DOCTORAL THESIS

RATIONAL DESIGN OF POLYMER-BASED NANOSYSTEMS FOR AN EFFICIENT TARGETING OF THE IMMUNE SYSTEM

Ana Sara Caetano Cordeiro

Doctoral Program in Drug Research and Development

Faculty of Pharmacy

SANTIAGO DE COMPOSTELA

2017

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

TESIS DOCTORAL

DISEÑO RACIONAL DE NANOSISTEMAS POLIMÉRICOS PARA VEHICULIZACIÓN AL SISTEMA INMUNE

Ana Sara Caetano Cordeiro

Programa de Doctorado en Investigación
y Desarrollo de Medicamentos

Facultad de Farmacia

SANTIAGO DE COMPOSTELA

2017

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Prof. María José Alonso, full Professor of the Department of Pharmacology, Pharmacy and Pharmaceutical Technology of the University of Santiago de Compostela,

Dra. María de la Fuente Freire, Miguel Servet researcher, Instituto de Investigación Sanitaria de Santiago de Compostela, Servizo Galego de Saúde,

Dr. Jose Crecente Campo, research scientist of the Department of Pharmacology, Pharmacy and Pharmaceutical Technology of the University of Santiago de Compostela,

As supervisors of the doctoral thesis entitled **"Rational design of polymer-based nanosystems for an efficient targeting of the immune system"** presented by **Ana Sara Caetano Cordeiro** under the Doctoral Program in Drug Research and Development,

Authorize its presentation considering it gathers the necessary requirements of article 34 of the Doctoral Studies regulation and that as supervisors of this thesis they do not incur in the abstention causes established by the law 40/2015.

At Santiago de Compostela, on July 13th 2017,

Prof. María José Alonso	Dr. María de la Fuente	Dr. Jose Crecente

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Prof. María José Alonso, Catedrática del Departamento de Farmacología, Farmacia y Tecnología Farmacéutica de la Universidad de Santiago de Compostela,

Dra. María de la Fuente Freire, investigadora Miguel Servet, Instituto de Investigación Sanitaria de Santiago de Compostela, Servizo Galego de Saúde,

Dr. Jose Crecente Campo, investigador del Departamento de Farmacología, Farmacia y Tecnología Farmacéutica de la Universidad de Santiago de Compostela,

Como Directores de la Tesis Doctoral titulada **"Diseño racional de nanosistemas poliméricos para vehiculización al sistema inmune"**, presentada por **Ana Sara Caetano Cordeiro**, alumna del Programa de Doctorado en Investigación y Desarrollo de Medicamentos,

Autorizan la presentación de la tesis indicada, considerando que reúne los requisitos exigidos por el artículo 34 de la normativa de Estudios de Doctorado, y que como Directores de la misma no incurren en las causas de abstención establecidas en la ley 40/2015.

En Santiago de Compostela, a 13 de julio de 2017,

Prof. María José Alonso	Dr. María de la Fuente	Dr. Jose Crecente

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

À minha família,

e a todos os que vivem no meu coração.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

“Recomeça…
Se puderes
Sem angústia
E sem pressa.
E os passos que deres,
Nesse caminho duro
Do futuro
Dá-os em liberdade.
Enquanto não alcances
Não descanses.
De nenhum fruto queiras só metade.”

(Miguel Torga)

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Index

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Resumen/Abstract

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Resumen

El objetivo principal de esta tesis es el desarrollo de nanosistemas poliméricos para vehiculización de antígenos al sistema inmune. Con el fin de ampliar el conocimiento sobre la influencia de parámetros fisicoquímicos específicos de los nanosistemas en su biodistribución al sistema inmune, hemos estudiado el comportamiento de distintas nanocápsulas poliméricas. Así, hemos optimizado dos prototipos de nanocápsulas de quitosano con tamaño distinto y sus nanoemulsiones correspondientes, además de otras nanocápsulas con distinta cubierta polimérica (poliarginina, inulina y carboximetil-β-glucano). Los resultados han revelado que los prototipos de tamaño inferior a 100 nm han drenado más fácilmente desde el punto de inyección y se han acumulado más en los nódulos linfáticos. Sin embargo, la carga superficial de los nanosistemas y las especificidades de los polímeros utilizados en la cubierta también han mostrado tener un papel relevante en este proceso.

Además, considerando las propiedades inmunomoduladoras de los polisacáridos y, en particular, de los beta glucanos, hemos desarrollado nanopartículas de quitosano y carboximetil-β-glucano (CS:CMβG) como sistemas innovadores para liberación de antígenos. Dichas nanopartículas, con un tamaño en torno a 180 nm y un potencia zeta de +30 mV mostraron una alta capacidad para la asociación del antígeno modelo ovalbumina (OVA) (carga final de 6.7% m/m). Además, su perfil de estabilidad durante su almacenamiento, tanto en forma de suspensión acuosa (9 semanas a 4 ºC) como de polvo seco (1 mes a 40 ºC) resultó muy favorable. Por último, la administración subcutánea de esta formulación a ratones dio lugar a una acumulación significativa de la misma en los nódulos linfáticos, específicamente co-localizando con las células dendríticas, que es la principal población de células presentadoras de antígeno. Esta interacción condujo a una presentación eficaz del antígeno a células T, estimulando su proliferación en niveles similares a los obtenidos con alum. Finalmente, hemos desarrollado con éxito una nanodispersión de las nanopartículas sólidas en un vehículo oleoso, con posible aplicación para inmunización por vías no parenterales.

En general, nuestros resultados muestran el potencial de los nanosistemas poliméricos para ser modulados en cuanto a su composición, con el fin de promover una respuesta inmune más eficaz.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Abstract

The main goal of this thesis is the engineering of polymer-based nanocarriers for antigen delivery to the immune system. To understand better the influence of specific physicochemical characteristics of nanosystems in their biodistribution to the immune system, we evaluated the behavior of different polymer-based nanocapsules. Therefore, we optimized two prototypes of chitosan nanocapsules with different particle size and their corresponding nanoemulsions, as well as other nanocapsules with different polymeric coatings (polyarginine, inulin and carboxymethyl-β-glucan). Results showed that prototypes with less than 100 nm were more easily drained from the injection site and accumulated more in lymph nodes. Nevertheless, the surface charge of the nanocarriers and the specific characteristics of the polymers used in their shell were also shown to play a relevant role in this process.

On the other hand, considering the described immunomodulating properties of polysaccharides and, in particular, of the beta glucan family, we prepared chitosan:carboxymethyl-β-glucan (CS:CMβG) nanoparticles as a novel beta glucan-based antigen delivery system. These nanoparticles were efficiently loaded with a model antigen, ovalbumin (OVA), and presented adequate storage stability, both in suspension (4 ºC) and in dry powder form (40 ºC). Moreover, subcutaneous administration of this formulation to mice led to its significant accumulation in the draining lymph nodes, specifically in co-localization with dendritic cells, which are the main antigen presenting cell population. In fact, this interaction was shown to drive antigen presentation to effector T cells, stimulating their proliferation in levels similar to those observed for alum-adsorbed antigen. Finally, a solid-in-oil nanodispersion of the antigen-loaded carriers was successfully developed and could be interesting for non-parenteral immunization approaches.

Overall, our results show the potential of polymer-based nanocarriers to be modulated at the formulation level, aiming at a more efficient immune response.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Resumen *in extenso*

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Resumen *in extenso*

Introducción

El descubrimiento de la vacuna de la viruela por Edward Jenner, pionero en la utilización de patógenos con virulencia atenuada como forma de prevenir enfermedades infecciosas, supuso una verdadera revolución en el campo de la inmunología. Desde entonces, la vacunación ha contribuido a la prevención y erradicación de un gran número de enfermedades, convirtiéndola en una de las intervenciones más efectivas y económicas en salud. Sin embargo, la investigación en este campo no ha cesado de evolucionar, enfocada en la búsqueda de soluciones a algunas de las limitaciones de las vacunas actualmente disponibles. Estas limitaciones incluyen la baja resistencia a temperatura elevadas, la necesidad de múltiples dosis de una misma vacuna para lograr un efecto protector y, principalmente, la reducida inmunogenicidad de los antígenos modernos, particularmente proteínas y péptidos. Estos, desarrollados gracias a los impresionantes avances de la biotecnología en las últimas décadas, han evitado el riesgo asociado a los patógenos atenuados de revertir a su forma virulenta, siendo por ello más seguros. No obstante, su capacidad de generar una respuesta inmune eficiente es también más limitada y, por lo tanto, requieren del uso de potentes adyuvantes en la formulación de la vacuna.

Los adyuvantes se definen como compuestos que potencian la respuesta inmune, la alargan en el tiempo y/o la modulan hacia una determinada vía (celular o humoral), independientemente de su estructura o mecanismo de acción. Estos compuestos se agrupan habitualmente en dos categorías: sistemas de liberación de antígenos y compuestos con actividad inmunomoduladora. Los primeros adyuvantes desarrollados para uso humano fueron las sales de aluminio, a las que se les atribuye la capacidad de retener el antígeno en el sitio de inyección durante largos períodos de tiempo, formando un “depot” que favorece la atracción de células inmunes a esa zona y la consecuente generación/iniciación de la respuesta inmune. Sin embargo, limitaciones de este adyuvante como la elevada sensibilidad a cambios de temperatura o la aparición de reacciones adversas locales, han conducido a la búsqueda de otras alternativas que puedan potenciar la inmunogenicidad de los antígenos más modernos.

La nanotecnología fue aplicada por primera vez al transporte y liberación de antígenos a finales de los años 70, con el uso de nanopartículas a base de poliésteres en vacunas contra el virus de la gripe. Las ventajas de la utilización de estos sistemas como transportadores de antígenos incluyen su similitud estructural con los agentes

patógenos, su versatilidad tecnológica que permite su modificación para el direccionamiento específico hacia el tejido o célula diana y la posibilidad de incluir otras moléculas adyuvantes en su composición que puedan potenciar su efecto global. Además, los nanosistemas permiten aumentar la estabilidad del antígeno frente a la degradación, controlar su liberación en el tiempo y facilitar su administración por vías no parenterales como las mucosas, que han sido ampliamente estudiadas en este ámbito.

A pesar del potencial demostrado por este tipo de partículas para actuar como adyuvantes en formulaciones de vacunas, las limitaciones de los poliésteres, particularmente en relación al efecto de su propia degradación sobre la estabilidad de los antígenos, han conducido a la búsqueda de nuevas alternativas. Polímeros naturales, en particular los polisacáridos, han destacado como componentes de nanopartículas transportadoras de antígenos, principalmente debido a sus características estructurales y a la baja toxicidad presentada por estos biomateriales. Los polisacáridos están presentes en la estructura de diversos patógenos y, por este motivo, pueden simular un patrón reconocible por las células inmunes como una "señal de peligro" para iniciar la respuesta inmune. En este sentido, cabe destacar el quitosano como uno de los polisacáridos más estudiados para la preparación de sistemas de liberación de fármacos y antígenos. Sus propiedades mucoadesivas han permitido su aplicación en diversos tipos de nanosistemas para administración de antígenos por vía mucosa, con resultados prometedores. Otros polisacáridos con interés en este campo incluyen polímeros de manosa, dextrano, inulina y beta glucanos.

Considerando estas bases previamente descritas en la literatura sobre el papel de los nanosistemas poliméricos y de los polisacáridos como potenciales adyuvantes en inmunización, se ha propuesto como objetivo principal de esta tesis el desarrollo y optimización de nanosistemas a base de polímeros para la vehiculización de antígenos al sistemas inmune. Además, se ha estudiado la influencia de los principales parámetros fisicoquímicos de estos nanosistemas en su biodistribución e interacción con las principales poblaciones de células inmunes.

En detalle, los objetivos específicos de la tesis son:

1. Estudio de la influencia de parámetros fisicoquímicos de nanoemulsiones y nanocápsulas, incluyendo tamaño de partícula, carga superficial y composición, en su comportamiento *in vitro* e *in vivo* frente a células inmunes.
2. Diseño y desarrollo de una nueva formulación de nanopartículas a base de carboximetil-β-glucano y quitosano para el transporte y liberación de un antígeno modelo;

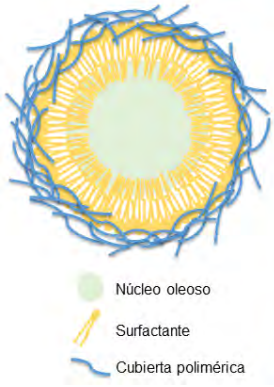
3. Estudio de la biodistribución al sistema linfático de dichas nanopartículas y de su interacción con la células inmunes y potencial adyuvante.

Modulación de los parámetros fisicoquímicos de nanocápsulas poliméricas para evaluación de su biodistribución e interacción con el sistema inmune

La influencia de los parámetros fisicoquímicos de los nanosistemas en su interacción con los sistemas biológicos, particularmente en el campo de las nanovacunas, es uno de los temas más estudiados a lo largo de las últimas décadas en el campo de la nanomedicina. La realización de estudios sistemáticos que permitan comparar exhaustivamente distintos nanosistemas podrá contribuir a un diseño más racional de nanovehículos transportadores de antígenos, mejorando también su eficacia como adyuvantes. En este sentido, hemos propuesto comparar la biodistribución e interacción con células inmunes de nanocápsulas con distintos tamaños y distintas cubiertas poliméricas. Para ello, hemos seleccionado las nanocápsulas de quitosano, previamente desarrolladas por nuestro grupo y con demostrado potencial adyuvante, como punto de partida del estudio comparativo. Este prototipo, preparado a través del método de desplazamiento de disolvente, está compuesto por un núcleo de escualeno, estabilizado por una capa de lecitina y a su vez recubierta por quitosano.

A partir de este sistema, que presenta un tamaño de partícula de 220-250 nm y una carga superficial de +30 mV, hemos optimizado la preparación de nanocápsulas con la misma composición pero con tamaño de partícula inferior a 100 nm. Para ello, hemos combinado dos técnicas: (i) la reducción de las cantidades de cada componente hasta un 25% de los valores iniciales y (ii) la inyección de la fase oleosa directamente en la fase acuosa a través de jeringa y aguja. Además, utilizando el mismo método, hemos preparado nanoemulsiones de composición similar pero sin la cubierta de quitosano. Finalmente, con el objetivo de comparar el efecto de diferentes cubiertas poliméricas y, consecuentemente, de distintas cargas superficiales en el comportamiento biológico de las nanocápsulas, hemos seleccionado otros tres polímeros para recubrir los nanosistemas. En particular, hemos elegido la poliarginina (polímero catiónico), la inulina (neutra) y el carboximetil-β-glucano (aniónico), considerando su potencial inmunomodulador y su potencial para formar micro y nanosistemas. La preparación de estos nanosistemas se ha llevado a cabo a través de la previamente mencionada técnica de desplazamiento de disolvente y todos los sistemas fueron caracterizados a nivel fisicoquímico usando técnicas de dispersión de luz dinámica y espectroscopía de correlación fotónica. Además, todos los prototipos han sido marcados con la molécula

fluorescente DiD a través de su encapsulación en el núcleo oleoso de las distintas nanocápsulas. La eficacia de esta encapsulación, así como la estabilidad de las nanocápsulas en almacenamiento y la estabilidad del propio marcaje fluorescente, han resultado adecuados para los estudios a realizar. La **figura 1** resume las características de todos los prototipos desarrollados.



