuge (Hettich, Tuttlingen, Germany). The filtrate (about 0.3 ml) was recovered for the determination of the free peptide. The nanoparticles retained in the filter were recovered with 2 cycles of reverse centrifugation of 30 seconds in a microcentrifuge. To disrupt the nanoemulsion and nanocapsule, the formulation was diluted a ten-fold in an ACN 100% solution and then incubated one hour under agitation (300 rpm) in a plate agitator at room temperature.

The peptide concentration was determined by Ultra Performance Liquid Chromatography (UPLC), using standard curves of the peptides in the two media depending if realizing direct (nanoparticles degraded matrix) or indirect evaluation (nanoparticles suspending medium). The UPLC analysis was performed on an Acquity H-UPLC Class system with a Tunable UV (TUV) detector equipped with an Acquity UPLC BEH C18 1.7 µm 130 Å 2.1 x 100 mm Column (Waters Corporation, Milford Massachusetts, USA). The mobile Phase A consisted of 0.1% TFA (v/v) in HPLC gradient water and the Phase B of 0.1% TFA (v/v) in HPLC gradient ACN. The chromatography conditions are described in the **Annex 3**.

3. RESULTS AND DISCUSSION

The main objective of the present work was to design nanosystems, constituted by Lysophosphatidylcholine (LS), Linoleic Acid (LA) and Squalene (SQ), as vaccine adjuvant systems for peptide based antigens. These components were selected because of their chemotactic (LS and LA) and immunostimulant properties (LS) and the possibility to synergize these properties by combining them in the form of nanoemulsions. Based on previous work from our group [40], Chitosan (CS) nanocapsules derived from these nanoemulsions were also developed to be used for intranasal administration. Hence, two nanoemulsion prototypes (LA:LS and SQ:LA:LS) and two CS nanocapsule prototypes (LA:LS:CS and SQ:LA:LS:CS) were prepared by solvent displacement technique (**Figure 1**) and characterized in terms of physicochemical characteristics (size, surface charge and pH), stability in storage conditions (4 ºC and RT) and in simulated physiological mediums (DMEM and RPMI w/FBS). Additionally, the systems were characterized for their capacity to initiate the complement and stimulate the production of cytokines and ROS, as well as their cytotoxicity. Finally, the capacity of the SQ-containing formulations to associate peptide antigens was also evaluated.

Table 1. Model peptide antigens molecular weight (MW) and estimated isoelectric point (pI), charge at pH 4 (Charge at pH 4) and grand average of hydropathy (GRAVY).

Peptide	MW (Da)	pI	Charge at pH 4*	GRAVY
68-4	2264.3	4.18	- 0.5	-1.665
69-2	2258.5	12.1	+ 2.4	-0.455
69-4	2461.6	7.1	+ 1.6	-1.995

*MW, pI and GRAVY were calculated with online software [41]. * The calculation of the charge at pH 4 is shown in the **Annex 2**.*

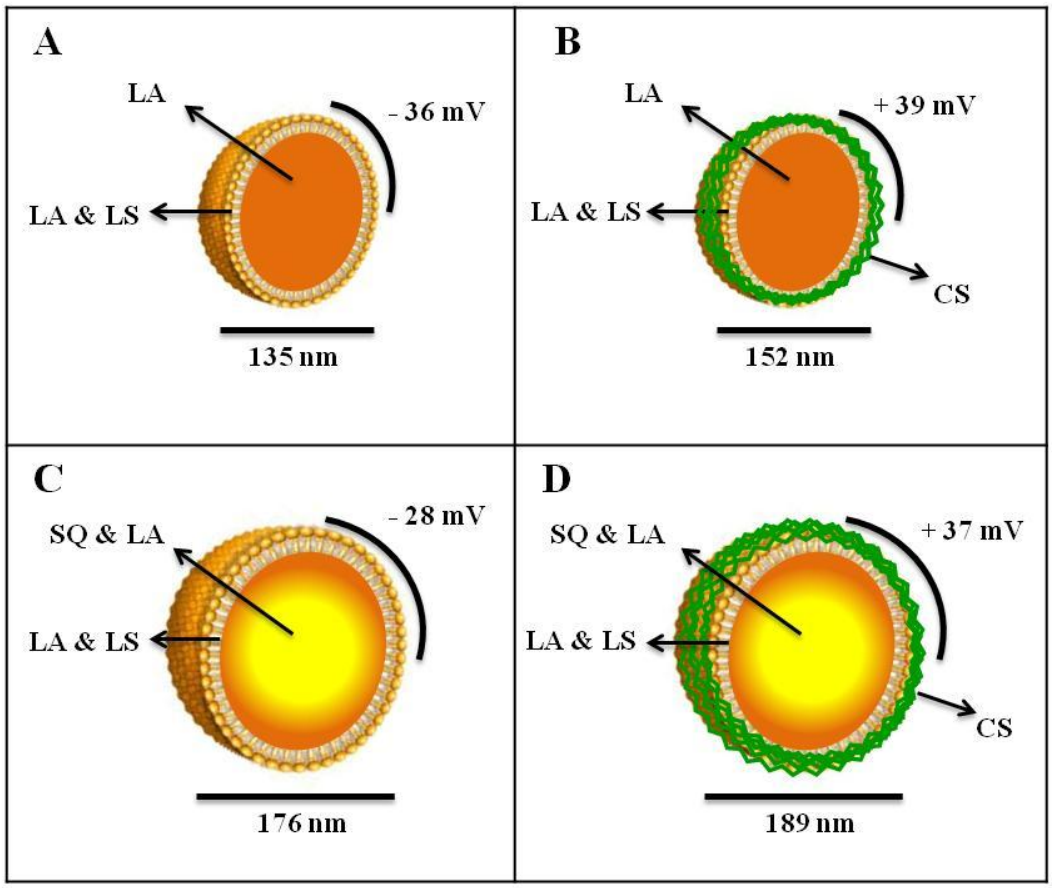


Figure 1. *Representation of the LA:LS (**A**), LA:LS:CS (**B**), SQ:LA:LS (**C**) and SQ:LA:LS:CS (**D**) formulations based on Lysophosphatidylcholine (LS), Linoleic Acid (LA), Squalene (SQ) and Chitosan (CS) with indication of particle size distribution values determined by DLS and surface charge by ELS.*

3.1. Physicochemical characterization of blank nanoemulsions and nanocapsules

From the rational combination of the mention components it was possible to form four different nanometric systems through the solvent displacement technique. Accordingly, both the linoleic acid:lysophosphatidylcholine (LA:LS) and squalene:linoleic acid:lysophosphatidylcholine (SQ:LA:LS) nanoemulsions, as well as the nanocapsules linoleic acid:lysophosphatidylcholine:chitosan (LA:LS:CS) and squalene:linoleic acid:lysophosphatidylcholine:chitosan (SQ:LA:LS:CS), presented a nanometric size (< 200 nm) and small PdI (**Table 2**). The particles without CS had a negative surface charge probably due to the negatively charged LA (pKa = 4.77 [42]). The pKa of LA also helps explains the low pH of the formulation (< 5).

Table 2. Physicochemical characterization of the formulations

Formulation	Ratio (w/w)	Size (nm)	PdI	ζ (mV)	pH	Conc. (mg/ml)
LA:LS	10/2	135 ± 21	0.2	- 36 ± 3	4.2 ± 0.1	8 ± 3
LA:LS:CS	10/2/1	152 ± 11	0.2	+ 39 ± 5	3.7 ± 0.3	11 ± 3
SQ:LA:LS	10/4/4	176 ± 16	0.2	- 28 ± 7	4.2 ± 0.4	9 ± 1
SQ:LA:LS:CS	10/4/4/1	189 ± 15	0.2	+ 37 ± 8	4.1 ± 0.3	7 ± 1

Ratio: mass ratio (w/w), PdI: Polydispersity Index, ζ: surface charge; Conc.: particle concentration (mg/ml).

Their nanometric size and round shape of the developed nanosystems was further confirmed by TEM evaluation (**Figure 2**).

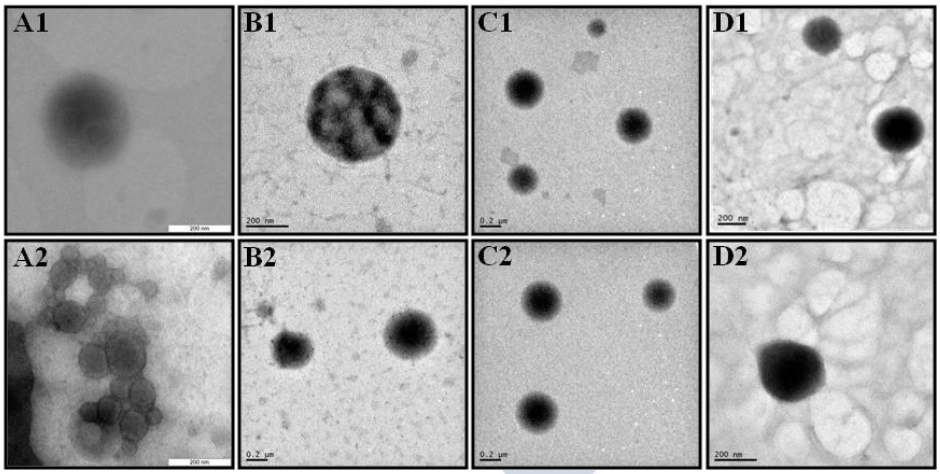


Figure 2. *TEM images showing the morphology of LA:LS (**A1** & **A2**), LA:LS:CS (**B1** & **B2**), SQ:LA:LS (**C1** & **C2**) and SQ:LA:LS:CS formulations (**D1** & **D2**). In all cases the scale bar is of 200 nm*

3.2. Stability of the nanoemulsions and nanocapsules

3.2.1. Stability in supplemented DMEM and RPMI with FBS

The SQ:LA:LS and SQ:LA:LS:CS maintained their nanometric size and PdI in DMEM (**Figure 3A**) and RPMI with FBS (**Figure 3B**) for up to 24 hours, while the LA:LS and LA:LS:CS aggregated soon after their dilution in the mediums. The SQ-containing

formulations seem to resist better in these mediums which indicates that SQ is contributing for the stability of the systems in these conditions.

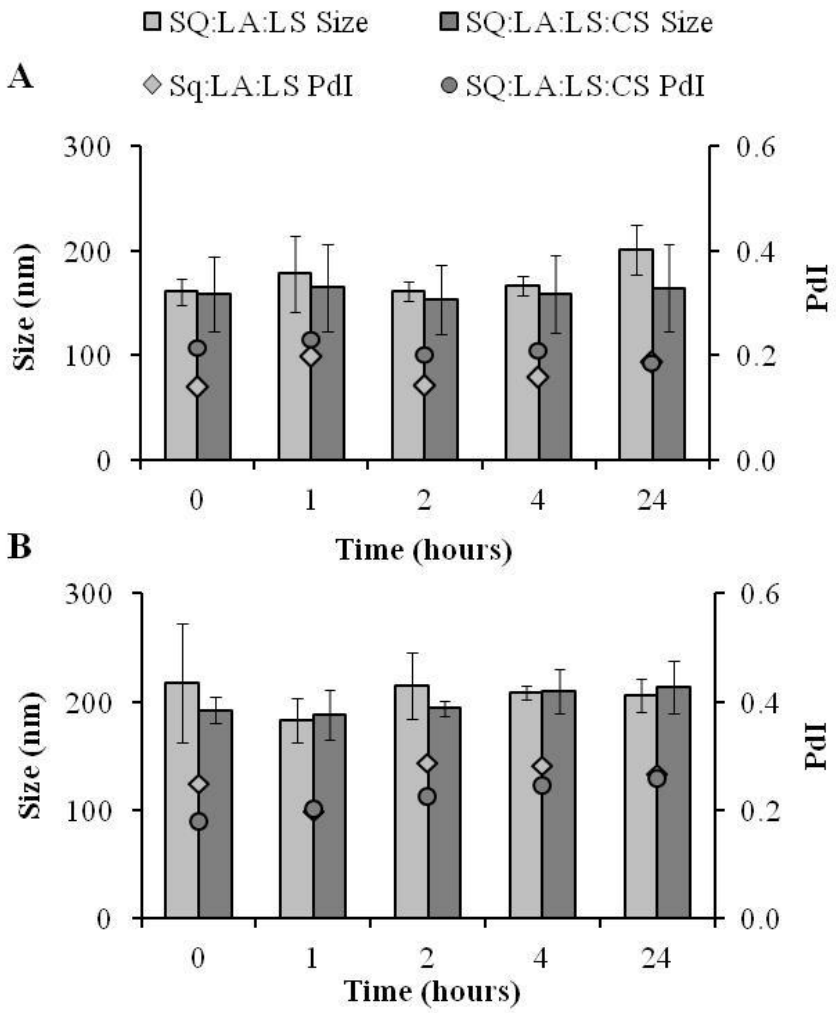


Figure 3. *Evaluation of particle size distribution values (Size) and Polydispersity Index (PdI) over time in DMEM (**A**) and RPMI with FBS (**B**) of SQ:LA:LS and SQ:LA:LS:CS formulations.*

3.2.2. Stability in aqueous suspension

All formulations maintained their nanometric size for up to three months at 4 ºC (**Table 3**), with the exception of LA:LS that aggregated after one month. After three months, however, the SQ:LA:LS already showed a drastic decrease in the surface charge (-42%), which could be related with alterations in the LA disposition and/or ionization, and after six months this formulation had aggregated. The nanoemulsion SQ:LA:LS:CS was remarkably stable, as its physicochemical characteristics were almost unchanged for up

to six months (decrease of size and zeta potential of about 6% and 1% respectively, and a rise in PdI of 12%).

Table 3. Physicochemical characterization of the systems over time at 4 ºC.

Formulation	After 1 month at 4 °C			After 3 months at 4°C			After 6 months at 4°C		
	Size (nm)	PdI	ζ (mV)	Size (nm)	PdI	ζ (mV)	Size (nm)	PdI	ζ (mV)
LA:LS:CS	140 ± 11	0.2	+ 39 ± 1	163 ± 19	0.3	+ 36 ± 6	193 ± 24	0.4	+ 36 ± 4
SQ:LA:LS	164 ± 24	0.2	- 19 ± 16	190 ± 53	0.3	- 8 ± 25	Aggr.	Aggr.	N/A
SQ:LA:LS:CS	158 ± 11	0.2	+ 39 ± 5	182 ± 48	0.2	+ 40 ± 10	177 ± 33	0.2	+ 33 ± 8

PdI: Polydispersity Index; ζ: Surface charge; Aggr.: Formulation Aggregated. N/A: Not Available

At room temperature, the SQ:LA:LS:CS was again the most stable formulation (**Table 4**), while the LA:LS nanoemulsion had already aggregated after fifteen days in these conditions. The fragility of this system compared with the SQ:LA:LS:CS indicates that both SQ and CS contribute for the nanoemulsion stability. The CS possibly helps maintains the structure through complexation with ionized species in the nanoemulsion surface while the SQ provides a highly hydrophobic core in which the LA and LS are more soluble than in water, decreasing the migration of these molecules from the emulsion and/or the Ostwald ripening process [43].

Table 4. Physicochemical characterization of the systems over time at room temperature (RT)

Formulation	After 15 days at RT			After 30 days at RT		
	Size (nm)	PdI	ζ (mV)	Size (nm)	PdI	ζ (mV)
LA:LS:CS	144 ± 5	0.3	N/A	Aggr.	Aggr.	N/A
SQ:LA:LS	171 ± 10	0.2	N/A	145 ± 9	0.1	- 18 ± 3
SQ:LA:LS:CS	187 ± 19	0.2	N/A	180 ± 21	0.2	+ 35 ± 7

PdI: Polydispersity Index; ζ: Surface charge; Aggr.: Formulation Aggregated. N/A: Not Available

3.3. Freeze drying studies

As expected from previous works [44], the use of trehalose was fundamental for the successful resuspension of these particles. The optimal trehalose concentration for SQ:LA:LS and for LA:LS:CS and SQ:LA:LS:CS was 2.5 and 5%, respectively (**Figure 4**). The presence of SQ in the formulation was found to improve the dispersibility of the freeze-dried formulation, a result that confirms the stabilizing role of the component.

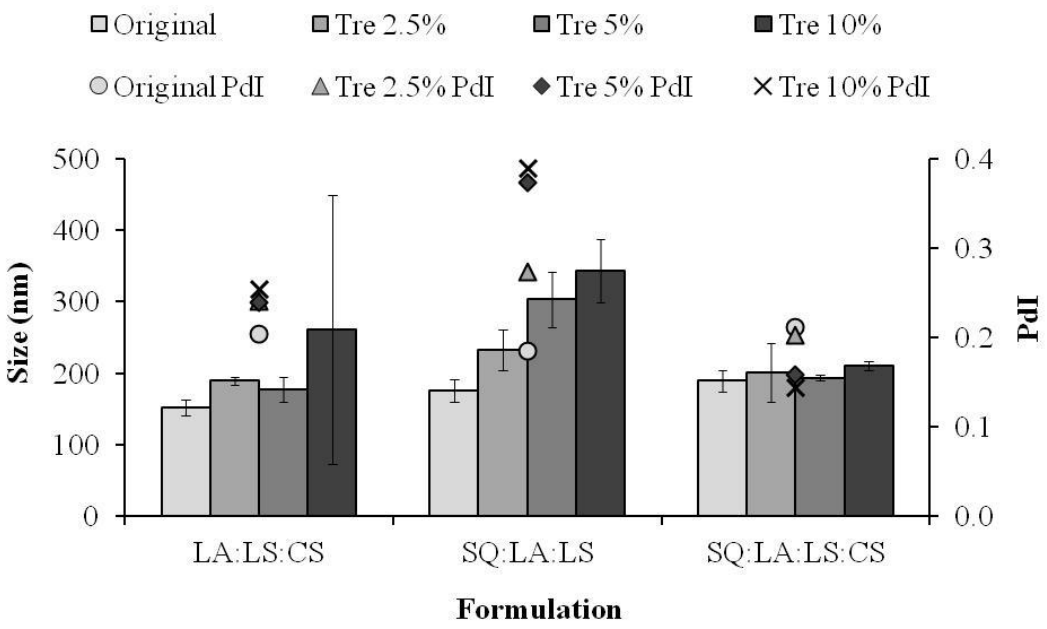


Figure 4. *Evaluation of particle size distribution values (Size) and Polydispersity Index (PdI) after resuspension of freeze dry formulations at different Trehalose (Tre) concentrations percentage (w/v).*

3.4. Complement cascade activation by the nanoemulsions and nanocapsules

The complement system is a fundamental component of the innate immune system and it is constituted by a complex network of plasma proteins that play an important role in the host defense due to its capacity to elicit and potentiate the immune system activation [45, 46] and even enhance both B [47] and T cell [48] responses. The complement system has three known cascades of activation (classical, lectin and alternative), which differ in the initial stimuli and following reactions but all share a common step that is the cleavage of the complement protein C3 into the anaphylatoxin C3a and the opsonin C3b factors. The total scheme leading to C3 hydrolysis and following reactions, is rather complex and have been thoroughly described elsewhere [45, 46]. As all

complement cascades share this step, it is possible to evaluate the capacity of a formulation to activate the complement through the determination of the C3 hydrolysis by Western-blot. Accordingly, when the formulations were incubated at 1000 µg/ml in human plasma for 1 hour at 37 ºC, it was observed that all, except the LA:LS, were able to induce the C3 cleavage (**Figure 5**). The C3 hydrolysis product (MW~43) bands, correspondent to these formulations, presented a two-fold signal intensity compared with the negative control, which corresponds to the C3 basal degradation. Given that the complement is activated by interaction with the particles surfaces, it would be expectable that the CS presence would be a major determinant in this process. However, both SQ:LA:LS and SQ:LA:LS:CS activate the complement with the same intensity.

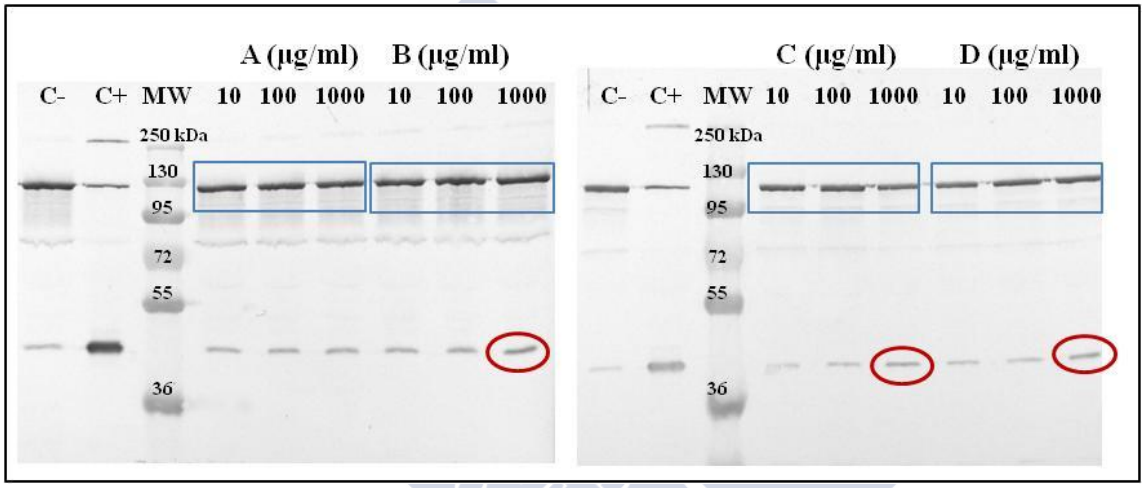


Figure 5. *Analysis by Western-blot of C3 complement factor split products in human plasma pool after incubation with LA:LS (**A**), LA:LS:CS (**B**), SQ:LA:LS (**C**) and SQ:LA:LS:CS (**D**) at 10, 100 and 1000 µg/ml. **C−**: pool of human plasma containing complement protein incubated with PBS; **C+**: pool of human plasma containing complement protein incubated cobra venom factor. Marked on light blue is the C3 protein (about 115 kDa). Marked on red are the C3 split products (about 43 kDa). MW: molecular weight of the protein marker (kDa).*

This result suggests that the CS might not be involved in the complement activation, which is in agreement with previous work with CS covered nanocapsules [39] or nanoparticles [49], where it was not observed complement activation. Therefore, the most probable trigger of the complement is the LS and/or LA although this would imply that the nanocapsule surface is not completely covered with CS. It has been already described that the LS exposure to IgM can start a complement cascade by the classical

route [50] and it is a possible cause for the results observed in here. The lack of complement activation by the LA:LS could result of its poor stability in the physiological simulated mediums. The rapid nanoemulsion disaggregation could possibly disrupt the complement adsorption and avoid the cascade reaction.