	Núcleo	Surfactante(s)	Cubierta	Tamaño (nm)	PdI	Potencial Zeta (mV)
SQL NE S	Escualeno	Lecitina	---	87 ± 12	0.2	-45 ± 9
CS NC S			Quitosano	93 ± 9	0.2	+22 ± 2
SQL NE M			---	166 ± 13	0.1	-66 ± 3
CS NC M			Quitosano	238 ± 19	0.2	+32 ± 5
PArg NC	Vitamina E	TPGS Colato Sódico	Poliarginina	90 ± 11	0.2	+55 ± 4
Inulin NC	Vitamina E	Glicocolato Sódico	Inulina	80 ± 5	0.2	-23 ± 5
CMβG NC	Escualeno	Tween 80 CTAB	Carboximetil-β-glucan	96 ± 8	0.2	-38 ± 3

Figura 1. *Representación esquemática de las nanocápsulas desarrolladas y resumen de su composición y características fisicoquímicas (tamaño de partícula, índice de polidispersión (PdI) y potencial zeta). SQL, escualeno; NE, nanoemulsiones; S, pequeñas; CS, quitosano; NC, nanocápsulas; M, medianas; PArg, poliarginina; CMβG, carboximetil-β-glucano.*

Conociendo la importancia de la interacción de los nanosistemas con las células presentadoras de antígeno en su eficacia como potenciales adyuvantes, hemos estudiado la internalización *in vitro* por macrófagos de las nanocápsulas de quitosano de ambos tamaños y de sus nanoemulsiones control. Asimismo, hemos evaluado la participación de los receptores scavenger (SR) y TLR-4 en dicho proceso de internalización, las rutas de internalización utilizadas por los macrófagos y el perfil de citocinas generado tras dicha interacción. La internalización de los nanosistemas fue bastante elevada en todos los casos, independientemente del tamaño de partícula y de la presencia o ausencia de cubierta polimérica. Sin embargo, comparando el perfil de citocinas generado tras la internalización de los cuatro nanosistemas por macrófagos derivados de médula ósea de ratones wildtype y TLR-4 knockout (TLR-4 -/-), hemos podido observar diferencias en relación al tamaño. En este caso, las nanocápsulas de menor tamaño han generado niveles significativamente más elevados de la citocina MCP-1 que las nanocápsulas de más de 200 nm, particularmente en células TLR-4 -/-. Estos resultados pueden indicar una participación específica de este receptor en el reconocimiento e internalización de estas nanocápsulas, aunque serían necesarios más estudios para comprobar esta hipótesis. Para evaluar la participación de los receptores

scavenger en la internalización, hemos usado una línea celular derivada de la Raw 264.7, en la cual la expresión de estos receptores se encontraba silenciada. Los resultados han mostrado una intervención significativa de los SR en la internalización de todos los prototipos, particularmente en el caso de los de menor tamaño. Este hecho puede estar relacionado con el recubrimiento de las nanocápsulas por proteínas presentes en el medio de cultivo celular, dada la descrita preferencia de los SR por ligando polianiónicos.

Para completar el estudio *in vitro* de estas formulaciones, hemos evaluado el efecto de la inhibición química de cuatro rutas de internalización: fagocitosis, macropinocitosis, endocitosis mediada por clatrinas y mediada por caveolas. A través de este estudio, hemos verificado que la internalización se ha visto reducida con cualquiera de los cuatro inhibidores, particularmente en el caso de la fagocitosis. Además, esta ruta fue la única en la cual se han verificado diferencias significativas en la internalización según el tamaño de las nanopartículas. Contrariamente a lo que habitualmente se ha descrito para la fagocitosis, hemos observado una importante influencia de esta vía en la internalización de los prototipos de menor tamaño, especialmente las nanocápsulas. Así, a pesar de la predominancia de la fagocitosis como ruta principal de internalización de los nanosistemas, podemos concluir que este proceso se produce por una combinación de rutas, sin una correlación específica con el tamaño de partícula o la presencia de una cubierta polimérica. En la **tabla 1** se describen de forma resumida los resultados obtenidos en esta parte del trabajo.

Tabla 1. *Resumen de los resultados obtenidos en la evaluación in vitro de las vías de internalización y de la influencia de los receptors scavenger (SR) en la internalización de las nanocápsulas de quitosano (CS NC) e nanoemulsiones control (SQL NE) por macrófagos de la línea celular RAW 264.7. Las flechas indican inhibición de la internalización tras la incubación con el inhibidor respective; (SR) indica una inhibición más relevante en las células cuya expresión de SR se encontraba silenciada, en comparación con células wild-type.*

Formulación	Fagocitosis	Macropinocitosis	Mediada por clatrinas	Mediada por caveolas
SQL NE S	↓	-	↓	↓
CS NC S	↓↓	-	↓	↓
SQL NE M	↓	-	↓ (SR)	↓
CS NC M	↓	-	↓ (SR)	↓

Considerando que la mayoría de las vacunas son administradas por vías parenterales, como la subcutánea o la intramuscular, es de especial relevancia conocer el comportamiento de los prototipos de nanovacunas tras su administración por una de estas vías. En el caso de la vía subcutánea, que hemos seleccionado para nuestros estudios, la formulación administrada encuentra primeramente una matriz compleja que debe atravesar, la matriz intersticial, para poder llegar a los vasos linfáticos que se ocuparán de su drenaje hasta los nódulos linfáticos, donde se concentran las células inmunes que pueden desencadenar la respuesta inmune adecuada. La mayoría de las vacunas, por sus características intrínsecas, forma un “depot” en el punto de inyección, que atrae células inmunes por respuesta inflamatoria que transportan el antígeno hasta los nódulos linfáticos. Sin embargo, es posible a través de la nanotecnología lograr un drenaje de los nanosistemas transportadores del antígeno directamente a los nódulos linfáticos, dependiendo de sus características fisicoquímicas como el tamaño de partícula y la carga superficial. Por este motivo, hemos utilizado los prototipos de nanocápsulas previamente descritos, para estudiar el efecto de estos parámetros en su drenaje a los nódulos linfáticos tras administración subcutánea. Con este fin, hemos utilizado la microscopía de excitación de dos fotones, que permite la observación de procesos biológicos a nivel subcelular con alta resolución, minimizando el riesgo de daño tisular. Además, hemos cuantificado la acumulación de los nanosistemas en los nódulos linfáticos y su co-localización con poblaciones celulares de interés utilizando citometría de flujo.

En primer lugar, las nanocápsulas de quitosano de dos tamaños y las correspondientes nanoemulsiones control, todas ellas marcadas con DiD, han sido administradas por vía subcutánea en la almohadilla de la pata de ratones C57/B6. Con el fin de normalizar los resultados obtenidos y eliminar la interferencia de cualquier problema en el momento de la inyección, hemos co-administrado partículas de poliestireno marcadas con un fluoróforo distinto. Además, para permitir el marcaje de distintos tipos celulares en los nódulos linfáticos, hemos administrado por la misma vía un conjunto de anticuerpos marcados con distintos fluoróforos, dirigidos a macrófagos y células dendríticas. Doce horas después de las administraciones, los ratones han sido sacrificados y sus nódulos linfáticos poplíteales y lumbares han sido recogidos para observación microscópica y obtención de suspensiones celulares para citometría.

Los resultados de este estudio han permitido observar una diferencia significativa en la distribución y acumulación de las nanocápsulas de distintos tamaños. Las nanocápsulas de menor tamaño de partícula (< 100 nm) mostraron una acumulación significativamente más elevada que las de mayor tamaño (> 200 nm) (**figura 2**), lo que se corresponde con lo que se había previamente descrito para otro tipo de nanosistemas y su distribución en el sistema linfático.

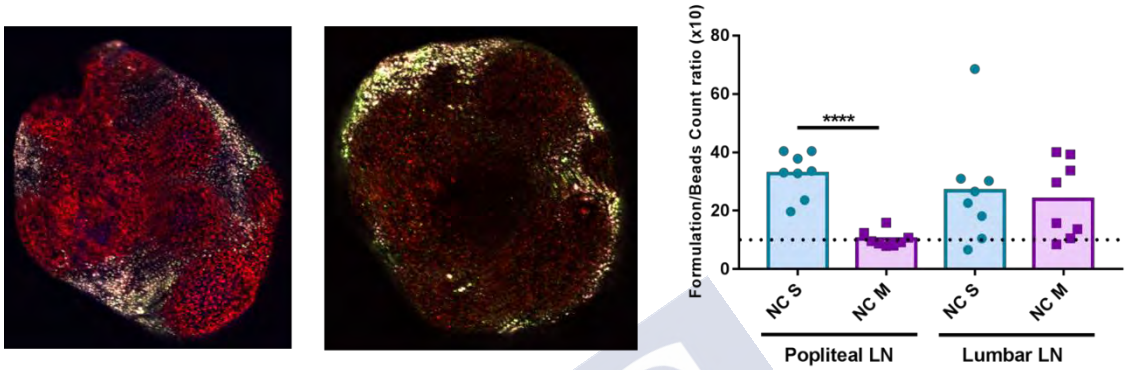


Figura 2. *Distribución de nanocápsulas de quitosano de dos tamaños (NC S, inferior a 100 nm y NC M, superior a 200 nm) marcadas con DiD en los nódulos linfáticos popliteales y lumbares, tras inyección por vía subcutánea. Las imágenes ex vivo de microscopía de excitación de dos fotones muestran la acumulación de los nanosistemas (NC S a la izquierda y NC M al centro) en el nódulo popliteal. Los macrófagos se ven en rojo (CD169-PE), las nanocápsulas en blanco (DiD) y las partículas de poliestireno utilizadas como control en verde (Firefli™ Fluorescent Green). La intensidad de fluorescencia se ha cuantificado a través de citometría de flujo (gráfica a la derecha). Los resultados se han normalizado considerando la señal obtenida para las partículas control (la línea discontínua señala una igual acumulación de nanocápsulas y partículas control). **** p<0.0001.*

Además, estas formulaciones también han demostrado niveles superiores de co-localización con las principales poblaciones celulares presentes en los nódulos linfáticos popliteales (macrófagos medulares, macrófagos del seno subcapsular y células dendríticas), en comparación con las nanocápsulas de mayor tamaño de partícula. Finalmente, la comparación de los resultados obtenidos con las nanocápsulas de ambos tamaños y sus correspondientes nanoemulsiones control ha permitido observar un posible efecto combinado de ambos parámetros – tamaño de partícula y carga superficial (o presencia/ausencia de cubierta polimérica). Mientras las nanocápsulas de menor tamaño se han acumulado más en el nódulo popliteal que las correspondientes nanoemulsiones, lo contrario ha ocurrido con las formulaciones de mayor tamaño,

sugiriendo que en este caso el tamaño de partícula no es el único determinante del perfil de biodistribución de este tipo de formulaciones. Estos resultados de citometría se confirmaron con la técnica de microscopía previamente descrita donde se obtuvo un patrón de comportamiento similar.

De forma complementaria, se han obtenido vídeos de la llegada y acumulación de los dos prototipos de nanocápsulas de quitosano en el nódulo linfático popliteal, a través de la misma técnica de microscopía pero en condiciones *in vivo*. Para ello, se ha inyectado una mezcla de ambas nanocápsulas, en igual cantidad, por vía subcutánea en la almohadilla de la pata del ratón y se ha observado su llegada al nódulo linfático. En este caso, se ha podido observar la llegada de las nanocápsulas de menor tamaño aproximadamente 15 minutos tras el inicio de la adquisición de las imágenes, mientras que la llegada de las nanocápsulas de mayor tamaño sucede bastante más tarde y con menor grado de acumulación en el nódulo linfático. Estos resultados corroboran los anteriormente descritos y soportan, de manera innovadora, el perfil de biodistribución previamente trazado para este tipo de prototipos.

Con el objetivo de estudiar el efecto de la cubierta polimérica y de la carga superficial de las partículas, hemos realizado un estudio similar de biodistribución utilizando los cuatro prototipos de nanocápsulas anteriormente descritos, todos ellos con tamaño inferior a 100 nm, marcados con DiD y cubiertas de quitosano, poliarginina, inulina y carboximetil-β-glucano. En este caso, los resultados mostraron una acumulación preferencial de las nanocápsulas de carga superficial positiva en relación a las de carga superficial negativa, en el nódulo linfático popliteal, particularmente en el caso de las nanocápsulas de poliarginina. En el nódulo linfático lumbar esta diferencia entre las formulaciones catiónicas y aniónicas no ha sido tan marcada, ya que tanto las nanocápsulas de poliarginina como las de carboximetil-β-glucano se han acumulado significativamente más que las de quitosano y de inulina, respectivamente. Así, es posible observar que no sólo la carga superficial de las partículas pero también las características propias de los materiales que las constituyen pueden influenciar en su biodistribución en el sistema linfático.

En general nuestros resultados han permitido observar una influencia importante del tamaño de partícula y de la carga superficial de los nanosistemas en su biodistribución al sistema linfático. Además, la naturaleza de los polímeros utilizados en la cubierta de las nanocápsulas parece tener también un papel relevante, posiblemente dotando dichos nanosistemas de propiedades específicas que faciliten su interacción con las células inmunes. Así, nuestros estudios muestran la importancia de seleccionar

racionalmente los materiales a utilizar en la preparación de nanosistemas para liberación de antígenos, y de controlar cuidadosamente sus características fisicoquímicas con el objetivo de lograr una biodistribución y un efecto biológico lo más eficaces posible.

Desarrollo de nanopartículas a base de polisacáridos para transporte de antígenos

Los polisacáridos son polímeros de especial interés para el desarrollo de nanosistemas, en particular los destinados al transporte y liberación de antígenos, dada su amplia presencia en la estructura de distintos agentes patógenos. Así, estos polímeros pueden dotar a los nanosistemas de una similitud estructural con dichos patógenos que facilite su reconocimiento por células inmunes y aumentar su eficacia como adyuvantes en potenciales formulaciones de vacunas. En este trabajo en particular, nos hemos enfocado en el carboximetil-β-glucano (CMβG), un polímero aniónico de la familia de los beta glucanos, que han sido descritos en la literatura como inmunomoduladores. Especialmente, estos polisacáridos han sido estudiados en el transporte de antígenos en forma de micropartículas, obtenidas por disolución del contenido celular de *Saccharomyces cerevisiae*. Con el objetivo de desarrollar una formulación innovadora con propiedades inmunomoduladoras y capaz de asociar eficazmente un antígeno modelo, hemos combinado el carboximetil-β-glucano con quitosano (CS), ampliamente descrito en la literatura por sus propiedades mucoadesivas y también inmunomoduladoras. Asimismo, este material ha sido utilizado en una variedad de nano y microsistemas transportadores de antígenos, muchos de ellos desarrollados originalmente en nuestro grupo de investigación.

Para la preparación de nanopartículas utilizando estos dos polisacáridos se ha realizado un cribado de condiciones de preparación, variando la proporción en masa de ambos y manteniendo constantes las concentraciones iniciales y la proporción en volumen de ambas disoluciones (1:1). Así, hemos podido desarrollar nanopartículas con una proporción en masa CS:CMβG 2:1, tamaño nanometrico (180 nm) y carga superficial positiva (+30 mV). Además, hemos logrado la asociación de un antígeno modelo, ovalbumina (OVA), a las partículas desarrolladas, en una cantidad de aproximadamente 7% (m/m), con una eficacia de asociación de aproximadamente 50%. Los sistemas con OVA presentaron características fisicoquímicas similares a las de las formulaciones blancas y la liberación del antígeno en agua a 37 ºC fue despreciable. La observación de estos nanosistemas a través de técnicas de microscopía electrónica de transmisión y de barrido ha permitido confirmar la estructura esférica prevista y un tamaño de

partícula ligeramente inferior al determinado por dispersión de luz dinámica, lo cual está probablemente relacionado con las condiciones de preparación de las muestras para microscopía.

Uno de los principales retos en el desarrollo de nuevas formulaciones de vacunas es lograr su estabilidad en almacenamiento y en condiciones menos favorables de temperatura y humedad. Para este fin, tras verificar la estabilidad de las nanopartículas desarrolladas y la integridad de la OVA asociada en condiciones convencionales de almacenamiento (4 ºC) hasta 9 semanas, hemos desarrollado una forma de presentación de dichas partículas en polvo seco, a través de un proceso de liofilización. Tras la optimización de este proceso, seleccionando los crioprotectores más adecuados y la concentración necesaria de los mismos, hemos podido preparar una formulación susceptible de ser liofilizada, en presencia de 15% (m/v) de trehalosa. Esta formulación ha sido estable a 25 ºC y 40 ºC durante un mes, con humedad controlada a 60% y a 75%, respectivamente (**figura 3**). Además, el antígeno asociado no ha visto afectada su integridad en este proceso, según lo observado a través de técnicas de Western blot. Sin embargo, esta estabilidad debe ser comprobada a través de ensayos de eficacia *in vivo*, que permitan garantizar el mantenimiento de las propiedades iniciales de la formulación.