3.5. *In vitro* assays

3.5.1. Citotoxicity of the nanoemulsions and nanocapsules

The xCELLigence® system permitted to monitor the cell viability reduction in a time and dose-dependent manner upon contact with the formulations (**Figure 6**) and determine the formulations IC50 at the experimental endpoint (48 hours) (**Table 6**).

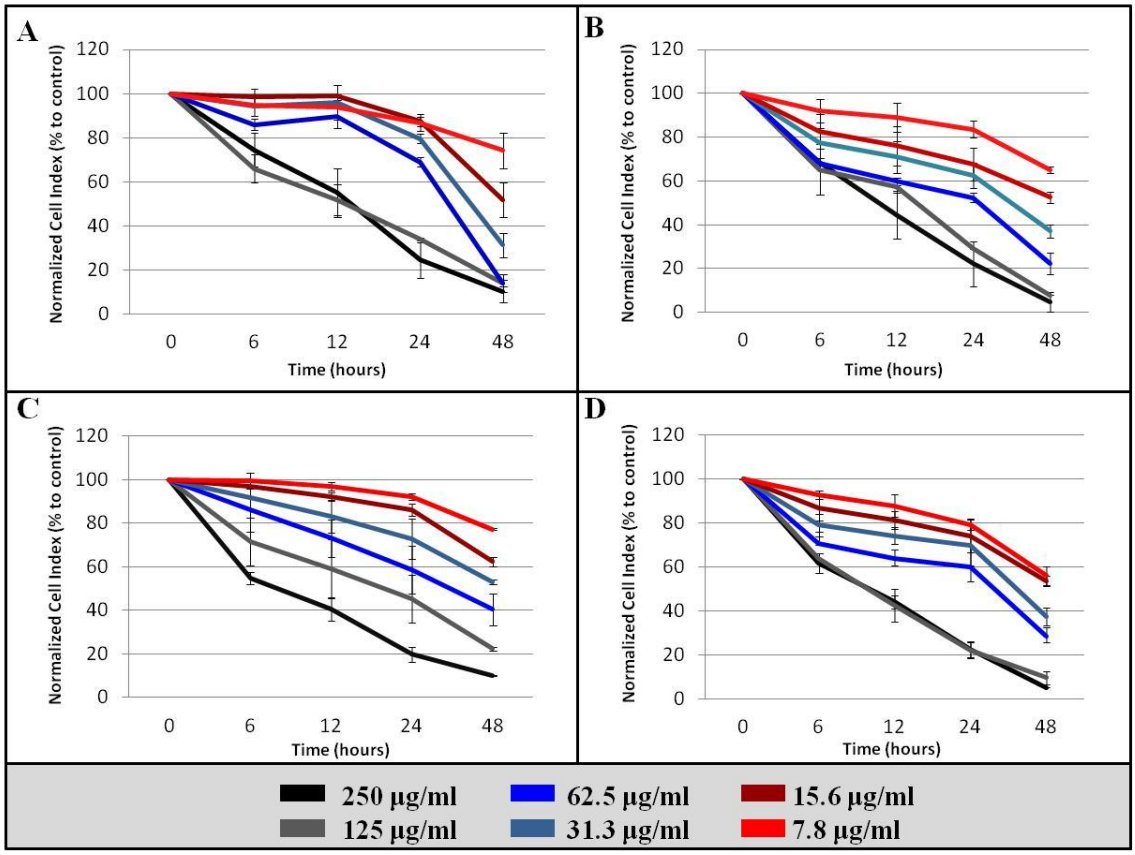


Figure 6. *Raw 264.7 cells were incubated with LA:LS (**A**), LA:LS:CS (**B**), SQ:LA:LS (**C**) and SQ:LA:LS:CS (**D**) formulations at six different concentrations (250, 125, 62.5, 31.25, 15.625 and 7.81 µg/ml — black, grey, dark blue, light blue, red, light red lines, respectively) for 48 h. The normalized Cell Index is the cell viability relative to the negative control (% viable cells) and is expressed as percentage.*

The formulations were relatively toxic (IC 50 < 50 μg/ml), with the LA:LS as the most toxic one and the SQ:LA:LS:CS as the less toxic at the experimental endpoint (LA:LS > LA:LS:LCS > SQ:LA:LS > SQ:LA:LS:CS). In all cases, the main mechanism of cell death identified was apoptosis (data not shown), which is less harmful for the tissues than necrosis.

The greater toxicity of the LA:LS and LA:LS:CS could be related to the greater concentration of LA in these formulations (4 mg/ml) compared with the other formulations (0.8 mg/ml). A reduction of the LA concentration in future compositions can possible decrease the toxicity of the formulations and also permit a rise in the final pH to values more adequate for *in vivo* administration. It is also possible that the formulations with SQ were less toxic because of a stronger attachment of the LA and LS to the highly hydrophobic nucleus of SQ. Nevertheless, one should be aware that these *in vitro* conditions do not simulated perfectly the *in vivo* conditions, as hardly such high concentration of nanoemulsion or nanocapsule would remain confined to a region and interacting with the same immune cells for 48 hours.

3.5.2. Cytokine production by PMBC upon incubation with nanoemulsions and nanocapsules

In order to evaluate the capacity of the developed systems to activate and stimulate the immune cells, these formulations were incubated at 10 and 100 μg/ml with a PBMC sample (which is constituted by lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells) for 24 hours at 37 ºC. The immune cells cytokines expression profile was determined using the Human Th1/Th2 11plex FlowCytomix™ Multiplex Kit, which allows a simultaneous quantification of several cytokines (IL-12p70, IFN γ, IL-2, TNF β, IL-10, IL-4, IL-6, IL-5, IL-8, IL-1β and TNF α). Surprisingly, from all the cytokines evaluated, the formulations only stimulated the secretion of interleukin-8 (IL-8 or CXCL8) (**Table 6**), a pro inflammatory cytokine. While the LA:LS and SQ:LA:LS were capable to stimulate this secretion at 10 μg/ml, the other formulations only had effect at the 100 μg/ml concentration. The IL-8 has chemoattractant properties and also stimulates the respiratory burst in phagocytes [51], which can be useful for the objective intended. Considering previous work with LS, it would be expectable an increased secretion of IFN-γ, TNF-α [21] and IL-1β [23]. However, it is possible that the formulations cytotoxicity did not allow the normal

function of the cells, preventing the full expression of cytokines. Optimally, the developed lipidic systems should permit a slow release of LS and LA, as their hydrophobic character would delay its dispersion in water. This slow release would diminish the free LA and LS in solution decreasing their potential toxicity and extending their adjuvant action. These molecules would act as danger signals that would provoke the migration of leucocytes towards the adjuvant systems. The nanometric size of the systems would then enhance its uptake by the phagocytes, increasing the recognition of the associated antigen by the immune cells. Finally, the LS presence and the nanometric size itself would provoke the activation of these cells against the associated antigens. However, in this *in vitro* model the nanoparticles are confined already with the immune cells, making it impossible to assess the potential chemotactic properties of the system. Such confinement and high concentration of the formulation could also potentiate the formulation toxicity and decrease its immunostimulant properties.

3.5.3. ROS production

The capability of the formulations to trigger the respiratory burst was evaluate in a HL60 cell line, a human promyelocytic leukemia cell line that has been widely used for the study of cells differentiation in the presence of specific stimulus [52]. The ROS production can be a result of cellular stress [53], induced apoptosis [54] or immune cells activation [55]. The cytometry evaluation indicated that all prototypes at the concentration of 100 μg/ml, stimulated the ROS production after 1 hour of incubation but after 12 hours there was no signal of ROS (**Table 6**). A hypothesis for such results is that the initial ROS production is due to cellular stress and eventual apoptosis (as it was observed in the cytotoxicity assays) leading to decreased ROS levels after 12 hours. However, the most toxic prototype, LA:LS, generated lower levels of ROS than the rest of the systems, which suggest additional causes for this respiratory burst profile.

Table 6. Resume of the formulation's complement activation, half maximal inhibitory concentration (IC50) after 48 hours, Interleukin-8 (IL-8) secretion and Reactive Oxygen Species (ROS) production.

Formulation	LA:LS	LA:LS:CS	SQ:LA:LS	SQ:LA:LS:CS
Complement Activation	-	+	+	+
IC50 (µg/mL) after 48 h	16 ± 0	27 ± 4	33 ± 7	47 ± 15
IL-8 release at 10 ug/ml	+	-	+	-
IL-8 release at 100 ug/ml	+	+	+	+
ROS after 1 h	+	++	++	++
ROS ater 12 h	-	-	-	-

Results with a 2-fold (+) or 4-fold (++) signal compared with the signal of the negative control were considered positive.

3.6. Peptide-based antigen association

Based on their stability profile, SQ-containing formulations were considered the most promising ones and were selected for the association of the model peptide-based antigens. The loading method was designed to permit the interaction of both peptide and particles at maximum possible concentration, in order to increase the probability of interaction between both elements. In other hand, this two steps method also avoids that the antigen enters in contact with the organic phase (that could denature the antigen).

As model antigens, we selected three peptides (68-4, 69-2 and 69-4) that present amino acid sequences homologous to specific epitopes from the simian immunodeficiency virus (SIV), which makes them interesting antigen models, as this virus is an exceptional model for HIV vaccine research [56]. These three peptides present similar molecular weight (MW) but different hydrophobic character (estimated by the grand average of hydropathy (GRAVY) index) and isoelectric point (pI) (**Table 1**). This was designed to permit the assessment of the capacity of the developed systems to associate peptide-based antigens with different characteristics. The SQ:LA:LS particles withstood well the filtration process, with only a small variation in size or surface charge (7% and 4% decrease, respectively) and even a substantial decrease in the PdI (37%) (**Figure 7**).