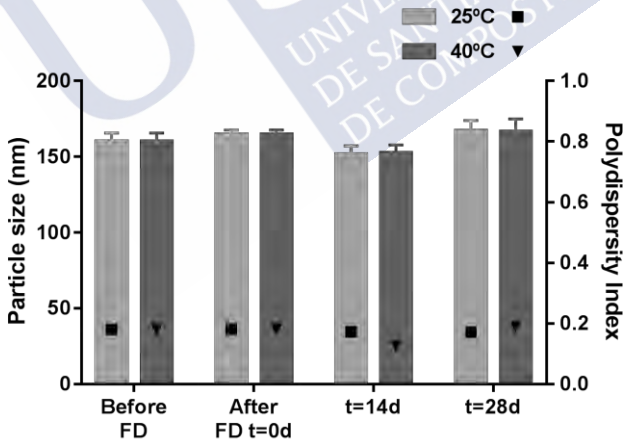


Figura 3. *Estabilidad de las nanopartículas de CS:CMβG con OVA, liofilizadas en presencia de 15% de trehalosa (m/v) (tamaño de partícula - barras; índice de polidispersión - símbolos) tras almacenamiento a 25 ºC / 60% de humedad y 40 ºC / 75% de humedad.*

Finalmente, considerando otro de los importantes retos en el desarrollo de las vacunas, que es la inmunización no parenteral, hemos desarrollado una formulación "solid-in-oil" que pudiera ser administrada por dichas vías no parenterales. Estas formulaciones, que consisten en una dispersión en aceite de nanopartículas liofilizadas en condiciones

específicas, han sido ya descritas como eficaces en el transporte de antígenos, especialmente por vía transdérmica. Así, hemos liofilizado las nanopartículas de quitosano y carboximetil-β-glucano con OVA, en presencia de sacarosa y ésteres de este sacárido. Tras el proceso de liofilización, hemos utilizado una mezcla de escualeno, un aceite con demostradas propiedades inmunomoduladoras y utilizado como adyuvante en vacunas para influenza, y 10% (m/m) de Span 80 (oleato de sorbitano). Esta mezcla ha permitido la redispersión completa de las nanopartículas, obteniéndose una formulación de aspecto límpido y cuyas propiedades fisicoquímicas se han mantenido en relación a las originalmente determinadas para las nanopartículas. Estos resultados abren la puerta al empleo de esta formulación como potencial adyuvante en vacunas no parenterales siendo necesarios nuevos estudios que evalúen su eficacia biológica.

De acuerdo con lo previamente mencionado en relación a la importancia del drenaje linfático en la distribución de los nanosistemas transportadores de antígenos, hemos estudiado dicha distribución para las nanopartículas desarrolladas, con y sin OVA asociada. Para esto, hemos marcado el quitosano con el fluoróforo 5-TAMRA, un derivado de la rodamina, y utilizado este polímero modificado para preparar nanopartículas que pudieran ser utilizadas en los estudios de microscopía y citometría de flujo anteriormente descritos. De manera idéntica, las nanopartículas han sido administradas por vía subcutánea en la almohadilla de la pata de los ratones y los nódulos linfáticos popliteales y lumbares han sido recogidos doce horas después de esta administración. Los resultados han mostrado niveles de acumulación similares para las formulaciones con y sin antígeno en el nódulo drenante (popliteal) y una localización preferencial de dichas nanopartículas en la zona medular de los nódulos. Además, en el caso de las partículas cargadas con antígeno, su co-localización con células dendríticas ha sido significativamente más elevada que con macrófagos, posiblemente debido a la alta capacidad de reconocimiento de antígenos de estas células.

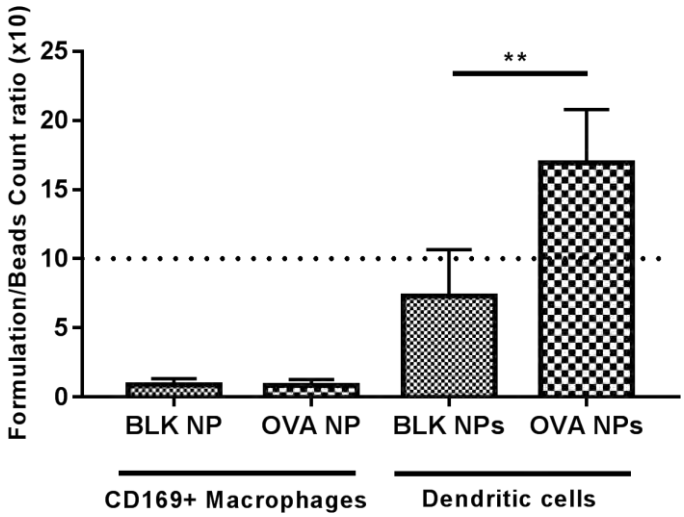


Figura 4. *Distribución de las nanopartículas de CS:CMβG (blancas, BLK, o cargadas con ovalbumina, OVA) en el nódulo linfático popliteal, tras inyección por vía subcutánea. La intensidad de fluorescencia se ha cuantificado por citometría de flujo, en dos poblaciones celulares, macrófagos y células dendríticas. Los resultados se han normalizado considerando la señal obtenida para las partículas control (la línea discontínua señala una igual acumulación de nanocápsulas y partículas control).* ** *p<0.01.*

Considerando este interesante perfil de distribución y co-localización con células presentadoras de antígeno de esta formulación, hemos llevado a cabo un estudio preliminar de respuesta inmune que permitiera evaluar el potencial adyuvante de las nanopartículas desarrolladas. Para ello, hemos utilizado un modelo animal OT II HZ, del cual hemos extraído células T CD4+, que en este animal presentan receptores específicos para el reconocimiento de OVA presentada por moléculas MHCII. Así, estas células han sido marcadas con un fluoróforo intracelular que permite controlar su proliferación y posteriormente han sido transferidas por vía intravenosa a animales C57/B6, previamente inmunizados con las nanopartículas cargadas con OVA o con OVA asociada a un adyuvante convencional (alum). Tras un período de tres días, los nódulos linfáticos de estos ratones han sido extraídos y se ha cuantificado la proliferación de las células T en estos órganos, necesariamente asociada a la presentación del antígeno por las células dendríticas tras la inmunización. Los resultados han mostrado que las nanopartículas que hemos desarrollado han generado niveles de proliferación celular similares a los obtenidos con el control positivo (alum), evidenciando una capacidad al menos similar a este adyuvante para presentar el antígeno a las células dendríticas del animal y para crear el ambiente inflamatorio necesario para la producción de una respuesta inmune adecuada. Sin embargo, son necesarios más estudios para

determinar el potencial de esta formulación para generar respuestas humorales adecuadas y/o para ser administrada por vías no parenterales.

Conclusiones

El trabajo experimental desarrollado en esta tesis, atendiendo a los objetivos planteados inicialmente, ha permitido estudiar la influencia de los parámetros fisicoquímicos de los nanosistemas en su biodistribución al sistema linfático y en su interacción con las células inmunes. En este ámbito, hemos podido concluir que el papel del tamaño de partícula es uno de los factores más importantes en el drenaje linfático de los nanosistemas, ya que las partículas con tamaño inferior a 100 nm han mostrado ser más eficientes en este proceso que las de tamaño superior a 200 nm. Sin embargo, es relevante mencionar la importancia de otros factores estudiados, como la composición de la cubierta polimérica y la carga superficial por ella atribuida, en este proceso. Así, es posible que una combinación de estos factores sea responsable por la mayor o menor eficacia de los nanosistemas a nivel de su llegada al sistema linfático, por lo que estos parámetros deben ser racionalmente evaluados y seleccionados a la hora de desarrollar un nuevo prototipo de nanovacuna.

Además, hemos podido desarrollar y caracterizar un nuevo sistema incorporando dos polisacáridos con demostrado potencial inmunomodulador, logrando la asociación y presentación eficaz de un antígeno modelo a células del sistema inmune. Tras su liofilización, estos sistemas han sido estables en condiciones de temperatura y humedad elevadas, manteniendo las propiedades coloidales y la integridad del antígeno. Adicionalmente, hemos podido desarrollar una forma innovadora de presentación de esta formulación, la nanodispersión “solid-in-oil”, que tiene particular interés para administración no parenteral. Finalmente, como prueba de concepto, hemos mostrado la capacidad de las nanopartículas cargadas con antígeno para transportarlo a células dendríticas y, así, generar una respuesta a nivel de la proliferación de células T específicas para dicho antígeno.

En suma, los resultados obtenidos en esta tesis han permitido destacar la importancia de la modulación de parámetros fisicoquímicos en el desarrollo de nanosistemas para vehiculización de antígenos, así como el potencial de las formulaciones basadas en polisacáridos para actuar como transportadores de antígenos y adyuvantes en nuevos abordajes de inmunización.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Introduction

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Introduction

1. Vaccine development: history and challenges

Edward Jenner first described the importance of vaccination over 200 years ago, in a pioneering work that led to an entire new field of scientific research. This initial discovery allowed the development of vaccines that eventually contributed to the prevention and eradication of numerous infectious diseases, including smallpox, polio, measles and tetanus [1]. Despite being one of the greatest milestones in healthcare, vaccination still holds a wide and interesting field for research and innovation. Several infectious diseases, due to specific characteristics of their etiological agents, cannot yet be fully controlled through vaccines, as is the case of HIV, malaria or tuberculosis [2]. In addition, currently available vaccines do not allow a global coverage of disease prevention, given, for example, their limited availability in developing countries, a major obstacle to the growth of these countries and to the eradication of life-threatening diseases that could be easily prevented [3]. Considering this, numerous public and private organizations, including the World Health Organization and the Bill and Melinda Gates Foundation, among others, have dedicated important investments and research programs to overcome this issue.

Historically, vaccines were firstly developed as killed or attenuated pathogens, with outstanding capacities to generate a strong and long-lasting immune response. However, this approach presented an important risk due to the potential of these pathogens to revert to the infectious form. For this reason, novel antigen forms such as inactivated microorganisms or toxoids have been developed, which are safer, though unable to yield a protective immune response without multiple dosing or an additional adjuvant component [4].

By definition, adjuvants are all compounds able to enhance the immune response in terms of type, strength and length, irrespective of their structure, composition or mode of action [5]. The first human vaccines to have an adjuvant included in the formulation were prepared with aluminum salts (also known as alum), in the beginning of the twentieth century. Alum is a very effective adjuvant for parenteral vaccination, given it forms a depot at the injection site that allows a slow release of the antigen, leading to a sustained immune response for a long time. Complementary mechanisms of action including direct interaction of this adjuvant with dendritic cell membrane lipids and activation of NALP3 inflammasome, have also been proposed in the last few years [6,7]. However, the main drawback of this adjuvant is related to its high sensitivity to temperature changes that

might compromise its activity, particularly freezing, then requiring the maintenance of a cold chain of storage [8,9]. This limitation led to the development of other adjuvants such as complete and incomplete Freund's adjuvant (CFA/IFA), which are water-in-oil emulsions. Nevertheless, the strong toxicity observed with these compounds mainly hampered their use in vaccination and raised the need for alternative strategies in adjuvant development. Moreover, progress in biotechnology field allowed the development of recombinant proteins and peptides with antigenic properties, as well as nucleic acids encoding for them. These novel antigens, though safer and promising, usually present low immunogenicity and also require stronger adjuvants to generate immunity [5,10]. In addition, conventional adjuvants usually elicit Th2-biased immune responses, meaning a predominantly antibody-mediated process that is usually ineffective against intracellular pathogens such as HIV and other diseases such as cancer. Accordingly, the role played by adjuvants in the modulation of the immune response towards the desired humoral or cellular branch, has been also a focus of attention in this field [11,12].

2. Nanotechnology for antigen delivery

Following the innovative work of Kreuter and Speiser using polyacrylic nanoparticles for influenza immunization [13], a significant number of articles have been published concerning the development of polymeric antigen delivery systems (**figure 1**) [14,15]. Biodegradable polymeric nanoparticles were among the first systems to be evaluated, given the advantages they might provide to vaccine formulations. These include their structural similarity to natural pathogenic agents, their flexibility for modification to gain specific targeting properties, as well as the possibility of including additional adjuvant molecules within a single nanostructure [16,17].

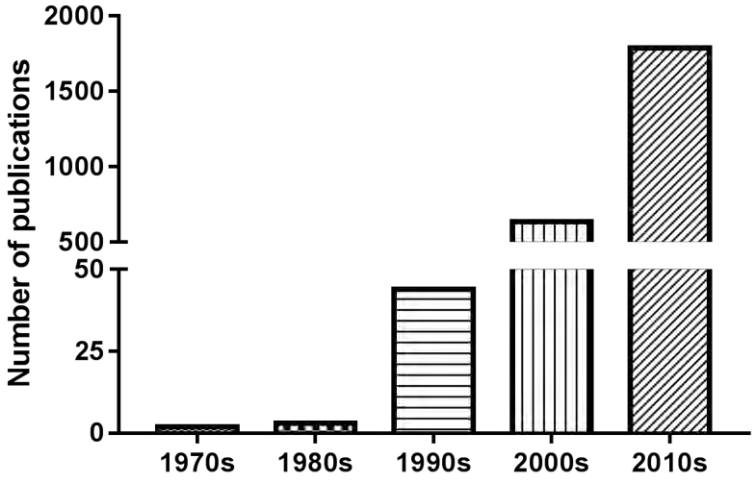


Figure 1. *Articles published since 1976 concerning polymer-based carriers for antigen delivery. Results were obtained from Scopus database using "(particle AND polymer) AND (vaccine OR antigen) AND (micro OR nano)" as keywords.*

One of the first demonstrated advantages of the use of polymeric nanocarriers for antigen delivery was their ability to control the release of the associated molecules. An outstanding work by Preis and Langer in 1979 established the interest of using antigen-loaded ethylene-vinyl acetate copolymer beads to achieve sustained immune responses that were comparable to those elicited by the antigen associated with the adjuvant CFA [18]. Following, PLGA microparticles were among the first prototypes studied for this purpose, with application for several antigens, including proteins, peptides and virus-like particles [19,20]. However, the harsh conditions used in the preparation of these systems, usually requiring organic solvents, and the acidic environment created by their degradation led to important loss of antigen activity [21]. To overcome these issues and limit the interaction between antigens and PLGA, interesting alternatives including PLGA-coated oil-based carriers [22], or the use of additional materials such as polyethylene oxides (PEG) and cationic polymers [23,24], were followed.

Researchers also began to consider the relevance of particle size with respect to the ability of polymeric nanocarriers to efficiently transport and deliver the associated antigens. For this reason, the first report concerning the use of polymeric nanoparticles for antigen delivery was published by Almeida *et al* at the beginning of the 90s [25]. In this article, the authors presented the potential of these polylactic acid (PLA)-based nanoparticles to be administered by intranasal route and elicit potent immune responses through this pathway. Physically, the reduction in particle size increases the surface area

of the particles, potentially allowing higher antigen loading. Moreover, authors have also shown the advantages of smaller particles in terms of lymphatic drainage and interaction with immune cells [26,27], reinforcing the adjuvant potential of these carriers.

Nanoparticles additionally offer an important advantage in vaccination. They can be tailored for administration through alternative routes to parenteral, towards the development of needle-free vaccines. Needle-free immunization presents several advantages, mainly in terms of patient compliance, cost-effectiveness and vaccine coverage in developing countries [28]. In fact, specific vaccines against poliomyelitis, rotavirus and cholera, among others, are already available for oral administration, as well as influenza vaccines administered intranasally [29]. Moreover, the potential of generating local immune responses that provide protection at the entrance site of most pathogens is an additional source of interest for research in this field. In this regard, nanoparticles may be useful in protecting the antigen against degradation, facilitating the crossing of the mucosal barriers and improving the antigen interaction with APCs, usually abundant in mucosal tissues [30]. Considering this, researchers presented PEGylation as a possible strategy to achieve proper mucosal stability of the nanocarriers. As an example, our group developed PLA-PEG nanoparticles for nasal administration, which were able to efficiently deliver tetanus toxoid to rats upon intranasal administration, eliciting strong and long-lasting antibody responses against this antigen [31,32].

The use of natural polymers in the preparation of antigen nanocarriers also been presented as an alternative to the previously used synthetic polyesters, considering the first are biodegradable, have low toxicity and high versatility, which are characteristics that can be exploited for the formulation of a wide variety of nanostructures [17]. In particular, polysaccharides are widely spread in the composition of several pathogenic agents, therefore providing recognizable patterns that allow immune cells to initiate a response against them. These structure resemblances, also known as pathogen-associated molecular patterns (PAMPs), act on pattern recognition receptors (PRRs) present in antigen presenting cells (APCs), such as Toll-like or Nod-like receptors (TLRs or NLRs, respectively), triggering cytokine cascades that ultimately lead to innate and adaptive immune responses [33–35].

Chitosan is undoubtedly the most studied polysaccharide in antigen delivery systems. This biodegradable polymer has been used in different compositions and types of structures, as a coating of oily-core based systems or as part of matrix-like nanoparticles [36–38]. Chitosan-based nanovaccines are particularly interesting given the mucoadhesive properties of this polymer, which may facilitate the development of

vaccines for mucosal administration. The first studies with chitosan-based nanoparticles for nasal antigen delivery were reported by Vila *et al* [39,40], showing an improved antigen transport across the nasal mucosa. In this work, the antibody titers in mucosal tissues of animals immunized with antigen-loaded chitosan nanoparticles were higher than the ones elicited by the soluble antigen. This approach was also applied to more complex antigens such as HBsAg, with mice receiving three doses of antigen-loaded chitosan nanoparticles through intranasal route and achieving seroprotective antibody levels [36]. Other chitosan derivatives, especially those based on the methylation of chitosan amine groups, were also explored for this purpose. N-trimethyl chitosan (TMC) is one of the most studied chitosan derivatives in intranasal antigen delivery, with several publications reporting the adjuvant potential of nanoparticles based on this polymer [41–44].

Other polysaccharides have also been explored as components of nanoparticles for vaccination including mannans, dextran, inulin and beta glucans. Given the presence of several mannan-specific receptors in APCs, mannans have been used as components of nanocarriers for antigen delivery, mainly as targeting moieties to direct them to immune cells [17,45]. With respect to dextran, its water solubility, high availability and versatility for functionalization facilitate its inclusion in a variety of particulate systems. Its most common derivative, dextran sulfate, has been studied as an immunostimulant, though its exact mechanism of action is still under research [17,46]. Inulin-based microparticles have been developed and studied as an adjuvant (Advax™) for a wide range of antigens [47–49] and are currently undergoing clinical trials for the prevention of different infectious diseases, for example influenza, Japanese encephalitis and HIV-1 [50]. In the case of beta glucans, known to stimulate the immune system through different receptors [51,52], their application in nanocarriers for antigen delivery has been poorly addressed [53,54]. The most recent advances of polysaccharide-based nanocarriers for antigen delivery are described in more detail in Chapter 1 of this thesis ("Nanoengineering of vaccines using natural polysaccharides").

Alternatively, polyaminoacids have also been evaluated as components of antigen delivery systems [55]. For example, protamine is a naturally occurring arginine-rich polymer, with cell-penetrating and gene delivery properties. Considering this, protamine-based nanoparticles [56] and nanocapsules [57] were successfully developed by our group and used for the delivery of hepatitis B and influenza antigens, respectively. *In vivo* results with these prototypes showed their ability to elicit efficient immune responses even at low antigen doses, comparable to those elicited by the alum-adsorbed antigens. Recently, we have also described the enveloping of hepatitis B surface antigen, which is

structurally a nanoparticle, with protamine, polyarginine and anionic polysaccharides, rendering different particulate prototypes that presented interesting preliminary results in terms of immune response triggering [38].

Despite the strong potential of polymeric systems in this field, lipid-based antigen carriers have been the most successful in terms of access to the market (**table 1**). In fact, oil-in-water nanoemulsions such as MF59™, AS03™ and AF03™, were among the first adjuvants of this kind to be successfully included in marketed vaccines against influenza [58–60]. Other lipid-based delivery systems, such as liposomes and virosomes have also been tested in vaccine research, with some prototypes reaching clinical trials and some marketed formulations [59,61]. Recently, a vaccine against malaria containing a liposome-based adjuvant (AS01™) and two additional immunostimulants (MPL – monophosphoryl lipid A – and QS-21, a natural saponin molecule) was approved in the European markets, though with limited efficacy (around 50%) [62,63]. In the case of virosomes, which are liposomes containing functional glycoproteins from the viral envelope, they were successfully included in commercialized vaccines against influenza and hepatitis A [64,65]. Other lipid-based formulations such as nanocapsules, a core-shell system containing an oily core stabilized by surfactants and coated with polymers, have also been studied in this field. In particular, chitosan-coated nanocapsules were successfully developed in our group for antigen delivery [66,67], with the advantage of allowing the incorporation of lipophilic adjuvant molecules such as imiquimod in the oily core of the nanosystems [37]. These antigen- and imiquimod-loaded nanocapsules were able to elicit seroprotective antibody levels against hepatitis B for a long period, upon intranasal administration to mice. Moreover, these levels were significantly higher than the ones achieved with the same nanocarriers lacking imiquimod.

Table 1. *Summary of marketed particle-based adjuvants [10,60,63]. O/W, oil-in-water; MPL, monophosphoryl lipid A. Note: Pandemrix™ was removed from the market due to association with narcolepsy.*

Adjuvant commercial name	Type of structure / composition	Vaccines	Diseases
MF59™	Squalene O/W nanoemulsion	Fluad™, Focetria™	Influenza
AF03™		Pandemrix™, Prepandrix™	Influenza
AS03™	Squalene + Vitamin E O/W nanoemulsion	Humenza™	Pandemic influenza
AS01™	Liposomes, MPL, QS-21	Mosquirix™	Malaria
n.a.	Virosomes	Epaxal™, Inflexal™ V	Hepatitis A, Influenza

Finally, other types of antigen delivery systems have been proposed in the literature and patented throughout the last decades. In particular, porous silica, iron oxide and gold particles have been described as materials for this application, as well as natural scaffolds such as pollen grains and bacterial-derived particles, loaded with different antigens [68]. Figure 2 proposes a classification of the currently studied antigen delivery systems as described in literature and patents.

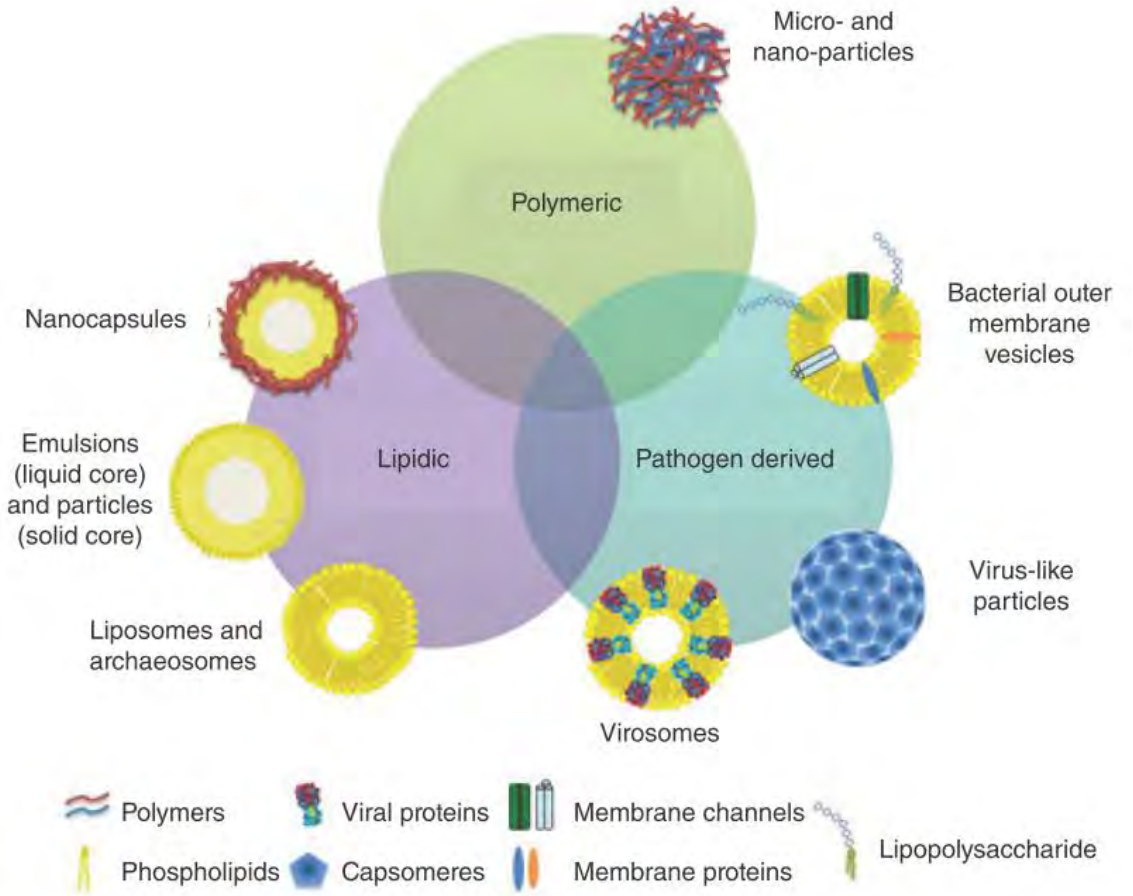


Figure 2. *Main antigen delivery systems, classified according to their main components (polymers or lipids) or to the structures from which they were developed, in the case of the pathogen-derived vehicles. Reproduced with permission from [68].*

Overview and future perspectives

The last century has witnessed a fascinating progress in antigen and adjuvant research, aiming at safer and more effective vaccine formulations. This has led to an increasing development of particulate delivery systems that may act as carriers and adjuvants for modern day antigens, which usually present low intrinsic immunogenicity. Besides, particle systems should protect the antigen from degradation, control its release and eventually be engineered to target specific immune cell populations of interest. Overall, nano and microparticles have shown potential to act as adjuvants in different immunization approaches, with promising results in a variety of model animals. Building on this knowledge, research is now centered in peptide- and gene-based antigens, and moving towards the combination of antigen delivery systems and immunomodulating small molecules such as imiquimod or CpG, with some approaches reaching clinical

trials [10]. Open questions such as the need for the simultaneous activation of multiple immune pathways, or the most effective formulation strategies for the control of vaccine biodistribution and kinetics, remain as challenges in this field.

References

[1] Koff WC, Burton DR, Johnson PR, Walker BD, King CR, Nabel GJ, et al. Accelerating next-generation vaccine development for global disease prevention. Science 2013;340:1232910. doi:10.1126/science.1232910.

[2] Delany I, Rappuoli R, De Gregorio E. Vaccines for the 21st century. EMBO Mol Med 2014;6:708–20. doi:10.1002/emmm.201403876.

[3] Rappuoli R, Mandl CW, Black S, De Gregorio E. Vaccines for the twenty-first century society. Nat Rev Immunol 2011;11:865–72. doi:10.1038/nri3085.

[4] Plotkin SA, Plotkin SL. The development of vaccines: how the past led to the future. Nat Rev Microbiol 2011;9:889–93. doi:10.1038/nrmicro2668.

[5] Reed SG, Orr MT, Fox CB. Key roles of adjuvants in modern vaccines. Nat Med 2013;19:1597–608. doi:10.1038/nm.3409.

[6] Eisenbarth SC, Colegio OR, O'Connor Jr W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 2008;453:1122–6. doi:10.1038/nature06939.

[7] Flach TL, Ng G, Hari A, Desrosiers MD, Zhang P, Ward SM, et al. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nat Med 2011;17:479–87. doi:10.1038/nm.2306.

[8] Lindblad EB. Aluminium adjuvants—in retrospect and prospect. Vaccine 2004;22:3658–68. doi:10.1016/j.vaccine.2004.03.032.

[9] Matthias DM, Robertson J, Garrison MM, Newland S, Nelson CM. Freezing temperatures in the vaccine cold chain: A systematic literature review. Vaccine 2007;25:3980–6. doi:10.1016/j.vaccine.2007.02.052.

[10] Brito LA, O'Hagan DT. Designing and building the next generation of improved vaccine adjuvants. J Control Release 2014;190:563–79. doi:10.1016/j.jconrel.2014.06.027.

[11] Gutjahr A, Tiraby G, Perouzel E, Verrier B, Paul S. Triggering Intracellular Receptors for Vaccine Adjuvantation. Trends Immunol 2016;37:573–87. doi:10.1016/j.it.2016.07.001.

[12] Irvine DJ, Hanson MC, Rakhra K, Tokatlian T. Synthetic Nanoparticles for Vaccines and Immunotherapy. Chem Rev 2015;115:11109–46. doi:10.1021/acs.chemrev.5b00109.

[13] Kreuter J, Speiser PP. New adjuvants on a polymethylmethacrylate base. Infect Immun 1976;13:204–10.

[14] De Koker S, Lambrecht BN, Willart MA, van Kooyk Y, Grooten J, Vervaet C, et al. Designing polymeric particles for antigen delivery. Chem Soc Rev 2011;40:320–39. doi:10.1039/b914943k.

[15] Sahdev P, Ochyl LJ, Moon JJ. Biomaterials for nanoparticle vaccine delivery systems. Pharm Res 2014;31:2563–82. doi:10.1007/s11095-014-1419-y.

[16] De Temmerman M-L, Rejman J, Demeester J, Irvine DJ, Gander B, De Smedt SC. Particulate vaccines: on the quest for optimal delivery and immune response. Drug Discov Today 2011;16:569–82. doi:10.1016/j.drudis.2011.04.006.

[17] Cordeiro AS, Alonso MJ, de la Fuente M. Nanoengineering of vaccines using natural polysaccharides. Biotechnol Adv 2015;33:1279–93. doi:10.1016/j.biotechadv.2015.05.010.

[18] Preis I, Langer RS. A single-step immunization by sustained antigen release. J Immunol Methods 1979;28:193–7. doi:10.1016/0022-1759(79)90341-7.

[19] Alonso MJ, Gupta RK, Min C, Siber GR, Langer RS. Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. Vaccine 1994;12:299–306. doi:10.1016/0264-410x(94)90092-2.

[20] Silva AL, Soema PC, Slütter B, Ossendorp F, Jiskoot W. PLGA particulate delivery systems for subunit vaccines: Linking particle properties to immunogenicity. Hum Vaccines Immunother 2016;12:1056–69. doi:10.1080/21645515.2015.1117714.

[21] Jiang W, Gupta RK, Deshpande MC, Schwendeman SP. Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Adv Drug Deliv Rev 2005;57:391–410. doi:10.1016/j.addr.2004.09.003.

[22] Sánchez A, Gupta RK, Alonso MJ, Siber GR, Langer RS. Pulsed controlled-release system for potential use in vaccine delivery. J Pharm Sci 1996;85:547–52. doi:10.1021/js960069y.

[23] Tobío M, Nolley J, Guo Y, McIver J, Alonso MJ. A Novel System Based on a Poloxamer/ PLGA Blend as a Tetanus Toxoid Delivery Vehicle. Pharm Res 1999;16:682–8. doi:10.1023/a:1018820507379.

[24] Martínez Gómez JM, Csaba N, Fischer S, Sichelstiel A, Kündig TM, Gander B, et al. Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis. J Control Release 2008;130:161–7. doi:10.1016/j.jconrel.2008.06.003.

[25] Almeida AJ, Alpar HO, Brown M. 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;45:198–203. doi:10.1111/j.2042-7158.1993.tb05532.x.

[26] Abellán-Pose R, Teijeiro-Valiño C, Santander-Ortega MJ, Borrajo E, Vidal A, García-Fuentes M, et al. Polyaminoacid Nanocapsules for Drug Delivery to the Lymphatic System: Effect of the Particle Size. Int J Pharm 2016;509:107–17. doi:10.1016/j.ijpharm.2016.05.034.

[27] Reddy ST, Rehor A, Schmoekel HG, Hubbell JA, Swartz MA. In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. J Control Release 2006;112:26–34. doi:10.1016/j.jconrel.2006.01.006.

[28] Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev 2006;58:68–89. doi:10.1016/j.addr.2005.12.003.

[29] Lycke N. Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol 2012;12:592–605. doi:10.1038/nri3251.

[30] Csaba N, García-Fuentes M, Alonso MJ. Nanoparticles for nasal vaccination. Adv Drug Deliv Rev 2009;61:140–57. doi:10.1016/j.addr.2008.09.005.

[31] Vila A, Sánchez A, Perez C, Alonso MJ. PLA-PEG nanospheres: new carriers for transmucosal delivery of proteins and plasmid DNA. Polym Adv Technol 2002;13:851–8. doi:10.1002/pat.280.

[32] Vila A, Sánchez A, Évora C, Soriano I, Vila-Jato JL, Alonso MJ. PEG-PLA Nanoparticles as Carriers for Nasal Vaccine Delivery. J Aerosol Med 2004;17:174–85. doi:10.1089/0894268041457183.

[33] Demento SL, Siefert AL, Bandyopadhyay A, Sharp FA, Fahmy TM. Pathogen-associated molecular patterns on biomaterials: a paradigm for engineering new vaccines. Trends Biotechnol 2011;29:294–306.

[34] Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. Int Rev Immunol 2011;30:16–34. doi:10.3109/08830185.2010.529976.

[35] Mahla RS, Reddy MC, Prasad DVR, Kumar H. Sweeten PAMPs: Role of sugar complexed PAMPs in innate immunity and vaccine biology. Front Immunol 2013;4:1–16. doi:10.3389/fimmu.2013.00248.