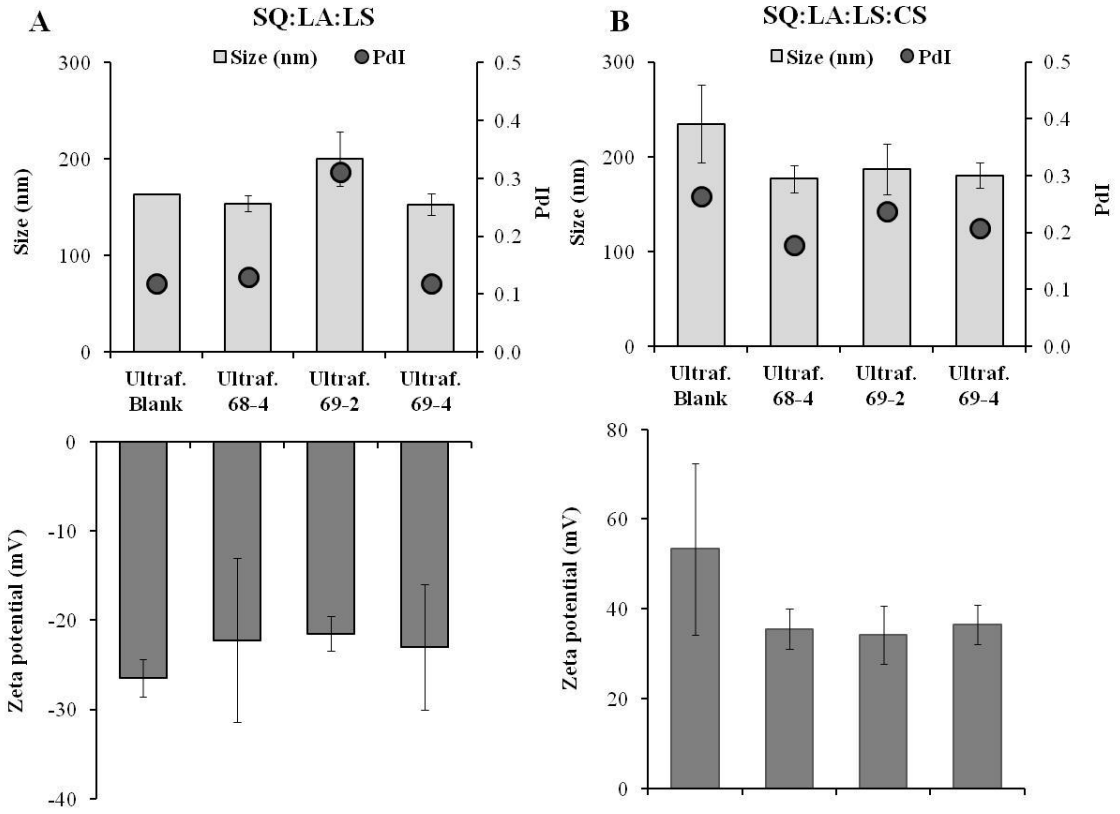


Figure 7*. Evaluation of particle size distribution values (Size), Polydispersity Index (PdI) and surface charge (Zeta potential) of the blank (Ultraf. Blank) and loaded (Ultraf. 68-4, 69-2 and 69-4) SQ:LA:LS (**A**) and SQ:LA:LS (**B**) formulations after ultrafiltration.*

When incubated with the peptides, the surface charge decreased slightly in all cases and even the size and PdI presented lower values, with the exception of the peptide 69-2, where it was observed an increase in size and PdI. The SQ:LA:LS:CS formulation after filtration presented a great increase in size, PdI and surface charge (24, 25 and 44% increase, respectively) but when incubated with the peptides and filtrated, these particles showed similar physicochemical properties to the original formulation (**Figure 6**). Preliminary stability studies indicate that the SQ:LA:LS and SQ:LA:LS:CS loaded and filtered formulations are stable for at least one week at room temperature (**Figure 8**).

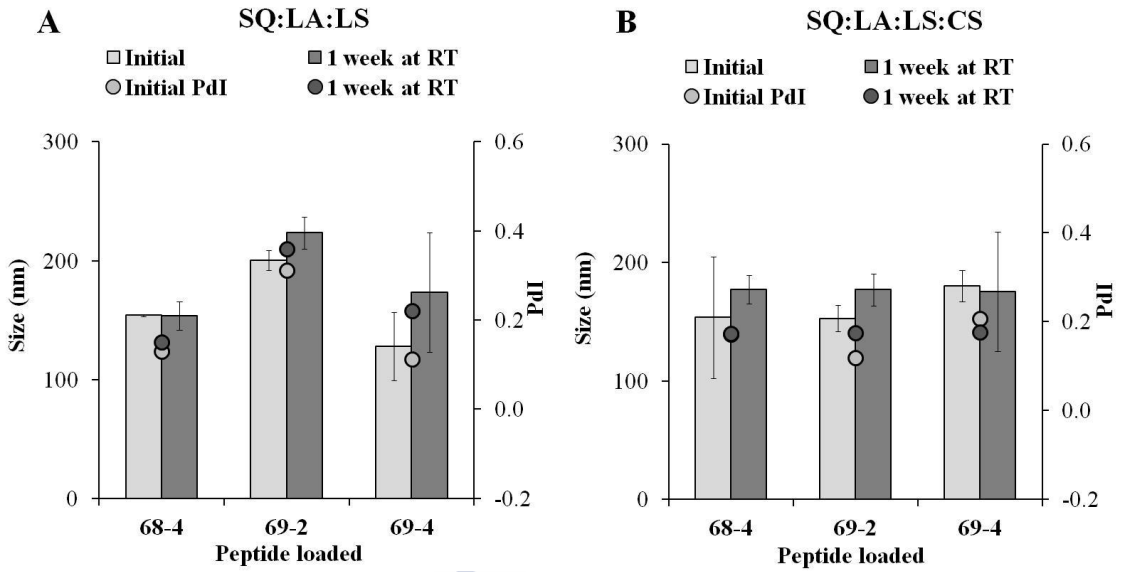


***Figure 8**. Preliminary evaluation of particle size distribution values (Size) and Polydispersity Index (PdI) over time of loaded and filtered SQ:LA:LS (**B**) and SQ:LA:LS:CS (**B**) formulations with different peptides (68-4, 69-2 and 69-4, respectively) at room temperature (RT).*

The association efficiency was relatively poor in both systems (**Table 7**). However, it is possible to identify a correlation between the GRAVY index (69-2 > 68-4 > 69-4) (**Table 1**) and the association (69-2 > 68-4 > 69-4). Most notably, the greatest association in both systems was achieved with the peptide 69-2, the most hydrophobic of the peptides, followed by the 68-4, the second most hydrophobic. This parameter seems to be more important for these systems than pI or the peptide charge, which is coherent with the formulation hydrophobic character. It would be expectable that the CS shell would decrease the association of the 69-2 and 68-4 peptides (due to their positive charge) but apparently this shell does not interfere with the association of positively charged peptides, which indicates the system is flexible enough to permit the association of differently charged peptides.

Table 7. Association efficiency of the different peptides (68-4, 69-2 and 69-4) to the SQ:LA:LS and SQ:LA:LS:CS nanoparticles

Formulation	Peptide	IPA (%)	DPA (%)
SQ:LA:LS	68-4	24 ± 10	16 ± 10
	69-2	33 ± 19	21 ± 14
	69-4	21 ± 1	7 ± 1
SQ:LA:LS:CS	68-4	37 ± 6	23 ± 5
	69-2	39 ± 10	45 ± 9
	69-4	27 ± 2	18 ± 3

IPA: Peptide association by indirect peptide evaluation; DPA: Peptide association by direct peptide evaluation

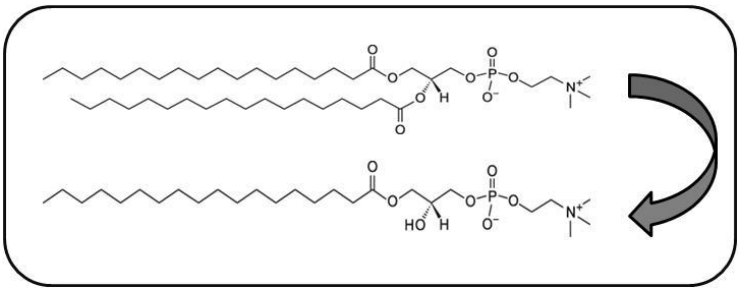
CONCLUSION

Here, we report the design and characterization of novel nanoemulsions and nanocapsules based on molecules with immune stimulant properties and chemotactic action (lysophosphatidylcholine and linoleic acid), as new vaccine adjuvant systems. The inclusion of squalene and chitosan in the composition seems to increase the formulations stability and can potentially further enhance the systems adjuvanticity.

The developed systems were capable of activating the complement system, however they were also found to be cytotoxic. The squalene based formulations presented lower toxicity and were capable to associate peptide-based antigens with different physicochemical characteristics albeit with low efficiency. Overall, the formulation composition should be optimized in order to improve the system toxicity profile while maintaining its adjuvant potential. Additional studies should also be realized to improve the formulations capability to associate antigens. The exceptional properties of the lysophosphatidylcholine as adjuvant material and surfactant should stimulate the further improvement of the exposed prototypes as vaccine adjuvant systems.

SUPPORTING INFORMATION

Annex 1. Illustration of lysophosphatidylcholine generation by hydrolysis of the phosphatidylcholine



Annex 2. Calculation of the peptide charge at pH 4

Charge at pH 4 is a predicted value calculated by the following equation:

$$Z = \sum_{i} Ni \frac{10^{pKai}}{10^{pH} + 10^{pKai}} - \sum_{j} Nj \frac{10^{pH}}{10^{pH} + 10^{pKaj}}$$

In which Z represents the Net charge of the peptide sequence, Ni the number of arginine, lysine and histidine residues and the N-terminus and pKai, their pKa. The Nj represents the number of aspartic acid, glutamic acid, cysteine and tyrosine residues and C-terminus and and pKaj, their pKa.

Annex 3. Chromatography conditions and peptide retention times

Gradient	Min.	A (%)	B (%)	Flow rate	0.3 ml/min	Peptide	RT (min)
	Initial	90	10	**Col. Temp.**	35 ºC	68-4	2.404
	2.00	0	100	**Inj. Vol.**	10 µl	69-2	2.141
	2.10	90	10	**λ**	210 nm	69-4	2.255

Min.: Time in minutes; A (%): Phase A (TFA 0.1% in H2O); B (%): Phase B (TFA 0.1% in ACN); Col. Temp.: Column Temperature; Inj. Vol.: Injection Volume; λ: Wavelength; RT (min): Retention Time in minutes

REFERENCES

1. Zepp, F., *Principles of vaccine design—Lessons from nature.* Vaccine, 2010. **28, Supplement 3**(0): p. C14-C24.

2. Siegrist, C.-A., *Vaccine immunology.* Vaccines. Saunders, 2008.

3. WHO, *Annex 1 - Guidelines for the production and quality control of synthetic peptide vaccies*. World Health Organ Technical Report Series. Vol. 889. 1999: WHO Expert Committee on Biological Standardization.

4. Chu, D.W.-S., et al., *Immunogenicity and tolerability of an AS03A-adjuvanted prepandemic influenza vaccine: A phase III study in a large population of Asian adults.* Vaccine, 2009. **27**(52): p. 7428-7435.

5. Khurana, S., et al., *Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus.* Sci Transl Med, 2010. **2**(15): p. 3000624.

6. Correia-Pinto, J.F., N. Csaba, and M.J. Alonso, *Vaccine delivery carriers: insights and future perspectives.* Int J Pharm, 2013. **440**(1): p. 27-38.

7. Fang, J.-H. and M. Hora, *The Adjuvant MF59: A 10-Year Perspective Gary Ott, Ramachandran Radhakrishnan*, in *Vaccine Adjuvants*, D. O'Hagan, Editor 2000, Springer New York. p. 211-228.

8. Morel, S., et al., *Adjuvant System AS03 containing alpha-tocopherol modulates innate immune response and leads to improved adaptive immunity.* Vaccine, 2011. **29**(13): p. 2461-73.

9. Foged, C., et al., *Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model.* Int J Pharm, 2005. **298**(2): p. 315-22.