[36] Prego C, Paolicelli P, Díaz B, Vicente S, Sánchez A, González-Fernández Á, et al. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. Vaccine 2010;28:2607–14. doi:10.1016/j.vaccine.2010.01.011.

[37] Vicente S, Peleteiro M, Díaz-Freitas B, Sánchez A, González-Fernández Á, Alonso MJ, et al. Co-delivery of viral proteins and a tlr7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy. J Control Release 2013;172:773–81. doi:http://dx.doi.org/10.1016/j.jconrel.2013.09.012.

[38] Correia-Pinto JF, Peleteiro M, Csaba N, González-Fernández Á, Alonso MJ. Multi-enveloping of particulated antigens with biopolymers and immunostimulant polynucleotides. J Drug Deliv Sci Technol 2015;30:424–34. doi:10.1016/j.jddst.2015.08.010.

[39] Vila A, Sánchez A, Tobío M, Calvo P, Alonso MJ. Design of biodegradable particles for protein delivery. J Control Release 2002;78:15–24.

[40] Vila A, Sánchez A, Janes KA, Behrens I, Kissel T, Vila-Jato JL, et al. Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice. Eur J Pharm Biopharm 2004;57:123–31. doi:10.1016/j.ejpb.2003.09.006.

[41] Sayın B, Somavarapu S, Li XW, Thanou M, Sesardic D, Alpar HO, et al. Mono-N-carboxymethyl chitosan (MCC) and N-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery. Int J Pharm 2008;363:139–48. doi:10.1016/j.ijpharm.2008.06.029.

[42] Hagenaars N, Verheul RJ, Mooren I, de Jong PH, Mastrobattista E, Glansbeek HL, et al. Relationship between structure and adjuvanticity of N,N,N-trimethyl chitosan (TMC) structural variants in a nasal influenza vaccine. J Control Release 2009;140:126–33. doi:10.1016/j.jconrel.2009.08.018.

[43] Mangal S, Pawar D, Garg NK, Jain AK, Vyas SP, Rao D, et al. Pharmaceutical and immunological evaluation of mucoadhesive nanoparticles based delivery system(s) administered intranasally. Vaccine 2011;29:4953–62. doi:10.1016/j.vaccine.2011.04.112.

[44] Slütter B, Bal SM, Keijzer C, Mallants R, Hagenaars N, Que I, 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;28:6282–91. doi:10.1016/j.vaccine.2010.06.121.

[45] Apostolopoulos V, Thalhammer T, Tzakos AG, Stojanovska L. Targeting antigens to dendritic cell receptors for vaccine development. J Drug Deliv 2013;2013:869718. doi:10.1155/2013/869718.

[46] Petrovsky N, Cooper PD. Carbohydrate-based immune adjuvants. Expert Rev Vaccines 2011;10:523–37. doi:10.1586/erv.11.30.

[47] Layton RC, Petrovsky N, Gigliotti AP, Pollock Z, Knight J, Donart N, et al. Delta inulin polysaccharide adjuvant enhances the ability of split-virion H5N1 vaccine to protect against lethal challenge in ferrets. Vaccine 2011;29:6242–51. doi:10.1016/j.vaccine.2011.06.078.

[48] Lobigs M, Pavy M, Hall RA, Lobigs P, Cooper P, Komiya T, et al. An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. J Gen Virol 2010;91:1407–17. doi:10.1099/vir.0.019190-0.

[49] Cristillo AD, Ferrari MG, Hudacik L, Lewis B, Galmin L, Bowen B, et al. Induction of mucosal and systemic antibody and T-cell responses following prime-boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. J Gen Virol 2011;92:128–40. doi:10.1099/vir.0.023242-0.

[50] www.clinicaltrials.gov. Last visited in May 2017. n.d.

[51] Huang H, Ostroff GR, Lee CK, Wang JP, Specht CA, Levitz SM. Distinct patterns of dendritic cell cytokine release stimulated by fungal beta-glucans and toll-like receptor agonists. Infect Immun 2009;77:1774–81. doi:10.1128/IAI.00086-09.

[52] Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, et al. Dectin-1 is required for β-glucan recognition and control of fungal infection. Nat Immunol 2007;8:31–8. doi:10.1038/ni1408.

[53] Huang H, Ostroff GR, Lee CK, Specht CA, Levitz SM. Robust Stimulation of Humoral and Cellular Immune Responses following Vaccination with Antigen-Loaded B-Glucan Particles. MBio 2010;1:e00164-10-e00164-16.

doi:10.1128/mBio.00164-10.

[54] De Smet R, Demoor T, Verschuere S, Dullaers M, Ostroff GR, Leclercq G, et al. β-Glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination. J Control Release 2013;172:671–8. doi:10.1016/j.jconrel.2013.09.007.

[55] González-Aramundiz JV, Lozano MV, Souza-Herves A, Fernández-Megía E, Csaba N. Polyaminoacids and Polypeptides in Drug Delivery. Expert Opin Drug Deliv 2012;9:183–201. doi:10.1517/17425247.2012.647906.

[56] González-Aramundiz JV, Olmedo MP, González-Fernández Á, Alonso Fernández MJ, Csaba NS. Protamine-based nanoparticles as new antigen delivery systems. Eur J Pharm Biopharm 2015;97:51–9. doi:10.1016/j.ejpb.2015.09.019.

[57] González-Aramundiz JV, Presas E, Dalmau-Mena I, Martínez-Pulgarín S, Alonso C, Escribano JM, et al. Rational design of protamine nanocapsules as antigen delivery carriers. J Control Release 2017;245:62–9. doi:10.1016/j.jconrel.2016.11.012.

[58] O'Hagan DT, De Gregorio E. The path to a successful vaccine adjuvant – "The long and winding road." Drug Discov Today 2009;14:541–51. doi:10.1016/j.drudis.2009.02.009.

[59] Correia-Pinto JF, Csaba N, Alonso MJ. Vaccine delivery carriers: Insights and future perspectives. Int J Pharm 2013;440:27–38. doi:10.1016/j.ijpharm.2012.04.047.

[60] Klucker M-F, Dalençon F, Probeck P, Haensler J. AF03, An Alternative Squalene Emulsion-Based Vaccine Adjuvant Prepared by a Phase Inversion Temperature Method. J Pharm Sci 2012;101:4490–500. doi:10.1002/jps.23311.

[61] Felnerova D, Viret J-F, Glück R, Moser C. Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. Curr Opin Biotechnol 2004;15:518–29. doi:10.1016/j.copbio.2004.10.005.

[62] The RTS.S Clinical Trials Partnership. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet 2015;386:31–45. doi:10.1016/S0140-6736(15)60721-8.

[63] Didierlaurent AM, Laupèze B, Di Pasquale A, Hergli N, Collignon C, Garçon N.

Adjuvant system AS01: helping to overcome the challenges of modern vaccines. Expert Rev Vaccines 2017;16:55–63. doi:10.1080/14760584.2016.1213632.

[64] Moser C, Amacker M, Zurbriggen R. Influenza virosomes as a vaccine adjuvant and carrier system. Expert Rev Vaccines 2011;10:437–46. doi:10.1586/erv.11.15.

[65] Bovier PA, Bock J, Ebengo TF, Frösner G, Glaus J, Herzog C, et al. Predicted 30-year protection after vaccination with an aluminum-free virosomal hepatitis A vaccine. J Med Virol 2010;82:1629–34. doi:10.1002/jmv.21883.

[66] Vicente S, Díaz-Freitas B, Peleteiro M, Sanchez A, Pascual DW, González-Fernández Á, et al. A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach. PLoS One 2013;8:e62500. doi:10.1371/journal.pone.0062500.

[67] Vicente S, Peleteiro M, González-Aramundiz JV, Díaz-Freitas B, Martínez-Pulgarín S, Neissa JI, et al. Highly versatile immunostimulating nanocapsules for specific immune potentiation. Nanomedicine 2014;9:2273–89. doi:10.2217/nnm.14.10.

[68] Cordeiro AS, Alonso MJ. Recent advances in vaccine delivery. Pharm Pat Anal 2016;5:49–73. doi:10.4155/ppa.15.38.

Chapter 1

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter 1

Nanoengineering of vaccines using natural polysaccharides

Adapted from: Cordeiro AS, Alonso MJ, de la Fuente M (**2015**) *"Nanoengineering of vaccines using natural polysaccharides"*, Biotechnology Advances **33** *(2015)*, 1279-1293

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

ABSTRACT

Currently, there are over 70 licensed vaccines, which prevent the pathogenesis of around 30 viruses and bacteria. Nevertheless, there are still important challenges in this area, which include the development of more active, non-invasive, and thermo-resistant vaccines. Important biotechnological advances have led to safer subunit antigens, such as proteins, peptides, and nucleic acids. However, their limited immunogenicity has demanded potent adjuvants that can strengthen the immune response. Particulate nanocarriers hold a high potential as adjuvants in vaccination. Due to their pathogen-like size and structure, they can enhance immune responses by mimicking the natural infection process. Additionally, they can be tailored for non-invasive mucosal administration (needle-free vaccination), and control the delivery of the associated antigens to a specific location and for prolonged times, opening room for single-dose vaccination. Moreover, they allow co-association of immunostimulatory molecules to improve the overall adjuvant capacity.

The natural and ubiquitous character of polysaccharides, together with their intrinsic immunomodulating properties, their biocompatibility, and biodegradability, justify their interest in the engineering of nanovaccines. In this review, we aim to provide an overview of the state-of-the-art regarding the application of nanotechnology in vaccine delivery, with a focus on the most recent advances in the development and application of polysaccharide-based antigen nanocarriers.

1. Challenges and advances in vaccine development

Throughout the last decades, vaccination has played a fundamental role in the prevention of severe infectious diseases, and even in the eradication of some of them. Despite the advances achieved to date, significant challenges still need to be faced in order to gradually increase vaccine coverage. These include not only the development of new vaccines against certain pathogens such as human immunodeficiency virus (HIV), malaria and tuberculosis, among others, but also the development of single-dose and needle-free vaccines intended to improve patient compliance and reduce associated costs. Lastly, the production of formulations that can avoid the cold chain of transport represents a keystone to improve vaccination worldwide. Progress in both antigen and adjuvant development has led to the recognition of the value of nanotechnology to deal with the above indicated challenges. For the preparation of nanovaccines, different immunomodulating biomaterials have been proposed, including polysaccharides. This innovative approach is the main focus of this review and is summarized in figure 1.

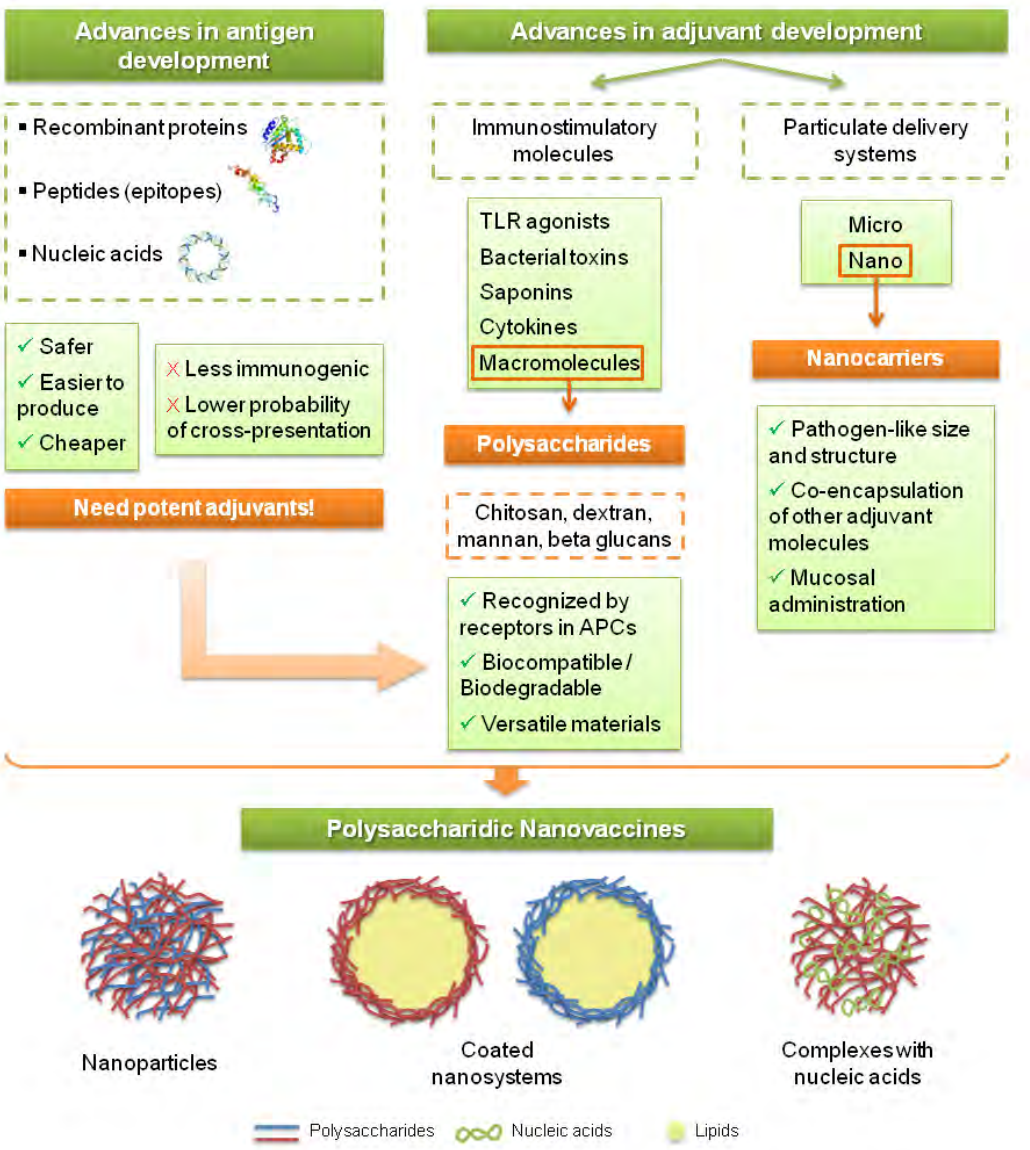


Figure 1. *Advances in biological and microbiological technologies have increased the knowledge of pathogens and led to the development of newer and safer subunit antigens. Nevertheless, these antigens are less effective in inducing protective immune responses and therefore require a parallel development of potent adjuvants such as immunomodulating molecules and particulate delivery systems. Among these, polysaccharide-based nanosystems have demonstrated potential to be successfully used in vaccine formulations.*

1.1. Biotechnology and antigen development

The first commercialised vaccines against rabies, poliomyelitis, tetanus and childhood tuberculosis, among others, were based on the attenuation of pathogens and toxins.

However, the potential toxicity and the difficulty in carrying out this process with complex pathogens, such as HIV and hepatitis C virus (HCV), has led to the search for optimized antigens [1]. Advances in biotechnology and an increased knowledge of the pathogens characteristics have led to the development of newer and safer subunit antigens, in particular proteins, peptides and nucleic acids [2].

1.1.1. Recombinant proteins

Recombinant DNA technology has allowed the production of several proteins with antigenic activity, using expression vectors such as bacteria or yeast. A well-known example of this application is the production of the hepatitis B surface antigen (rHBsAg) in *Escherichia coli*, which has led to the first recombinant protein-based vaccines reaching the market using alum as adjuvant (Engerix-B® from GlaxoSmithKline Biologicals and Recombivax HB® from Merck & Co., Inc.). Similarly, antigens of the human papillomavirus (HPV), expressed in *Saccharomyces cerevisae* and *Trichoplusia ni*, are commercialised as Gardasil® (Merck & Co., Inc.) and Cervarix® (GlaxoSmithKline Biologicals), with alum and AS04® (a combination of alum and monophosphoryl lipid A (MPLA)) as adjuvants, respectively. Other pathogens for which recombinant protein antigens have been identified and studied include hepatitis C and E and rotavirus [3].

The concept of “reverse vaccinology”, focussed on the scan of the whole genome of the pathogen for identification of antigenic protein candidates, represented an important advance towards the development of new vaccines. This strategy has led to the development, for example, of a new meningococcal vaccine, commercialised in Europe under the brand name of Bexsero® (by Novartis Vaccines). Vaccines against pathogens such as *Streptococcus pneumoniae* and *Leishmania infantum*, among others, have also been investigated using this approach [4].

1.1.2. Peptides

The above indicated antigenic proteins still have important limitations such as complex production processes, difficult purification steps, and instability in a liquid form. More importantly, protein-based vaccines may induce autoimmunity and allergic reactions, as reported, for example, in the development of a vaccine against group A *Streptococcus* (GAS) [5,6]. For these reasons, efforts are currently focussed on the production of small peptides as antigens. These antigenic peptides can be identified through the analysis of specific antibody-inducing regions within larger proteins (epitopes), and are generally obtained through simple synthesis with high purity, in large scale, and at a lower cost [6–9]. Several peptide-based vaccine formulations have already reached the clinical

development phase, as it is the case for some preventive HIV vaccines [10] as well as some therapeutic anticancer vaccines (Mohit et al. 2014; Ott et al. 2014).

Overall, it can be stated that proteins and peptides have improved the vaccines safety profile in comparison with live attenuated pathogens, however, their poor immunogenicity is definitely hindering their development and success [7,8]. For this reason, it is essential to advance in the design of novel adjuvants that may help providing these vaccines with a robust immune response [13].

1.1.3. Genetic vaccination

In the last decades, nucleic acids-based vaccines have gained increasing attention [14,15]. Essentially, nucleic acids such as plasmid DNA (pDNA) and messenger RNA (mRNA) allow *in situ* production of the antigen by the cellular machinery of the host. This strategy mimics the natural infection by intracellular pathogens as it leads to the local formation of the antigenic molecule. Moreover, nucleic acids can be tailored to express antigens that are chemically or structurally different from their native form, with the intention of improving their immunogenicity [14,16,17]. The main limitation of this approach is the low level of gene expression achieved upon administration; a limitation that can be overcome by designing effective viral and non-viral transfection vectors [16,18].

Plasmid DNA vaccination, based on the administration of selected antigen-encoding DNA through a plasmid vector, has been applied to prevent diseases such as malaria and HIV and also as a therapeutic approach in cancer immunotherapy. Some of these formulations have already reached the clinical development phase, as recently reviewed by Mazid et al. [18,19]. Nevertheless, the risk of genome integration and long-term effects of these vaccines are yet to be clarified [20]. As an alternative, messenger RNA has raised particular attention, with preclinical proofs-of-concept described for prophylactic influenza vaccination and some formulations in clinical development for anticancer immunotherapy [14,17].

1.2. Vaccine adjuvants and antigen nanoengineering

Alum has been the traditional adjuvant of choice for vaccines, though its mechanism of action is not yet completely understood. It also has specific limitations such as the necessity to be stored at low temperature, a limited efficacy for peptide antigens, and inability to generate Th1 (cellular) immune responses [21–23]. The need to overcome these limitations has stimulated the search for new adjuvants. As a consequence, there

is currently a large variety of adjuvants, which for the purpose of this review we have classified in two groups, molecular adjuvants or immunostimulatory molecules and antigen delivery systems produced by the nanoengineering of antigens.

1.2.1. Molecular adjuvants

Small molecules, targeted at specific receptors present on immune cells (pattern recognition receptors (PRRs)), such as Toll-like receptors (TLRs), have the ability to trigger stronger immune responses [24]. The most studied molecules are agonists for TLRs, as for example CpG oligonucleotides (TLR 9), poly(I:C) (TLR 3) or imiquimod (TLR 7/8), which have already been evaluated for their adjuvant properties in vaccines against malaria, hepatitis B, influenza, as well as in different therapeutic anticancer vaccines [25].

Other molecules such as bacterial toxins (cholera toxin, *E. coli* heat-labile toxin and others), saponins (Quil-A or QS-21) and cytokines, are also used as immunostimulants in vaccine formulations. In particular, bacterial toxins are known to enhance the immune response by targeting the antigen to the M cells in the intestinal tract, thereby boosting a strong humoral response at a mucosal level. An example of these toxins is cholera toxin (recombinant B subunit), which is used as an adjuvant for a commercialized oral cholera vaccine [26]. Another example refers to a transdermal patch containing heat-labile *E. coli* enterotoxin for the enhancement of the immune response against pathogens such as *E. coli* and influenza [27–29]. Nevertheless, a major limitation of these toxins is related to the immune response that can be generated against themselves rather than against the associated antigen [30–32].

In the case of saponins, which are plant-derived triterpene glycosides, a detoxified derivative (QS-21) of Quil-A (from *Quillaja saponaria)* has been successfully included in particulate formulations such as AS01™ (liposomes based on monophosphoryl lipid A (MPLA)) and AS02™ (squalene oil-in-water emulsion also containing MPLA), currently under clinical development for malaria vaccines [33,34]. Also, the inclusion of saponins in immunostimulating complexes (ISCOMs) has been evaluated in vaccination against influenza, toxoplasmosis or Epstein-Barr virus-induced tumours, among others, achieving protective immunity in clinical studies [35,36].

With respect to cytokines, IL-2, IL-12 and IFN-γ are some of the molecules that have been studied for immune response modulation, both at preclinical and clinical levels, though some toxic effects have been observed with high doses in human studies [37–39].

1.2.2. Nanoengineering of antigens: antigen delivery systems

Macromolecules such as polymers, among them polysaccharides (as reviewed in section 3), lipids such as MPLA and squalene, as well as several phospholipids, have also been used in some cases for the nanoengineering of antigens, leading to the formation of nanocarriers or antigen delivery systems [40,41]. Specific moieties, such as pathogen-associated molecular patterns (PAMPs) that can be recognized by PRRs, are in some occasions naturally present in these macromolecules, or can be synthetically included in their structures to potentiate their function [24,42]. In the case of MPLA and squalene, their recognized immunomodulation features have led to their inclusion in marketed vaccines, or as components of approved adjuvants such as MF59, AS03™ (in the case of squalene) and AS04™ (in the case of MPLA), as well as in other formulations still in preclinical and clinical development [43].

Nanoengineering approaches can be used to associate antigens to delivery carriers made of specific biomaterials, normally recognized for their adjuvant properties. These delivery carriers are able to transport and control the release of antigens to the cells where they should exert a biological activity. Polymeric nanoparticles, ISCOMs, liposomes and lipid nanoparticles, among others, are included in this category [21,44–46].

In general, it is recognized that the depot effect generated by the majority of antigen delivery systems after subcutaneous injection, allowing their uptake by the antigen presenting cells, is an attractive feature of this type of adjuvants [47,48]. Moreover, they are able to mimic the particulate nature of pathogens, therefore increasing the possibilities of an effective immune response [49]. In this context, nanotechnology is expected to have a significant impact, as will be discussed in the following section.

2. The potential of nanotechnology for vaccine delivery

The use of technologies and biomaterials at nanometric scale in therapeutics and diagnostics is a growing research field since the second half of the 20th century [50]. A large variety of nanoparticulate systems has been developed throughout the past decades to improve the delivery, targeting and efficacy of drugs, biomolecules, nucleic acids and antigens [51]. Depending on the components and methodology chosen, it is possible to develop a wide range of nanostructures, as for example (i) polymeric nanoparticles, based on a matrix-type entanglement of selected polymers, (ii) oil-in-water (O/W) nanoemulsions, consisting in oil nanodrops stabilized by adequate surfactants,

(iii) nanocapsules, which are polymer-coated nanoemulsions, forming a core-shell nanostructure, and (iv) lipid-based nanosystems, such as liposomes and solid lipid nanoparticles, among others.

In the area of vaccination, nanotechnology has led to the development of nanostructures holding specific advantages for antigen delivery. First of all, as mentioned before, due to their particulate structure and nanometric size, similar to the ones of virus and bacteria, nanoparticles can mimic the natural infection process and be taken-up by the antigen presenting cells (APCs), thereby leading to enhanced immune responses [48,52–54]. A number of authors have reported that particles in the nanometric range are particularly suitable for their interaction with the immune system [55–57]. In addition, in our lab, working with PLA-PEG micro and nanoparticles, we have also observed that 200 nm-nanoparticles can enhance the transport of the antigen through the nasal mucosa more efficiently than microparticles, (either 1 or 5 μm) after intranasal administration [58]. However, there is still some controversy regarding this issue, with other works supporting the idea that microparticles can elicit stronger immune responses [59,60]. This disagreement may come from the difficulty to compare different studies, as many variables account for the total outcome of the immune response, such as the constituting biomaterials, the nature and doses of antigen, and the route of administration [61]. It has also been hypothesized that using nanoparticles may favour cellular immune responses through optimal interactions with $CD8^+$ dendritic cell (DC) subsets [48]. For example, using model carboxilated polystyrene micro (2 μm) and nanoparticles (40 nm), loaded with ovalbumin (OVA) [57], it was shown that nanoparticles were able to elicit significantly higher IgG and T-cell responses. Finally, it has been reported that a size below 100 nm is desirable if the purpose is to facilitate the transport of nanoparticles from the subcutaneous tissue up to the lymph nodes, where the antigens will be presented to mature immune cells for an adaptive immune response [55,62].

Nanostructures have also shown an interesting potential for mucosal antigen delivery, due to their ability to interact and get across mucosal barriers [63,64]. This property is mainly related to the particle size [65,66], and also to the composition, being favoured when the systems include mucoadhesive materials such as chitosan [67]. Moreover, our group was pioneer in demonstrating that the modification of nanocarriers with polyethylene glycol (PEG) units, in different degrees, was also responsible for improved transport of those systems across the nasal mucosa [68]. Overall, the increased interaction of these systems with the mucosae boosts the antigen presentation in those areas, where the natural entrance of several pathogens usually happens, therefore mimicking the natural infection process. Mucosal antigen delivery, or needle-free

vaccination, allows overcoming important limitations associated to parenteral immunization such as the high cost of preparation, the need for specific administration materials (needles and syringes) and specialized technical staff, and is also more likely to be well accepted by patients, altogether resulting in an improved vaccine coverage [69,70].

Finally, another important advantage of nanosystems in vaccine delivery is the possibility to co-encapsulate additional immunostimulatory molecules, such as the ones described in section 1.2.1, with the purpose of increasing the overall adjuvant capacity. As an example, TLR agonists such as CpG, poly(I:C) [71] or imiquimod [72], have been associated to dextran nanoparticles and chitosan nanocapsules, respectively. These molecules promote specific receptor-based recognition of the nanovaccines and the consequent cell activation, strengthening the elicited immune response.

To highlight the potential of nanoparticles in vaccination, it is worth mentioning the formulations that have already reached the market and others in advanced clinical stages of development. Nanoemulsions, such as AS03™ (an oil-in-water nanoemulsion containing squalene, DL-α-tocopherol and Tween® 80) [73] or MF59™ (also squalene oil-in-water nanoemulsion stabilized with Tween® 80 and Span® 85) [74], are commercialized for influenza vaccines, namely Pandemrix™ (from GlaxoSmithKline Biologicals), and Fluad™ and Focetria™ (from Novartis), respectively. Virosomes, composed of a phospholipid membrane incorporating viral glycoproteins, are present in commercialized Hepatitis A and influenza vaccines, Epaxal™ and Inflexal™ (both from Crucell), respectively [75]. Other nanometric formulations such as AS01™ (liposomes based on MPLA) [76], AS02™ (squalene oil-in-water emulsion also containing MPLA) [77], and one polymeric carrier based on PLGA, among others, are currently under clinical development for different vaccines, as described in Table 1. Considering this, it is clear that nanometric delivery systems are in the spotlight for their potential in vaccination.

Table 1. Nanoengineered antigen formulations in clinical development.

Delivery System	Antigen	Phase	End	Identifier
MF59	Influenza (H5N1 inactivated virus)	n.d.	2014	NCT01578317
	Influenza (H7N9 inactivated virus)	I	ongoing	NCT02251288
		II	2014	NCT01938742
		II	ongoing	NCT02213354
	Influenza (killed virus, trivalent subunit vaccine)	I	ongoing	NCT02126761
		II	2013	NCT01879540
	RSV (RSV F protein)	I	ongoing	NCT02298179
	CMV (gp B)	II	2013	NCT00133497
MF59 & AS03	Influenza (H7N9 inactivated virus)	II	2015	NCT01942265
AS03	Influenza (H5N1 inactivated virus)	I/II	2012	NCT01353534
		I	2014	NCT01573312
		II	ongoing	NCT01910519
AS03 & AS01	Dengue (inactivated virus)	I	ongoing	NCT01702857
		I	ongoing	NCT01666652
AS01	Malaria (FMP012; FMP2.1; RTS,S proteins)	I	ongoing	NCT02174978
		I/II	2014	NCT02044198
		I/II	2014	NCT01883609
		II/III	ongoing	NCT00872963
AS02	Malaria (FMP1; RTS,S proteins)	I/II	2014	NCT01556945
ISCOM	HSV (GEN-003 protein)	II	ongoing	NCT02114060
		II	ongoing	NCT02300142
	Malaria (viral vector with ME-TRAP protein)	I	2014	NCT01669512
	Influenza (H7N9 VLP)	I/II	ongoing	NCT02078674
Iscomatrix	Cancer (tumour cell lysates)	I/II	ongoing	NCT02054104
Hydrogel	Influenza (Act-HIB® vaccine)	I/II	2012	NCT01578070
Virosomes	Hepatitis A (inactivated virus)	II	2013	NCT01405677
	Candidiasis (n.d.)	I	2012	NCT01067131

PLGA microspheres	Cancer (HER-2/Neu peptide)	I	2012	NCT00005023

Abbreviations: RSV, respiratory syncytial virus; CMV, cytomegalovirus; gp B, glycoprotein B; HSV, herpes simplex virus; VLP, virus-like particle; n.d., not disclosed.

3. Nanoengineering of vaccines using polysaccharides

In the late 70s, the work of Kreuter and Speiser opened the way for the specific use of polymers, such as polymethylmethacrylate, as materials for the engineering of antigen nanocarriers [78]. Since then, a significant number of studies have put in evidence the potential of nanoparticles to enhance the immune response against different antigens in a sustained and prolonged way [45,46,52,79,80]. Our group, being particularly active in the field, pioneered the encapsulation of model proteins and antigens within poly(lactic-co-glycolic acid) (PLGA) [81] and polylactic acid-polyethylene glycol (PLA-PEG) nanoparticles [68]. Interestingly, this work was followed by a great number of authors [82], whose contributions have led to the clinical development of PLGA-based nanovaccines [83]. All along this engineering trajectory, it became clear that a major inconvenience of this biomaterial was the degradation of the antigen encapsulated in the course of the polymer degradation [84,85]. Although specific formulation strategies were found to significantly reduce this effect over the encapsulated antigens [86,87], overall the results achieved using PLGA-based nanoengineering persuaded us and others to search for new biomaterials which might have a mild interaction with antigens.

Naturally occurring polymers, in particular polysaccharides attracted our attention in the mid 90's as biomaterials for antigen nanoengineering. With this goal in mind, we reported for the first time the production of nanoparticles consisting of assemblies of proteins and chitosan [88], as described in the following section. Following this, other authors have proposed the use of polysaccharides, i.e. dextran, mannan, and beta glucans for the nanoengineering of vaccines [89]. These latter biomaterials are found in the cell walls of several pathogens such as bacteria or yeast, a characteristic that provides them with intrinsic targeting abilities to APCs (acting as PAMPs on the PRRs present in these cells) and, consequently, a natural capacity to enhance the immune response against the associated antigens [24,42,89,90]. Other important features such as high biocompatibility and low toxicity make polysaccharides particularly interesting for pharmaceutical development purposes.

Another specific advantage associated to the use of polysaccharide-based antigen nanocarriers is related to the technologies used to produce them. These technologies rely on physicochemical processes such as ionic gelation [91], complexation [92] and solvent displacement [93], among others. These are generally simple and mild techniques, which minimize the use of solvents and high-energy sources, easy to scale-up, and importantly, suitable for the association of labile biomolecules [94]. Apart from selecting an appropriate technology, other relevant technical aspects for the development of nanovaccines, i.e. the stability of the antigen, in terms of biological activity, and the stability of the formulation during storage, are to be considered at early stages of development [95,96]. Selection of raw materials with pharmaceutical quality, i.e., produced according to specific criteria that assure their high purity and adequate characteristics for use in humans and with good inter-batch reproducibility, are also important topics to take into consideration in the process of nanovaccine design and manufacturing.

Nevertheless, the potential of these biomaterials in this field deserves a deeper analysis of the published material concerning polysaccharide-based nanosystems in vaccination. For this reason, the characteristics of the main polysaccharides with described adjuvant properties, as well as their application in the development of nanovaccines, are detailed as following.