10. Scheerlinck, J.P. and D.L. Greenwood, *Virus-sized vaccine delivery systems.* Drug Discov Today, 2008. **13**(19-20): p. 882-7.

11. Mehling, T., et al., *Estimation of LPC/water partition coefficients using molecular modeling and micellar liquid chromatography.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2013. **431**(0): p. 105-113.

12. Lauber, K., et al., *Clearance of Apoptotic Cells: Getting Rid of the Corpses.* Molecular Cell, 2004. **14**(3): p. 277-287.

13. Perrin-Cocon, L., et al., *Lysophosphatidylcholine is a natural adjuvant that initiates cellular immune responses.* Vaccine, 2006. **24**(9): p. 1254-63.

14. Radu, C.G., et al., *T cell chemotaxis to lysophosphatidylcholine through the G2A receptor.* Proc Natl Acad Sci U S A, 2004. **101**(1): p. 245-50.

15. Ryborg, A.K., et al., *Lysophosphatidylcholine: a chemoattractant to human T lymphocytes.* Arch Dermatol Res, 1994. **286**(8): p. 462-5.

16. Quinn, M.T., N. Kondratenko, and S. Parthasarathy, *Analysis of the monocyte chemotactic response to lysophosphatidylcholine: role of lysophospholipase C.* Biochim Biophys Acta, 1991. **3**(3): p. 293-302.

17. Lauber, K., et al., *Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal.* Cell, 2003. **113**(6): p. 717-30.

18. Yang, L.V., et al., *Gi-independent macrophage chemotaxis to lysophosphatidylcholine via the immunoregulatory GPCR G2A.* Blood, 2005. **105**(3): p. 1127-34.

19. Ryborg, A.K., et al., *Intracutaneous injection of lysophosphatidylcholine induces skin inflammation and accumulation of leukocytes.* Acta Derm Venereol, 2000. **80**(4): p. 242-6.

20. Asaoka, Y., et al., *Role of lysophosphatidylcholine in T-lymphocyte activation: involvement of phospholipase A2 in signal transduction through protein kinase C.* Proceedings of the National Academy of Sciences, 1992. **89**(14): p. 6447-6451.

21. Huang, Y.H., et al., *Lysophosphatidylcholine (LPC) induces proinflammatory cytokines by a platelet-activating factor (PAF) receptor-dependent mechanism.* Clin Exp Immunol, 1999. **116**(2): p. 326-31.

22. Sakata-Kaneko, S., et al., *Lysophosphatidylcholine upregulates CD40 ligand expression in newly activated human CD4+ T cells.* FEBS Letters, 1998. **433**(1–2): p. 161-165.

23. Liu-Wu, Y., E. Hurt-Camejo, and O. Wiklund, *Lysophosphatidylcholine induces the production of IL-1beta by human monocytes.* Atherosclerosis, 1998. **137**(2): p. 351-7.

24. Lotteau, V. and P. Andre, *Use of L-alpha-lysophosphatidylcholine to obtain the differentiation of monocytes into mature dendritic cells in vitro*, 2008, Google Patents.

25. Coutant, F., et al., *Sensing environmental lipids by dendritic cell modulates its function.* J Immunol, 2004. **172**(1): p. 54-60.

26. Illum, L., et al., *Enhanced nasal absorption of insulin in rats using lysophosphatidylcholine.* International Journal of Pharmaceutics, 1989. **57**(1): p. 49-54.

27. Wanten, G.J. and P.C. Calder, *Immune modulation by parenteral lipid emulsions.* The American Journal of Clinical Nutrition, 2007. **85**(5): p. 1171-1184.

28. Juttner, B., et al., *Unsaturated long-chain fatty acids induce the respiratory burst of human neutrophils and monocytes in whole blood.* Nutrition & metabolism, 2008. **5**(1): p. 19.

29. Pereira, L.M., et al., *Effect of oleic and linoleic acids on the inflammatory phase of wound healing in rats.* Cell Biochem Funct, 2008. **26**(2): p. 197-204.

30. Mena, J., et al., *Linoleic acid increases adhesion, chemotaxis, granule release, intracellular calcium mobilisation, MAPK phosphorylation and gene expression in bovine neutrophils.* Veterinary Immunology and Immunopathology, 2013. **151**(3–4): p. 275-284.

31. Hatanaka, E., et al., *Systematic study on ROS production induced by oleic, linoleic, and gamma-linolenic acids in human and rat neutrophils.* Free Radic Biol Med, 2006. **41**(7): p. 1124-32.

32. Hennig, B., et al., *Linoleic acid activates nuclear transcription factor-kappa B (NF-kappa B) and induces NF-kappa B-dependent transcription in cultured*

endothelial cells. The American Journal of Clinical Nutrition, 1996. **63**(3): p. 322-8.

33. Tripathi, P. and A. Aggarwal, *NF-kB transcription factor: a key player in the generation of immune response.* Curr. Sci., 2006. **90**(Copyright (C) 2013 American Chemical Society (ACS). All Rights Reserved.): p. 519-531.

34. Fox, C.B., *Squalene Emulsions for Parenteral Vaccine and Drug Delivery.* Molecules, 2009. **14**(9): p. 3286-3312.

35. Marttin, E., et al., *Acute histopathological effects of benzalkonium chloride and absorption enhancers on rat nasal epithelium in vivo.* International Journal of Pharmaceutics, 1996. **141**(1–2): p. 151-160.

36. Cury-Boaventura, M.F., et al., *Comparative toxicity of oleic and linoleic acid on human lymphocytes.* Life Sciences, 2006. **78**(13): p. 1448-1456.

37. Baldrick, P., *The safety of chitosan as a pharmaceutical excipient.* Regul Toxicol Pharmacol, 2010. **56**(3): p. 290-9.

38. Soane, R.J., et al., *Evaluation of the clearance characteristics of bioadhesive systems in humans.* International Journal of Pharmaceutics, 1999. **178**(1): p. 55-65.

39. Vicente, S., et al., *Co-delivery of viral proteins and a TLR7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy.* Journal of Controlled Release, 2013. **172**(3): p. 773-781.

40. Vicente, S., et al., *A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach.* PLoS ONE, 2013. **8**(4): p. e62500.

41. Artimo, P., et al., *ExPASy: SIB bioinformatics resource portal.* Nucleic Acids Research, 2012. **40**(W1): p. W597-W603.

42. Serjeant, E.P. and B. Dempsey, *Ionisation constants of organic acids in aqueous solution*1979, Oxford ; New York: Pergamon Press.

43. Taylor, P., *Ostwald ripening in emulsions.* Advances in Colloid and Interface Science, 1998. **75**(2): p. 107-163.

44. Lozano, M.V., et al., *Intracellular delivery of docetaxel using freeze-dried polysaccharide nanocapsules.* J Microencapsul, 2013. **30**(2): p. 181-8.

45. Walport, M.J., *Complement.* New England Journal of Medicine, 2001. **344**(15): p. 1140-1144.

46. Dunkelberger, J.R. and W.C. Song, *Complement and its role in innate and adaptive immune responses.* Cell Res, 2010. **20**(1): p. 34-50.

47. Carter, R.H. and D.T. Fearon, *CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes.* Science, 1992. **256**(5053): p. 105-7.

48. Kim, A.H., et al., *Complement C5a receptor is essential for the optimal generation of antiviral CD8+ T cell responses.* J Immunol, 2004. **173**(4): p. 2524-9.

49. Bertholon, I., C. Vauthier, and D. Labarre, *Complement Activation by Core–Shell Poly(isobutylcyanoacrylate)–Polysaccharide Nanoparticles: Influences of Surface Morphology, Length, and Type of Polysaccharide.* Pharmaceutical Research, 2006. **23**(6): p. 1313-1323.

50. Kim, S.J., et al., *I-PLA(2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation.* J Exp Med, 2002. **196**(5): p. 655-65.

51. Baggiolini, M. and I. Clark-Lewis, *Interleukin-8, a chemotactic and inflammatory cytokine.* FEBS Letters, 1992. **307**(1): p. 97-101.

52. Birnie, G.D., *The HL60 cell line: a model system for studying human myeloid cell differentiation.* Br J Cancer Suppl, 1988. **9**: p. 41-5.

53. Kawiak, A., et al., *Induction of Apoptosis in HL-60 Cells through the ROS-Mediated Mitochondrial Pathway by Ramentaceone from Drosera aliciae.* Journal of Natural Products, 2012. **75**(1): p. 9-14.

54. Hu, D.-E. and K.M. Brindle, *Immune cell-induced synthesis of NO and reactive oxygen species in lymphoma cells causes their death by apoptosis.* FEBS Letters, 2005. **579**(13): p. 2833-2841.

55. KOHCHI, C., et al., *ROS and Innate Immunity.* Anticancer Research, 2009. **29**(3): p. 817-821.

56. Letvin, N.L. and N.W. King, *Immunologic and Pathologic Manifestations of the Infection of Rhesus Monkeys with Simian Immunodeficiency Virus of Macaques.* JAIDS Journal of Acquired Immune Deficiency Syndromes, 1990. **3**(11): p. 1023-1040.

OVERALL DISCUSSION

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OVERALL DISCUSSION

Nanotechnology holds great potential for the improvement of the present and future vaccines through the development of safer and more efficient adjuvant systems. Supported by the great expertise of our group in this field, distinct strategies with different biomaterials were evaluated in the present work for diverse antigens, all based on a common element: nanometric delivery systems.

The nanometric delivery systems in described here, were developed through the combination of polysaccharides, polyamino acids, polynucleotides and lipids with intrinsic adjuvant properties. The objective was to explore a synergistic effect between these components when organized into nanostructures with adequate physicochemical characteristics, with the final goal of increasing the efficiency of antigen delivery and presentation to the immune system.

In terms of polysaccharides, we used both cationic polymers like chitosan (CS), a biodegradable and mucoadhesive polymer [1], and anionic polymers such as dextran sulfate (DS) and alginate (ALG), which present immunostimulant properties [2, 3]. As polyaminoacids polyarginine (PARG) and protamine (PR) were used due to their ability to enhance mucosal delivery and increase cellular internalization [4, 5]. As polynucleotide, a synthetic analog of the viral double-stranded RNA (dsRNA) poly (I:C) was used as a TLR3 agonist [6] to potentiate both humoral and cellular immune responses [7, 8] and finally, as lipids we used squalene (SQ), an oil present in nanoemulsion adjuvant systems in the market, which attest for its safety and immunoadjuvant properties [9], linoleic acid (LA) that presents chemotactic action humans [10] and lysophosphatidylcholine (LS) that presents not only chemotactic action, but also immunostimulant action and surfactant properties [11, 12].

The referred biomaterials were combined through specific nanotechnology formulation techniques like ionic gelation, layer by layer and solvent displacement techniques, according to the biomaterials physicochemical characteristics and the nature of the antigen of interest. In total, it were developed three types of different nanocarriers: i) Nanoparticles based on the gelification of chitosan with TPP in the presence of poly (I:C) (CS/TPP/pIC); ii) Multi-enveloping systems based on virus-like particles (VLP);

iii) Nanoemulsions and nanocapsules based on lysophosphatidylcholine (LS) and linoleic acid (LA) (**Figure 1**).

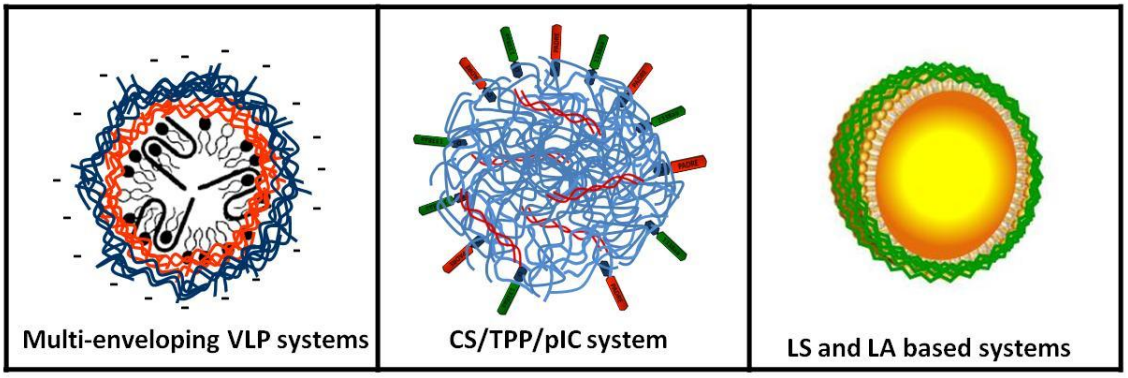

Figure 1. *Illustration of the different nanocarriers developed as vaccine adjuvant systems.*

As model antigens, we chose two different classes of subunit antigens with increasing presence in the field of vaccination due to their exceptional safety profile [13]: Virus-like particles (VLP) and peptide-based antigens (**Table 1**). Concretely, as VLP-type antigen, the hepatitis B recombinant surface antigen (HB) was selected and the peptide-based antigens were those from the Human Papillomavirus (HPV) and three other model peptides with different physicochemical characteristics.

Table 1. Summary of the type, origin, molecular weight (MW), isoelectric point (pI) and grand average of hydropathy (GRAVY) of the different antigens used in this study.

Antigen type	Virus	Code	MW (kDa)	pI	GRAVY
VLP	**Hepatitis B**	**HB**	> 3000	4.6	N/A
Peptide-Based	**HPV**	**1338aa**	3.4	3.94	N/A
	N/A	**68-4**	2.3	12.2	-1.665
		69-2	2.3	3.8	-0.455
		69-4	2.5	7.1	-1.995

1. Delivery systems for VLP antigens - Development and physicochemical characterization

Inspired by the nanoparticulated nature of the VLP that presents an exceptional immunogenicity as compared to other subunit antigen, we have designed a strategy specifically intended to modulate the immune response by enveloping the VLP antigen with a number of biomaterials with immunoadjuvant properties.

The VLP model antigen selected was the hepatitis B antigen (HB), which presents a nanometric size (22 nm) and negative surface charge (-33 mV). Based on this negative charge, the cationic polyamino acids protamine and polyarginine were adsorbed first to its surface to form the HB:Protamine (HB:PR) and HB:Polyarginine (HB:PARG) structures and, then, the anionic polymers dextran sulfate (DS) and alginate (ALG) or poly (I:C) (pIC) were adsorbed over the positive polymers, to originate HB:protamine: dextran sulfate (HB:PR:DS), HB:polyarginine:alginate (HB:PARG:ALG) or HB:polyarginine:poly (I:C) (HB:PARG:pIC) structures, respectively (**Table 2**).

Table 2. Physicochemical characterization of the Hepatitis B (HB) virus-like particle (VLP) antigen alone or multi-enveloped by different polymers.

Formulation	Code	Size (nm)	PdI	ζ (mV)	Antigen (%)
HB VLP	**HB**	22 ± 1	0.4	- 33 ± 2	N/A
HB:Protamine	**HB:PR**	24 ± 3	0.3	+ 32 ± 5	67 ± 2
HB:Protamine: Dextran sulfate	**HB:PR:DS**	35 ± 4	0.3	- 50 ± 0	67 ± 2
HB:Polyarginine	**HB:PARG**	27 ± 4	0.3	+ 52 ± 4	65 ± 3
HB:Polyarginine: Alginate	**HB:PARG:ALG**	39 ± 7	0.4	- 52 ± 0	65 ± 3
HB:Polyarginine: Poly (I:C)	**HB:PARG:pIC**	33 ± 6	0.4	- 41 ± 8	65 ± 3

PdI: Polydispersity Index; ζ: surface charge; Antigen (%): Antigen enveloping efficiency

The multi-enveloping strategy allowed the formation of very small systems (< 40 nm) with a variable surface charge depending on the final composition of the consecutive polymeric layers. These systems presented a high enveloping efficiency of the antigen (> 60%) and were stable in aqueous suspension at room temperature for at least one month and in PBS for at least 4 hours.

The humoral responses observed in mice after administration of the HB:PR, HB:PR:DS, HB:PARG:ALG and HB:PARG:pIC systems by the intramuscular (i.m.) (**Figure 2**) and intranasal (i.n.) (**Figure 3**) route, indicate that the multi-enveloping strategy could in general increase the immune response against the associated antigen and generate a protective antibody titer (>10 mIU/ml).

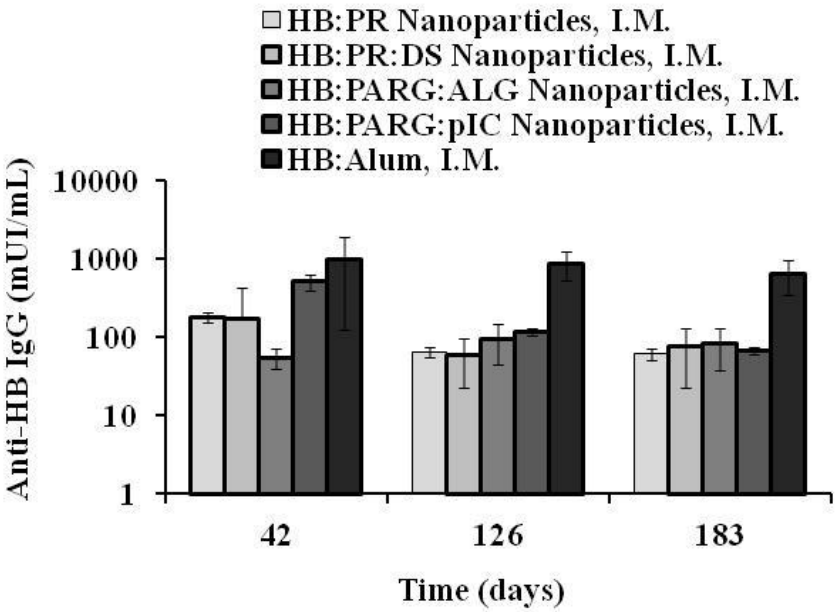

Figure 2. *Systemic humoral (IgG) immune response observed in mice upon intramuscular (10 µg, 0 and 4 weeks) immunization with Hepatitis B antigen coated with Protamine (HB:PR), Protamine and Dextran Sulfate (HB:PR:DS), Polyarginine and Alginate (HB:PARG:ALG) or Polyarginine and poly (I:C) (HB:PARG:pIC). Hepatitis B antigen adsorbed to Alum (HB:Alum) was used as positive control. The plot represents the anti-HB IgG average quantified at different time points after primary immunization (mean ± SEM, n = 10).*

However, despite their diverse composition and structure, the IgG titers achieved by the multi-enveloping antigen systems were lower than those obtained when using alum-adsorbed antigen, which suggests an intrinsic limitation of this multi-enveloping

strategy. It is possible that the coating of the antigen surface by the polymers and the very small size of these systems somehow hinders the antigen recognition by immune cells and leads to less intense immune responses.

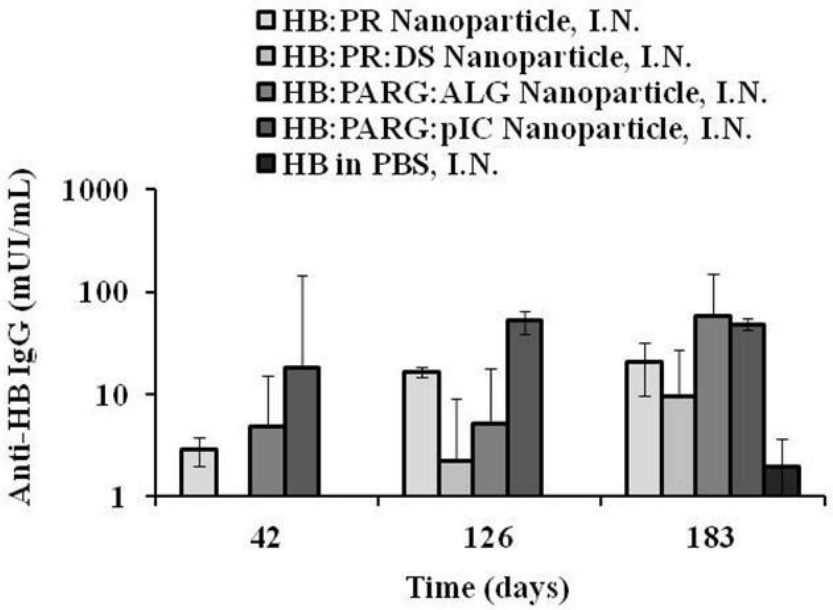

Figure 3. *Systemic humoral (IgG) immune response observed in mice upon intranasal (10 μg, 0, 4 and 16 weeks) immunization with Hepatitis B antigen coated with Protamine (HB:PR), Protamine and Dextran Sulfate (HB:PR:DS), Polyarginine and Alginate (HB:PARG:ALG) or Polyarginine and poly (I:C) (HB:PARG:pIC). Hepatitis B antigen dispersed in PBS (HB in PBS) was used as positive control. The plot represents the anti-HB IgG average quantified at different time points after primary immunization (mean ± SEM, n = 10).*

The highest antibody response was achieved with the HB:PARG:pIC composition. Interestingly, when comparing the IgG1/IgG2a ratio between the alum-adsorbed antigen and the HB:PARG:pIC formulation, it was observed that while the alum generated an T_h2 biased response, the HB:PARG:pIC generated a T_h1 biased response (**Figure 4**). This increased activation of T_h1 cells by the HB:PARG:pIC formulation can be an interesting advantage of this system, as these cells stimulate the development of cell-mediated immune responses capable of eliminating virus infected cells [14].