3.1. Chitosan as a biomaterial for antigen nanoengineering

Chitosan (CS) is a naturally occurring polymer composed of a linear backbone of D-glucosamine and *N*-acetyl-D-glucosamine monomers connected through β-(1,4) bonds. This polysaccharide is mainly obtained from the deacetylation of chitin, present in the exoskeleton of crustaceans and squids, though other sources such as fungal have also been reported [97,98]. One of the most relevant characteristics of chitosan is the possibility to modulate its degree of acetylation and therefore the number of amino groups [98]. Its cationic character allows electrostatic interactions with antigens that are negatively charged in physiological conditions, and is also responsible for its ability to interact with mucosal surfaces [99,100].

Some authors have indicated potential chitosan immunomodulatory properties based on some *in vitro* and *in vivo* studies performed using chitosan in solution. More precisely, Peluso *et al* found that chitosan solutions can activate peritoneal rat macrophages through the enhancement of nitric oxide secretion *in vitro* [101]. On the other hand, Porporatto *et al* have reported the ability of chitosan to trigger local and systemic immune

responses, evidenced by the enhancement of cytokine production upon oral administration of a single 3 mg dose of the polysaccharide in solution to rats [102]. Finally, Zaharoff *et al.* evaluated the adjuvant potential of a chitosan solution (1 mg dose), given subcutaneously, in comparison with phosphate buffered saline solution (PBS) and with common adjuvants such as alum and Incomplete Freund's Adjuvant (IFA), using a model protein antigen, β-galactosidase [103]. Results have shown the ability of chitosan to induce humoral and cellular responses, which were superior to the non-adjuvanted formulation and the alum-adjuvanted one and equivalent to the ones achieved using IFA. Nevertheless, these immunomodulatory properties are still under question and could be related to the uncontrolled precipitation of chitosan at physiological pH, and also to the source and quality and chitosan used [97].

With respect to the biocompatibility and biodegradability of chitosan, it is worth mentioning that chitosan has a "generally recognized as safe" (GRAS) status granted by the United States Food and Drug Administration (FDA). Chitosan has been used for a long time as a dietary supplement for the prevention of fat absorption, and as a component of wound dressings [104]. On the other hand, chitosan is present in new nasal vaccine formulations against meningitis and Norovirus, which are under clinical development [105,106]. In addition, it is present in a vaccine hydrogel formulation against influenza, for intramuscular injection [107]. Overall, chitosan is therefore considered as one of the most advanced polymers in the regulatory path for the indication of vaccination. Taking this into account, and also preclinical evidence of the potential of chitosan-based nanocarriers as adjuvants for mucosal and parenteral immunization (Table 2), it could be expected that chitosan nanoformulations could enter clinical trials in the oncoming years.

Table 2. *In vivo* evaluation of chitosan-based nanovaccines.

	Antigen	Administration route	*In vivo* efficacy results	Ref
Nanoparticles	OVA	IM, IN	After IM immunization (single dose), OVA-loaded TMC nanoparticles and TMC-OVA nanoconjugates provided higher IgG titres than the controls, and increased DC uptake and activation. IN immunization (2 doses) elicited strong and balanced IgG and IgA levels.	[121, 122]
	OVA		Higher IgG levels were achieved with TMC nanoparticles in comparison with PLGA nanoparticles (coated or not with TMC), irrespective of the administration route (3 doses).	[120]
		Intraduodenal	TMC or chitosan nanoparticles (2 doses), increased the IgG levels and induced DC maturation in comparison with OVA in solution.	[119]
		IN, ID	TMC nanoparticles co-encapsulating additional adjuvant molecules (2 doses) were compared. In terms of IgG and IgA levels, LPS was best in both routes, followed by MDP for IN route and CpG for ID route.	[125]
		IN, TD	Covalently-linked TMC:HA nanoparticles elicited higher IgG levels than free OVA and conventional TMC:HA nanoparticles based on electrostatic interactions (2 doses).	[124]
	rHBsAg	IM	Significantly higher IgG levels in comparison with alum-adsorbed antigen (2 doses).	[110]
		IP	Stronger and longer-lasting IgG levels elicited in a single-dose schedule in comparison with commercial vaccine. Results with the nanovaccine were comparable irrespective of IP, IM or SC administration route.	[112]
	rHBsAg	SC	Significantly higher IgG levels co-encapsulating CpG (2 doses) than with the antigen in solution. Coating the antigen-loaded nanoparticles with alginate and co-administering a CpG solution shifted the response towards Th1/Th2 balance and increased IFN-γ levels (cellular response).	[111]
	pRc/CMV-HBs (plasmid)	IN	Protective and Th1-biased IgG levels, as well as high IgA levels in nasal, salivary and vaginal secretions, elicited after 2 immunizations.	[130]

Nanoparticles	TT	IN	IgG levels upon 3 doses were higher than those reported with the antigen in solution, and comparable to IM alum-adsorbed vaccine.	[116]
			TMC nanoparticles (2 doses) elicited similar response than chitosan nanoparticles, which were significantly higher than the antigen in solution.	[123]
	Hemagglutinin		Two doses of the nanovaccine elicited high IgG and IgA levels, induction of IFN-γ production by spleen cells (cellular response) and an increased survival of challenged animals up to 100%.	[117]
	Streptococcus equi		Higher IgA levels, increased mucosal uptake and Th1/Th2 balanced responses in comparison with cationic liposomes (2 doses).	[118]
	Antigen-encoding plasmids	IM	Plasmid encoding *Chlamydia trachomatis* proteins. High local (mice thigh muscles) and systemic (mice spleens) protein expression levels after a single-dose administration.	[126]
			Plasmid encoding 3 T-cell epitopes of Esat-6 (*Mycobacterium tuberculosis* antigen). Strong Th1 and CTL responses as well as protection against challenge upon 3 immunizations.	[127]
		SC/IN	Plasmid encoding antigen 85B (*M. tuberculosis*). Co-encapsulation of another plasmid encoding an autophagy-inducing factor (myc-mTOR). Strong IgG and cytokine (IL-4 and IFN-γ) levels after SC prime and two IN boosts.	[129]
		IN	Plasmid encoding pHSP65pep (*M. tuberculosis*). Strong antibody and T-cell responses and increased protection against challenge after 4 immunizations.	[128]
			Plasmid encoding the SARS-CoV nucleocapsid protein. Particles functionalized with a protein vector for DC targeting (bfFP) and a DC maturation stimulus (aCD40) (2 doses) showed better targeting to DCs and increased mucosal response.	[131]
		Oral	Plasmid encoding Der p 2 (house dust mites allergen). Antibody (IgG2 and IgE) and cytokine (IFN-γ and IL-4) levels correlated with the minimization of the allergic process (2 doses).	[134]

Nanoparticles	Antigen-encoding plasmids	Oral	Plasmid encoding Rho1-GTPase (*Schistosoma mansoni* antigen). Significantly reduced liver granulomatosis and worm burden (after challenge) in comparison with controls (3 doses).	[135]
Nanocapsules	rHBsAg	IM, IN	A single IM dose of the vaccine prototype elicited similar IgG levels as two IM doses of alum-adsorbed antigen. Including imiquimod (TLR-7/8 agonist) enhanced a specific Th1-biased immune response through IN route.	[72,114]
Liposomes	Antigen-encoding plasmids	IN	Plasmid encoding HBsAg. Glycol chitosan-coated liposomes (2 doses) elicited seroprotection and increased IgA levels in nasal, vaginal and salivary secretions in comparison with controls.	[137]
			Plasmid encoding *Streptoccocus mutans* surface antigen. Chitosan-coated liposomes (2 doses) selectively released DNA at pH 7.4 (cellular cytoplasm), increased nasal residence time and enhanced IgA levels, in comparison with DNA-loaded chitosan nanoparticles.	[136]

Abbreviations: rHBsAg, recombinant hepatitis B surface antigen; OVA, ovalbumin; TT, tetanus toxoid; IM, intramuscular; IP, intraperitoneal; SC, subcutaneous; IN, intranasal; ID, intradermal; TD, transdermal; IgG, immunoglobulin G; IFN-γ, interferon gamma; IgA, immunoglobulin A; TMC, trimethylchitosan; DC, dendritic cells; PLGA, poly(lactic-co-glycolic acid); MDP, muramyl dipeptide; LPS, lipopolysaccharide; HA, hyaluronic acid; MCC, mono-N-carboxymethyl chitosan; CTL, cytotoxic T lymphocyte; IL-4, interleukin 4; SARS-CoV, severe acute respiratory syndrome coronavirus; JE, Japanese encephalitis; TLR, Toll-like receptor; IgE, immunoglobulin E.

3.1.1. Protein nanovaccines

3.1.1.1. Parenteral vaccination with chitosan-based nanovaccines

As indicated, chitosan nanoparticles, developed for the first time in the 90s [91], have been widely studied for the delivery of proteins and antigens (Arca et al. 2009; García-Fuentes and Alonso 2012). For example, chitosan nanoparticles associating rHBsAg were shown to exhibit an adjuvant effect that was higher than that of alum, after intramuscular administration to mice [110]. Other studies have been addressed to co-associate immunostimulant molecules, i.e. CpG, and rHBsAg in chitosan nanoparticles. This vaccine formulation increased the IgG titres in comparison with the antigen in solution, upon subcutaneous administration to mice [111]. Chitosan nanoparticles have

been additionally reported for their potential in a single-dose vaccination strategy for rHBsAg [112]. The results of this study indicated that after a single intraperitoneal, intramuscular or subcutaneous injection of the nanoparticles, the response achieved was stronger and lasted longer than the one elicited by a single dose of Recombivax HB® (alum-associated rHBsAg), given intraperitoneally.

A different type of chitosan-based nanocarrier, i.e. chitosan nanocapsules, originally disclosed by our group [113] was similarly proposed for a single-dose immunization schedule with rHBsAg [114]. Additionally, we developed a freeze-dried formulation in order to improve the preservation of the vaccine. The results observed upon a single intramuscular administration to mice indicate that the IgG levels were comparable to those obtained after vaccination with the alum-adsorbed antigen in a prime-boost scheme (weeks 0 and 4). We could also prove that these nanocapsules were adequate for the co-encapsulation of the TLR 7/8-agonist imiquimod [72], a formulation approach for nasal immunization, further discussed in the next section. More recently, we conducted experiments to assess the biodistribution of these nanocapsules after subcutaneous injection [115]. The results evidenced the formation of a depot, followed by a slow drainage of the nanocapsules towards the lymph nodes, where they accumulate (for illustration see figure 2). These results allowed us to conclude that this biodistribution profile was responsible for the long-lasting adjuvant effect observed for chitosan nanocapsules.

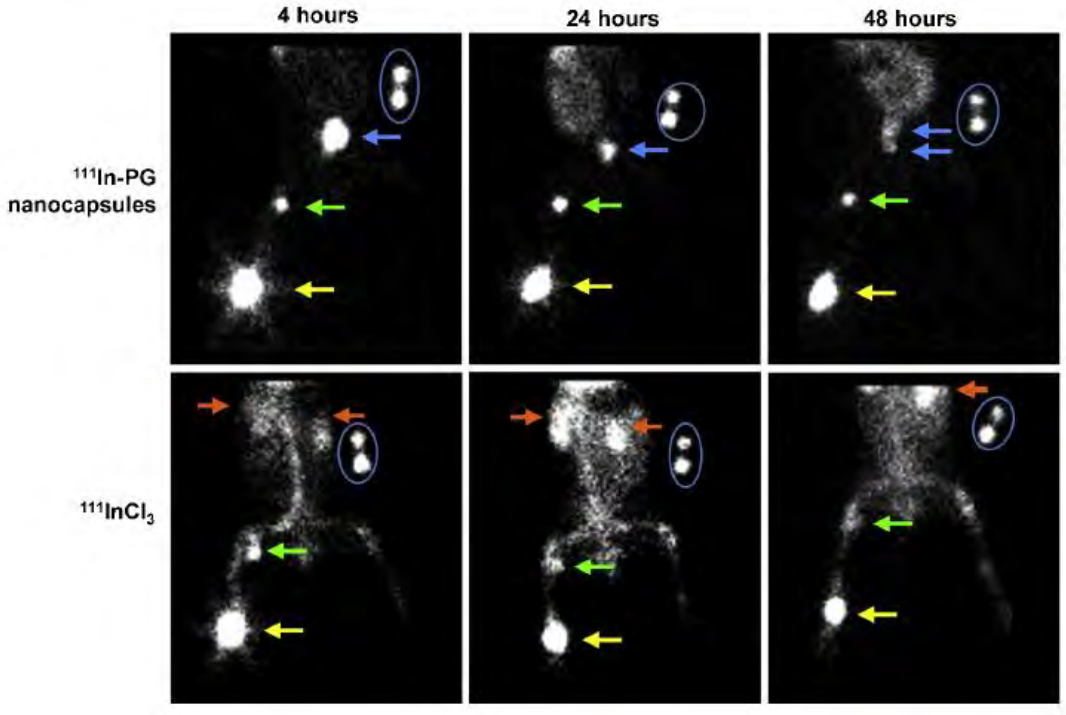


Figure 2. *Images of the lower body of rabbits injected with ^{111}In-radiolabelled chitosan nanocapsules (upper row) or with a control solution of $^{111}InCl_3$ (lower row), acquired 4, 24, and 48 hours post injection. A depot formation in the injection site, as well as a slow drainage and further accumulation in the popliteal lymph node, can be observed in the case of the nanocapsules. Yellow arrow: injection site (rear foot); green arrow: popliteal lymph node; blue arrow: iliac lymph nodes; orange arrows: kidneys; circle: external standard. Adapted with permission from [115].*

3.1.1.2. Mucosal vaccination with chitosan-based nanovaccines

Apart from the promising results obtained with parenteral immunization using chitosan nanovaccines, a great deal of effort has been addressed to the development of chitosan-based nanovaccines intended for mucosal vaccination. Our group pioneered the development of the first chitosan-based nanovaccine as a needle-free vaccination strategy [116]. Our results showed that after intranasal administration to mice, chitosan-based tetanus toxoid (TT) nanovaccine led to high and long-lasting IgG levels, which were comparable to those elicited by the alum-adsorbed vaccine administered intramuscularly [116]. More recently, chitosan nanoparticles were studied for the association of the hemagglutinin protein of H1N1 influenza virus. After intranasal administration of hemagglutinin-loaded chitosan nanoparticles to mice, both the systemic and mucosal antibody levels (IgG and IgA) were significantly enhanced with respect to the controls (antigen in solution), and a T cell response was also reported. Importantly, after being challenged through the same route with the virus, the animals receiving the nanovaccine presented higher survival rates [117]. In another study, chitosan nanoparticles were loaded with *Streptococcus equi* bacterial proteins and administered intranasally to mice. The results showed enhanced IgG and IgA responses as compared to those elicited by the antigen-loaded liposomes and the corresponding empty nanoparticles and liposomes[118].

Our group has also explored the potential of co-encapsulation of rHBsAg and the immunostimulant imiquimod into chitosan nanocapsules, for enhancing the immune response following intranasal vaccination [72]. The results obtained in mice evidenced an enhanced, specific and Th1/Th2 balanced immune response, which was significantly higher than the one observed for rHBsAg-loaded chitosan nanocapsules (without imiquimod) and the control rHBsAg-loaded nanoemulsion (figure 3). These results highlight the positive effect of co-delivering an additional immunostimulating molecule

with the antigen in a single nanostructure, and open room for the potential of modulating immunity towards the cellular pathway using a needle-free vaccination approach.