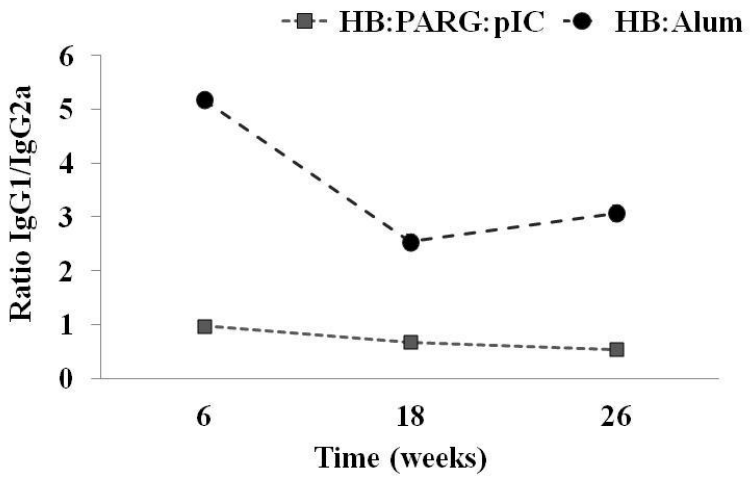

Figure 4. *Ratio of IgG1 and IgG2a subtypes in the serum of mice immunized with the multi-enveloped system comprising the Hepatitis B antigen coated with polyarginine and poly (I:C) (HB:PARG:pIC) (grey squares), and with the same antigen adsorbed to alum (HB:Alum) (black circle).*

2. Adjuvant systems for Peptide-based antigens - Development and physicochemical characterization

Peptide-based antigens present an enormous potential for the development of future vaccines due to their purity and highly precise composition [15]. However, on the contrary to VLP antigens, these antigens present in general poor immunogenicity [13, 16] and consequently need robust adjuvant systems in order to generate effective immune responses. In this sense, we have developed different adjuvant systems with increasing complexity to activate the immune responses against these antigens.

The great versatility of chitosan in the constitution of nanometric systems for the delivery of both polynucleotides [17] and proteins [18, 19] motivated us to design a novel nanostructure capable of co-deliver a potent immunostimulant (Poly (I:C)) and a T-helper peptide (PADRE) [20] as adjuvant system for the highly specific but poorly inmunogenic HPV-derived peptide antigen 1338aa. The gelification of the chitosan by TPP in the presence of Poly (I:C) at the mass ratio chitosan/TPP/pIC 4/1/04 permitted the constitution of a nanometric system (284 nm), with low PdI (0.2) and strong positive charge (> +40 mV) (**Table 6**) and with poly (I:C) encapsulation close to 100%. Furthermore, the poly (I:C) remained encapsulated after freeze drying and/or incubation with strong polyanions (heparin), and was released without observable damage after

enzymatic digestion of the nanoparticle with chitosanase (**Figure 5**). This mild and straightforward method was selected to avoid the degradation of the poly (I:C) and the peptides and in addition, to allow for the association of both peptides in different arrangements (i.e. surface adsorbed or entrapped inside the particles) and study the influence of these different peptide displays on the biological performance of the nanosystem.

Table 6. Physicochemical characterization of Chitosan/TPP/Poly (I:C) nanoparticles without peptide and with the peptides 1338aa and PADRE, either adsorbed onto the nanostructure surface or entrapped inside the matrix. (Means ± S.D., n=3).

Formulation	Code	Size (nm)	PdI	ζ (mV)	Antigen (%)
Chitosan/pIC without peptide (Blank)	**NP B**	**284 ± 15**	**0.2**	**+ 41 ± 1**	**N/A**
Chitosan/pIC-1338aa Adsorbed	**NP A1**	**299 ± 23**	**0.2**	**+ 49 ± 5**	**39 ± 1**
Chitosan/pIC/PADRE -1338aa Adsorbed	**NP A2**	**274 ± 9**	**0.2**	**+ 47 ± 5**	**50 ± 5**
Chitosan/pIC/PADRE -1338aa Entrapped	**NP E2**	**290 ± 28**	**0.2**	**+ 48 ± 4**	**67 ± 8**

PdI: Polydispersity Index; ζ: Surface charge; Antigen (%): Antigen association efficiency

By inducing the gelification of the chitosan with TPP in the presence of the Poly (I:C) and the peptides PADRE and 1338aa, it was possible to incorporate both peptides within the nanoparticle structure and constitute the Chitosan/Poly (I:C)/PADRE-1338aa Entrapped (NP E) formulation. On the other hand, by incubating the peptides PADRE and 1338aa together or the peptide 1338aa alone with preformed particles allowed the constitution of the particles Chitosan/Poly (I:C)/PADRE-1338aa Adsorbed (NP A2) and Chitosan/Poly (I:C)-1338aa Adsorbed (NP A1) formulations, respectively, in which the peptides were associated to the nanoparticle surface. Although presenting a different

peptide disposition, the size, PdI and surface charge of the three different systems did not change significantly (**Table 6**). This supports the robustness of the preparation method and indicates the versatility of the nanoparticles enabling the integration other immune stimulants in the future.

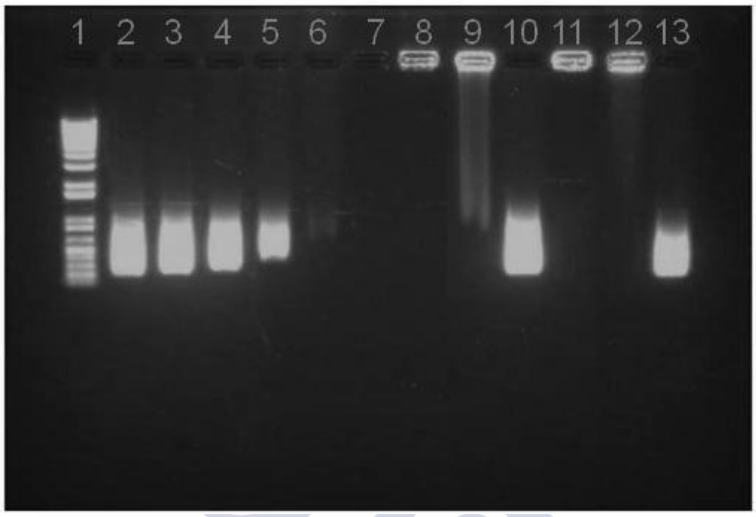

Figure 5. *Agarose gel assay on pIC release from NP B (Chitosan/TPP/Poly (I:C) without peptides): 1) DNA Ladder; 2) 8 μg pIC; 3) 4 μg pIC; 4) 2 μg pIC; 5) 1 μg pIC; 6) 0.5 μg pIC in solution; 7) NP B Supernatant; 8) NP B Pellet; 9) NP B Pellet incubated with Heparin; 10) NP B Pellet digested with chitosanase; 11) NP B Pellet Freeze dried; 12) NP B Pellet Freeze dried and incubated with Heparin; 13) NP B Pellet Freeze dried incubated with chitosanase.*

The final goal was to explore the synergistic effect of chitosan, poly (I:C) and PADRE peptide assembled together in a nanostructure but also the influence of the peptides disposition in the biological outcome.

The preliminary *in vivo* efficacy assay highlighted the importance of the peptide presentation form, with the Chitosan/Poly (I:C)/PADRE-1338aa Adsorbed particles, presenting the highest capacity to produce humoral responses (**Figure 6**). This capacity of inducing an immune response towards this particularly poor immunogenic antigen is in contrast to the lack of efficacy observed with alum salts. The results show the potential of this nanoparticle prototype for antigen delivery and the same time, also indicate the interest of our strategy to combine these nanometric antigen delivery systems with immunostimulants for the generation of an effective adjuvant systems.

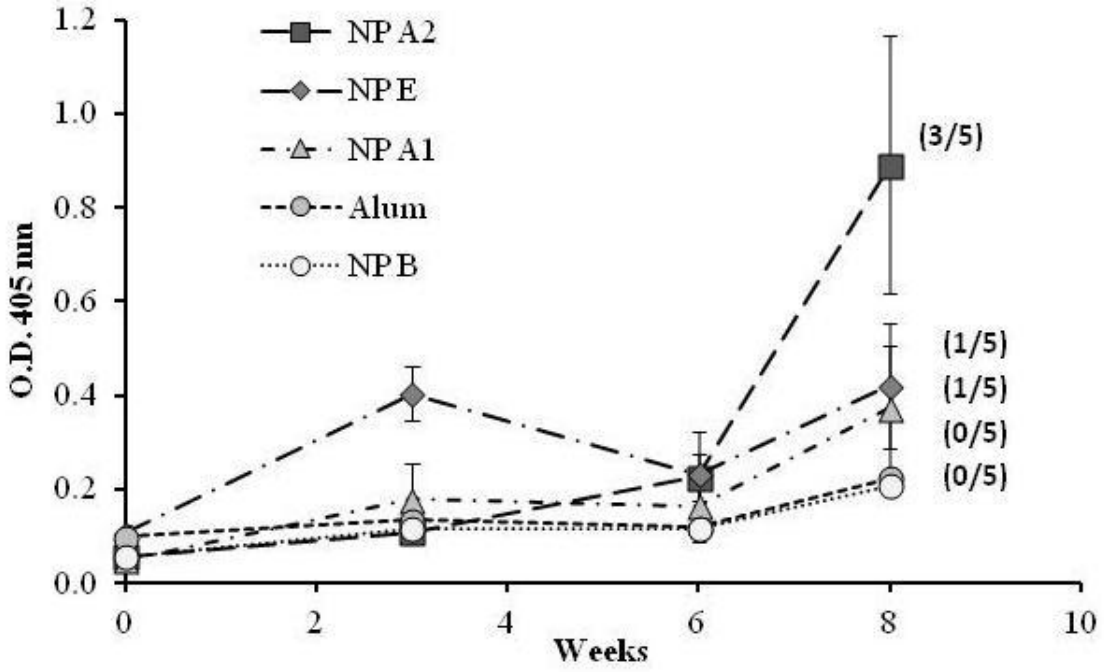

Figure 6. *1338aa peptide-specific IgG responses in the sera diluted 1/50 of mice immunized by the intramuscular route with nanoparticles without peptides (NP B), nanoparticles with surface adsorbed 1338a (NP A1), with surface adsorbed 1338aa and PADRE (NP A2), with both peptides entrapped in the matrix (NP E) or with alum-adsorbed 1338aa. (#/5): number of responsive mice in the total 5 mice per group; an O.D. 4 times higher than the mean O.D. values of NP B immunized mice serum (0.2) was considered a positive response.*

The general lack of immunogenicity of peptide-based antigens inspired us to further developed adjuvant systems for these antigens. Considering the success of already marketed nanoemulsion-based adjuvants like MF59 and ASO3 [9] and the promising results obtained chitosan based nanocapsules both for intramuscular [21] and intranasal route [22], we decided to develop novel nanoemulsions and nanocapsules based on materials with known adjuvant properties like squalene and chitosan, combined with materials less studied in this field such as linoleic acid and lysophosphatidylcholine, which present chemotactic and immunostimulant properties. With the objective to obtain a synergistic effect of these components through their organization in the form of nanostructures, we designed four lipid based nanosystems, namely:

(i) linoleic acid:lysophosphatidylcholine (LA:LS) and

(ii) squalene:linoleic acid:lysophosphatidylcholine (SQ:LA:LS) nanoemulsions and

(iii) linoleic acid:lysophosphatidylcholine:chitosan (LA:LS:CS) and
(iv) squalene:linoleic acid:lysophosphatidylcholine:chitosan (SQ:LA:LS:CS) nanocapsules.

These four systems were prepared by the solvent displacement technique and presented nanometric size (< 200 nm) with low polydispersity (< 0.2). The nanoemulsions presented a highly negative surface charge (< -20 mV) due to the presence of linoleic acid, and the nanocapsules had high positive surface charge (> + 30 mV) due to the surrounding chitosan shell (**Table 7**).

Table 7. Physicochemical characterization of the lysophosphatidylcholine and linoleic acid based nanoemulsions and nanocapsules. (Means ± S.D., n=3).

Formulation	Size (nm)	PdI	ζ (mV)
LA:LS	135 ± 21	0.2	- 36 ± 3
LA:LS:CS	152 ± 11	0.2	+ 39 ± 5
SQ:LA:LS	176 ± 16	0.2	- 28 ± 7
SQ:LA:LS:CS	189 ± 15	0.2	+ 37 ± 8

PdI: Polydispersity Index; ζ: Surface charge

With the exception of the LA:LS composition, the systems presented in general good stability in storage conditions (4 ºC and room temperature) and in simulated physiological media (DMEM and RPMI with FBS) and could be adequately converted to a dried form by lyophilization. Additionally, these particles were characterized for their capacity to initiate the complement system and stimulate the production of cytokines and ROS, as well as their cytotoxicity. The inclusion of squalene and chitosan in the composition seemed to increase formulation stability and is eventually expected to further enhance the systems adjuvanticity. Due to their lower toxicity, the squalene containing formulations were chosen to do preliminary association studies with three peptide-based model antigens (denoted as 68-4, 69-2 and 69-4) with similar molecular weights but different isoelectric points and hydrophobicity (see **Table 1**). Interestingly, it was found that the main characteristic affecting the efficiency of association was the

hydrophobicity of the peptides, which is compatible with the hydrophobic character of our lipid-based nanosystems and could be useful in the further optimization of peptide association (**Table 8**).

Table 8. Physicochemical characterization and loading efficiency of the SQ:LA:LS and SQ:LA:LS:CS formulations loaded with the peptide-based antigens 68-4, 69-2 and 69-4. (Means ± S.D., n=3).

Formulation	Peptide	Size (nm)	PdI	ζ (mV)	Antigen (%)
SQ:LA:LS	**68-4**	154 ± 8	0.2	- 22 ± 9	16 ± 10
	69-2	200 ± 29	0.3	- 22 ± 2	24 ± 14
	69-4	153 ± 11	0.1	- 23 ± 7	7 ± 1
SQ:LA:LS:CS	**68-4**	177 ± 14	0.2	+ 36 ± 5	23 ± 5
	69-2	187 ± 26	0.2	+ 34 ± 6	45 ± 9
	69-4	180 ± 13	0.2	+ 37 ± 4	18 ± 3

PdI: Polydispersity Index; ζ: Surface charge; Antigen (%): Antigen association efficiency

As a general conclusion from the results obtained during the present thesis: several types of polymeric and lipidic nanocarriers have been developed by the combination of different biomaterials in order to explore different strategies to enhance the efficacy of antigen delivery to and presentation by immunocompetent cells. These nanosystems have shown capacity to associate diverse immunomodulator molecules together with antigens of different nature such as virus-like particles or peptide-based antigens. The adjuvant properties of these nanostructures were explored by *in vitro* and *in vivo* studies and present great potential as future vaccine delivery systems. Altogether, this work reflects the high versatility of possibilities offered by nanotechnology for the development of new adjuvant systems.

REFERENCES

1. Soane, R.J., et al., *Evaluation of the clearance characteristics of bioadhesive systems in humans.* International Journal of Pharmaceutics, 1999. **178**(1): p. 55-65.

2. Kerlin, R.L. and D.L. Watson, *Effect of dextran sulphate on IgG subclass of antibody in efferent popliteal lymph of sheep.* Immunol Cell Biol, 1987. **5**: p. 411-7.

3. Yang, D. and K.S. Jones, *Effect of alginate on innate immune activation of macrophages.* J Biomed Mater Res A, 2009. **90**(2): p. 411-8.

4. Buschle, M., et al., *Transloading of tumor antigen-derived peptides into antigen-presenting cells.* Proceedings of the National Academy of Sciences, 1997. **94**(7): p. 3256-3261.

5. Reynolds, F., R. Weissleder, and L. Josephson, *Protamine as an efficient membrane-translocating peptide.* Bioconjugate Chemistry, 2005. **16**(5): p. 1240-1245.

6. Lundberg, A.M., et al., *Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems*. Vol. 110. 2007. 3245-3252.

7. Durand, V., et al., *Shaping of adaptive immune responses to soluble proteins by TLR agonists: a role for IFN-alpha/beta.* Immunol Cell Biol, 2004. **82**(6): p. 596-602.

8. Sloat, B. and Z. Cui, *Nasal Immunization with Anthrax Protective Antigen Protein Adjuvanted with Polyriboinosinic–Polyribocytidylic Acid Induced Strong Mucosal and Systemic Immunities.* Pharmaceutical Research, 2006. **23**(6): p. 1217-1226.

9. Correia-Pinto, J.F., N. Csaba, and M.J. Alonso, *Vaccine delivery carriers: insights and future perspectives.* Int J Pharm, 2013. **440**(1): p. 27-38.

10. Ryborg, A.K., et al., *Intracutaneous injection of lysophosphatidylcholine induces skin inflammation and accumulation of leukocytes.* Acta Derm Venereol, 2000. **80**(4): p. 242-6.

11. Mehling, T., et al., *Estimation of LPC/water partition coefficients using molecular modeling and micellar liquid chromatography.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2013. **431**(0): p. 105-113.

12. Lauber, K., et al., *Clearance of Apoptotic Cells: Getting Rid of the Corpses.* Molecular Cell, 2004. **14**(3): p. 277-287.

13. Claire-Anne Siegrist, *Vaccine immunology*, in *Vaccines (Sixth Edition)*, Offit Stanley A., Plotkin Walter A., and Orenstein Paul A., Editors. 2013, W.B. Saunders: London. p. 14-32.

14. Graham, M.B., V.L. Braciale, and T.J. Braciale, *Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection.* J Exp Med, 1994. **180**(4): p. 1273-82.

15. WHO, *Annex 1 - Guidelines for the production and quality control of synthetic peptide vaccies*. World Health Organ Technical Report Series. Vol. 889. 1999: WHO Expert Committee on Biological Standardization.

16. Lanzavecchia, A., *Identifying strategies for immune intervention.* Science, 1993. **260**(5110): p. 937-44.

17. Csaba, N., M. Koping-Hoggard, and M.J. Alonso, *Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery.* Int J Pharm, 2009. **382**(1-2): p. 205-14.

18. Prego, C., et al., *Chitosan-based nanoparticles for improving immunization against hepatitis B infection.* Vaccine, 2010. **28**(14): p. 2607-14.

19. Vila, A., et al., *Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice.* Eur J Pharm Biopharm, 2004. **57**(1): p. 123-31.

20. Wu, C.Y., et al., *Improving therapeutic HPV peptide-based vaccine potency by enhancing CD4+ T help and dendritic cell activation.* J Biomed Sci, 2010. **17**(88): p. 1423-0127.

21. Vicente, S., et al., *A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach.* PLoS ONE, 2013. **8**(4): p. e62500.

22. Vicente, S., et al., *Co-delivery of viral proteins and a TLR7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy.* Journal of Controlled Release, 2013. **172**(3): p. 773-781.

CONCLUSIONES/CONCLUSIONS

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CONCLUSIONES

El trabajo experimental recogido en la presente memoria se ha centrado en el diseño y desarrollo de nuevos nanosistemas transportadores de antígeno constituidos por diferentes biomateriales, y en la evaluación de su capacidad para vehiculizar antígenos, tras su administración por vía parenteral o por vía nasal. De los resultados obtenidos, se pueden extraer las siguientes conclusiones:

1. Utilizando como antígeno particulado modelo, el antígeno recombinante de superficie de la hepatitis B (rHBsAg), se ha logrado obtener sistemas de tamaño nanométrico por medio del recubrimiento secuencial del antígeno mediante protamina y sulfato de dextrano, mediante poliarginina y alginato o mediante poliarginina y poly (I:C). Esta estrategia ha permitido, en general, incrementar la respuesta humoral frente al antígeno recubierto, tras su administración por vía parenteral y nasal. . De entre las formulaciones evaluadas, aquélla que contiene el agente inmunoestimulante poly (I:C), es la que ha permitido alcanzar los niveles más elevados de IgG entre los sistemas testados y modular la respuesta inmune hacia un tipo predominantemente T_h1 (celular).

2. Se ha desarrollado un nuevo sistema adyuvante basado en la gelificación del quitosano con TPP, capaz de vehiculizar simultáneamente un inmunoestimulante (Poly (I:C)), un péptido T-helper (PADRE) y un antígeno peptídico (1338aa) y que permite tanto la encapsulación de los péptidos como su adsorción a la superficie de las nanopartículas. Los resultados obtenidos tras la evaluación *in vivo* de estas formulaciones llevaron a concluir que la disposición de los péptidos a la superficie de la nanopartícula es la configuración preferible para una inmunización efectiva

3. A través de la técnica de desplazamiento del solvente se han desarrollado nuevos nanoemulsiones y nanocápsulas con potencial adyuvante, basadas en lisofosfatidilcolina, acido linoléico, escualeno y quitosano, estables en distintas condiciones de acondicionamiento y soluciones fisiológicas simuladas y que pueden ser liofilizados y resuspendidos sin pérdida de sus características fisicoquímicas originales. El escualeno y el quitosano contribuyeron de manera significativa a incrementar la estabilidad de los sistemas. Los estudios de asociación de antígenos peptídicos pusieron

de manifiesto la capacidad de estos sistemas para vehiculizar antígenos peptídicos con distintas características fisicoquímicas.

CONCLUSIONS

The experimental work reported in this thesis has been oriented towards the design and development of new nanometric antigen delivery systems, composed by different biomaterials and the evaluation of their the capacity to deliver antigens upon parenteral or nasal administration. The results obtained led us to the following conclusions:

1. Using the recombinant hepatitis B surface antigen (rHBsAg) as a model virus-like particle (VLP) antigen, it was possible to develop nanometric systems through the sequential enveloping of the VLP antigen with protamine and dextran sulfate, with polyarginine and alginate or with polyarginine and poly (I:C). This strategy has led to a general increase of the humoral response against the multi-enveloped antigen and the generation of protective IgG titers (> 10 mIU/ml) upon parenteral and nasal administration. The association of the poly (I:C) to the system generated the highest IgG titers among the multi-enveloping systems and also permitted the modulation of the immune response towards a predominant T_h1 type response (cellular).

2. A new adjuvant system has been developed based on the ionic gelation of chitosan with TPP, capable of co-delivering an immunostimulant (Poly (I:C)), a T-helper peptide (PADRE) and a peptide-based antigen (1338aa). This system permits, either the entrapment or the adsorption of the antigenic peptide onto the nanoparticle surface. The results of the *in vivo* studies showed the capacity of this system to potentiate the humoral response against the associated antigen and indicated that the peptide display on the nanoparticle surface is critical for an effective immunization.

3. Using the solvent displacement technique, it was possible to develop new nanoemulsions and nanocapsules with adjuvant potential, based on lysophosphatidylcholine, linoleic acid, squalene and chitosan. These nanosystems were found to be stable under different storage conditions and suspension media and could be freeze dried and resuspended without losing their initial physicochemical properties. Squalene and chitosan contributed to the stability of the system. Preliminary studies indicated the ability of these systems to associate peptide-based antigen with different physicochemical characteristics.