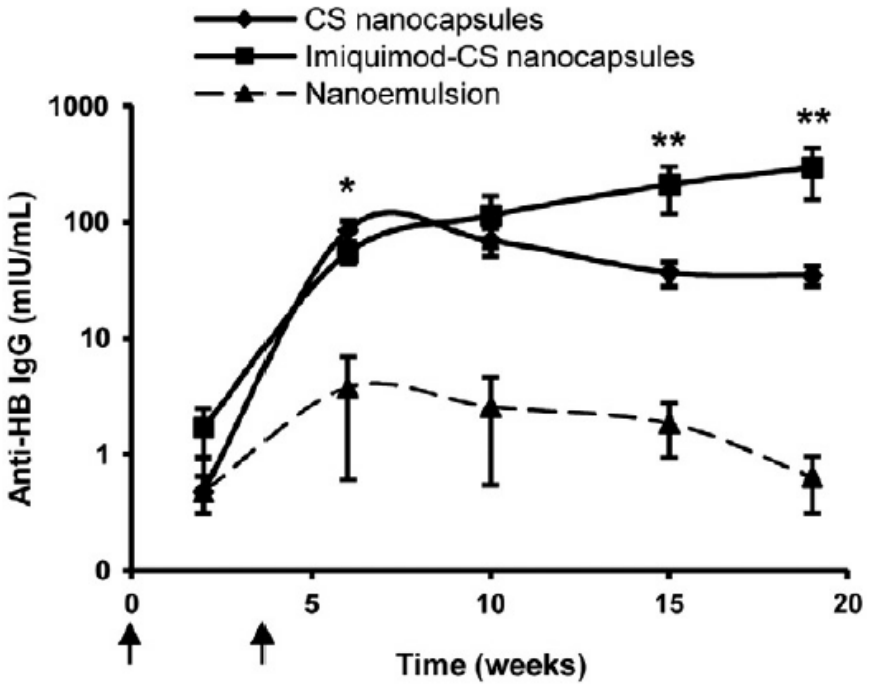


Figure 3. *Serum IgG levels achieved after intranasal immunization (two doses at 0 and 4 weeks, indicated by the arrows) of healthy mice with rHBsAg- imiquimod-loaded chitosan nanocapsules, rHBsAg-loaded chitosan nanocapsules (without imiquimod) and rHBsAg-loaded nanoemulsion (control group without chitosan). *p<0.05 between rHBsAg-loaded chitosan nanocapsules (with and without imiquimod) and the rHBsAg-loaded nanoemulsion; **p<0.05 between rHBsAg- imiquimod- loaded chitosan nanocapsules and the other two formulations. Reproduced with permission from [72].*

A chitosan derivative with higher water solubility and pH-independent cationic nature, N-trimethyl chitosan (TMC), has also been evaluated for the preparation of nanovaccines. The intraduodenal immunization of OVA-loaded TMC nanoparticles to mice, provided a similar immune response than conventional chitosan nanoparticles, in terms of IgG levels, and showed an improved ability to induce DC maturation [119]. The behaviour of OVA-loaded TMC nanoparticles was also compared with that of OVA-loaded PLGA and OVA-loaded TMC-coated PLGA nanoparticles, upon intranasal or intramuscular administration to healthy mice. While all formulations could enhance the immune response following intramuscular administration, only TMC nanoparticles were able to elicited an immune response after intranasal administration, a fact that was attributed to

their increased interaction with the nasal mucosa [120]. The efficacy of these nanoparticles was also compared with that of TMC-OVA conjugates, previously developed for intramuscular administration, which had shown adjuvant activity similar to that of TMC nanoparticles [121]. Upon intranasal administration of both TMC-OVA conjugates (30 nm) and OVA-loaded TMC nanoparticles (300nm), results have shown the conjugates superiority in terms of the IgG and IgA levels elicited, a fact attributed to the higher uptake of these structures by the nasal epithelium [122].

In another study, TT-loaded TMC nanoparticles were compared to TT-loaded chitosan nanoparticles, upon either intranasal or subcutaneous administration to mice, with the corresponding polymer solution as a control. The results evidenced the similar behaviour of both types of nanoparticles in terms of IgG response [123]. Finally, TMC has also been used in combination with hyaluronan (HA), [124]. Despite the observation of an immune response upon intranasal administration to mice, the benefits of incorporating HA to the formulation remain unclear, since comparative studies with plain TMC nanoparticles were not included in this work.

The last example corresponds to OVA-loaded TMC nanoparticles that co-encapsulate additional immunostimulant molecules, namely lipopolysaccharide (LPS, TLR-4 agonist), CpG (TLR-9 agonist), Pam_3CSK_4 (TLR-2 agonist), muramyl dipeptide (MDP, NOD-like receptor 2 ligand) and cholera toxin B subunit (CTB, GM1 ganglioside receptor ligand) [125]. Upon intranasal administration to healthy mice, a Th2-biased response was reported in most cases (except for CpG), being the highest response achieved with nanoparticles co-associating MDP.

3.1.2. Nucleic acid-based nanovaccines

3.1.2.1. Parenteral vaccination with chitosan/nucleic acid-based nanovaccines

Chitosan nanoparticles have also been developed for the delivery of plasmid DNA for immunization purposes [98]. In a couple of examples, chitosan nanoparticles have been loaded with plasmid DNA encoding for the recombinant major outer membrane protein of *Chlamydia trachomatis* [126] or for three T-cell epitopes of Esat-6, a *Mycobacterium tuberculosis* antigen critical for virulence [127]. In the first case, results showed high expression levels of the *Chlamydia trachomatis* protein after intramuscular administration to mice, both at the site of injection and also systemically (i.e. spleen) [126]. In the second example, the results obtained after intramuscular administration to

mice indicated a protection against a *Mycobacterium tuberculosis* challenge associated to strong Th1 and cytotoxic T lymphocyte (CTL) responses [127].

3.1.2.2. Mucosal vaccination with chitosan/nucleic acid-based nanovaccines

Chitosan nanoparticles have been tested for intranasal immunization against several pathogens. One example refers to chitosan nanoparticles that associate a plasmid encoding a multi-epitope protein against *M. tuberculosis* (pHSP65pep). This formulation provided the administered mice with an adequate protection against bacterial challenge, protection that was associated to significant T-cell responses [128]. In another study, also for *M. tuberculosis* immunization, chitosan nanoparticles were loaded with a plasmid encoding for antigen 85B together with a second plasmid encoding an autophagy-inducing factor (myc-mTOR) [129]. After immunization of mice with a subcutaneous prime and two intranasal boosts (2-week intervals), results proved a synergistic effect, due to the co-administration of both plasmids, in terms of immune response. Chitosan nanoparticles have also been evaluated against hepatitis B, upon association of a plasmid encoding the hepatitis B surface antigen (pRc/CMV-HBs) [130]. Results showed a Th1-biased response after intranasal administration to mice, which was accompanied of increased IgA levels in nasal, salivary, and vaginal secretions.

In an attempt to further improve their efficacy as DNA carriers for intranasal immunization, chitosan nanoparticles loaded with a plasmid encoding the nucleocapsid protein of severe acute respiratory syndrome coronavirus (pVAXN) were decorated with a bifunctional fusion protein (bfFP) targeted at the DC surface receptor DEC-205, and also with an antibody binding the DC receptor CD40, to stimulate these cells. Mice were immunized either intranasally or intramuscularly with (i) naked pVAXN, (ii) pVAXN-loaded nanoparticles, (iii) bfFP decorated pVAXN-loaded nanoparticles, and (iv) bfFP and aCD40 decorated pVAXN-loaded nanoparticles. The results indicated that the nanoparticles decorated with both molecules (bfFP/aCD40 approach), were more efficient in terms of IgG and IgA responses irrespective of the administration route [131]. The incorporation of immunostimulatory molecules in chitosan-based nanocarriers was also evaluated. In detail, Heuking *et al* [132,133] grafted TLR agonists to TMC and prepared nanoparticles with the model plasmid pGFP. *In vitro* results showed that the incorporation of Pam_3Cys (TLR-2 agonist) and 9-benzyl-8-hydroxyadenine (TLR-7 agonist) enhanced IL-8 release, a cytokine responsible for the attraction of leukocytes to the local of infection.

Chitosan nanovaccines have also been tested for oral immunization [134,135]. One interesting work refers to the association of plasmid DNA encoding Der p2, an allergen

from house dust mites, to chitosan nanoparticles, which were orally administered to healthy mice. The results evidenced high IgG2 and low IgE levels, together with high IFN-γ and low IL-4 secretion, in accordance with a reduction of the allergic response [134]. In another work, a chitosan derivative containing imidazole moieties (CSimi) was used in the preparation of nanoparticles for the delivery of a plasmid encoding a *Schistosoma mansoni* antigenic protein (Rho1-GTPase) [135]. After oral administration of three doses of pDNA-loaded CSimi nanoparticles to healthy mice (weeks 1, 3 and 5), the animals were challenged with the pathogen and results evidenced a reduction of the main pathogenic effect, liver granulomatosis, in comparison with the control animals that received PBS. Interestingly, one of the control groups that received blank chitosan nanoparticles (without antigen) also promoted a strong worm burden reduction in comparison with the control group, a fact that was attributed to an immune reaction against chitosan, since other similar carbohydrates are present in the parasite structure.

Chitosan-coated liposomes have also been described for mucosal immunization [136,137]. In detail, glycol chitosan-coated liposomes, which associated a plasmid encoding the surface hepatitis B antigen (pRc/CMV-HBs), were administered intranasally to mice [137]. The results, in terms of IgG responses, were similar to those observed in the control mice receiving the conventional alum-adsorbed encoded antigen (HBsAg) intramuscularly. However, the coated liposomes promoted a superior cell response as evidenced by the IL-2 and IFN-γ levels. Moreover, IgA levels in nasal, vaginal, and salivary secretions were only detected in the animals immunized with the chitosan-coated liposomes. In another example, chitosan-coated liposomes were loaded with a plasmid encoding a surface antigen of *Streptoccocus mutans,* for dental caries prevention, and their efficacy was compared to that of plasmid-loaded chitosan nanoparticles and plasmid in solution [136]. After intranasal administration to healthy mice, chitosan-coated liposomes elicited an improved immune response, which was related to the controlled antigen released and to the improved interaction of the plasmid-loaded nanoparticles with the nasal mucosa.

Globally, these results evidence the increasing interest in chitosan-based nanovaccines particularly for either parenteral or mucosal immunization strategies using both protein antigens and nucleic acid-based antigens. This potential is associated to the ability of chitosan-based nanosystems to interact with mucosal tissues and to their specific distribution into the lymphatic system. This accumulated information is firmly consolidating the potential of this polysaccharide as a biomaterial for the engineering of new vaccines.

3.2. Dextran as a biomaterial for nanoengineering antigens

Dextran is one of the most studied α-glucans in drug and antigen delivery. This polymer of α-(1,6)-glucan with α-(1,3) branches is obtained from bacteria, particularly from *Lactobacillus*, *Leuconostoc* and *Streptococcus* species [138]. Native bacterial dextran has high molecular weight and is water soluble, which is a useful characteristic for pharmaceutical formulation purposes. Also, its structure, particularly its hydroxyl groups, allow for easy functionalization and chemical modifications [139]. Finally, the biocompatibility and biodegradability of this biomaterial, together with its high availability and reduced cost of production, make it very attractive for the nanoengineering of antigens [138,139].

It is important to mention that dextran has GRAS status given by the FDA and has been used in humans for a very long time, mainly as plasma volume expander and anti-thrombotic agent [140]. Its sulfated derivative, dextran sulfate (DS), has also been approved by the FDA as a component of apheresis columns, used to remove low density lipoprotein (LDL) from the blood [141]. Since the 70s, the immunomodulating properties of dextran sulfate and its potential use as adjuvant have also been studied. Overall the results reported so far have shown its ability to increase the antibody- and cell-mediated immune responses in animal models [142,143]. This derivative, when administered "ad libitum" with drinking water at a 3% (w/v) concentration, has also been associated with strong pro-inflammatory effects, being used as an inducer of inflammatory diseases such as colitis in mice for the development of animal models of this disease [144]. Diethylaminoethyl (DEAE)-dextran is another derivative with adjuvant properties, studied mostly for veterinary vaccines [145]. Nevertheless, the mechanism of action of this derivative is still not well understood and requires further research [89,146].

3.2.1. Protein nanovaccines

As in the case of chitosan, the polysaccharide dextran has been explored as a biomaterial for nanoengineering of protein antigens. However, in this case, and despite its reported immunostimulant properties, the activity has been limited to a few studies, as described below.

3.2.1.1. Parenteral vaccination with dextran-based nanovaccines

Dextran nanoparticles containing OVA in combination with LPS were developed and administered intravenously to mice [147]. The results showed that these carriers were efficiently internalized by DCs, and induced OVA-specific T-cell proliferation ($CD4^+$ and $CD8^+$ T cells), as well as a stronger Th2-biased immune response, in comparison with

the controls (empty dextran nanoparticles, LPS-loaded nanoparticles and OVA-loaded nanoparticles). Dextran nanoparticles have also been evaluated for the encapsulation of immunostimulant molecules such as CpG or poly(I:C) [71]. *In vitro* studies show as, upon incubation with macrophages, these nanosystems elicited higher production of cytokines than the respective free molecules, demonstrating an improvement in the adjuvant potential of poly(I:C) and CpG.

The incorporation of dextran into chitosan-based nanosystems has recently been reported for the delivery of the capsid protein of HIV-1 (p24) and pertussis toxoid (PTX) [148,149]. Anionic and cationic chitosan/dextran nanoparticles were prepared by modulation of the mass ratio of both polysaccharides, and subsequently loaded with p24. After subcutaneous administration to healthy mice, both cationic and anionic nanoparticles rendered IgG titres that were comparable to those elicited by the Complete Freund's Adjuvant (CFA) [149]. In the case of chitosan/dextran nanoparticles loaded with PTX [148], the IgG response observed after subcutaneous administration to mice was significantly higher than the one elicited by the alum-adsorbed antigen.

Dextran nanoparticles were also prepared in combination with polyvinylalcohol (PVA), for the encapsulation of a recombinant *Bacillus anthracis* antigen (rPA), or resiquimod, a TLR 7/8 agonist [150]. These nanoparticles were subcutaneously administered to mice, either separately or in combination with the alum-adsorbed antigen. The first observation was a balanced Th1/Th2 immune response in animals receiving resiquimod. Indeed, the highest levels of IgG and cytokine production were observed with the co-administration of resiquimod-loaded nanoparticles in combination with rPA-loaded nanoparticles, or alum-adsorbed rPA; both combinations were responsible for 100% animal survival upon three challenges with the pathogen.

3.2.1.2. Mucosal vaccination with dextran-based nanovaccines

Dextran-based nanoparticles have been barely explored for intranasal vaccination. In a study [151], IgA was associated to dextran nanoparticles as a possible targeting moiety towards the M cells present in the nasal mucosa. After a single intranasal administration of fluorescent-labelled nanoparticles to healthy mice, the fluorescence levels for IgA-decorated dextran nanoparticles, analysed by confocal microscopy, were particularly high in the nasal-associated lymphoid tissue (NALT) cells in comparison with other nasal tissue areas. Therefore, these preliminary data suggest the potential of these nanoparticles for intranasal immunization.

3.2.2. Nucleic acid-based nanovaccines

Despite the number of studies reported on the use of chitosan nanoparticles for DNA vaccines, no studies have been identified incorporating dextran into the nanostructures or any other application of dextran-based nanocarriers in the development of genetic vaccines.

Overall, regardless of the limited number of studies reported so far, the results achieved with dextran-based systems open room for a deeper research in this field, particularly in terms of the combination of this polymer with other immunomodulatory molecules.

3.3. Mannans as biomaterials for nanoengineering antigens

The term “mannan” refers to storage polysaccharides very abundant in nature, consisting in D-mannose monomers connected by β-(1,4) bonds. The interest of these polysaccharides relies on their ability to interact with C-type lectins, a group of receptors expressed in APCs (DCs and macrophages). These receptors are responsible for the recognition of different carbohydrates present in the cell wall of certain pathogens and include the mannose receptor (MR), the mannose-binding lectin (MBL), and others more specific to DCs such as DC-SIGN or DEC-205 [152].

The recognition process described above leads to the endocytosis or phagocytosis of pathogens, with major importance in the innate immunity process, eliciting complement activation and triggering inflammation [89,153]. Moreover, interactions with specific DC C-type lectins have been correlated with DC trafficking and induction of both humoral and cellular responses [152].

The targeting of nanovaccines to APCs using this approach has been applied to different antigens, as discussed below. Nevertheless, it is important to be aware of a limitation of this strategy, the ubiquitous presence of mannan receptors in a variety of cells, including immune cells, epithelial cells (in the retina), mesangial cells (in kidney) and muscular (in trachea) cells [154].

3.3.1. Protein nanovaccines

The functionalization of nanovaccines with mannose residues for specific targeting of protein and peptide antigens to DCs, macrophages, and B cells, has been evaluated in murine and human *in vitro* models [155–157].

Polymeric nanoparticles based on a copolymer of poly-ε-caprolactone (PCL) and polyethyleneglycol (PEG), functionalized with mannan, were optimised for the encapsulation of the human basic fibroblast growth factor (bFGF), for cancer immunotherapy, and administered to healthy mice [158]. The fundamental role of mannan was evidenced in view of the significantly higher IgG levels achieved, in comparison with the control formulations, i.e. non-targeted nanoparticles and the alum-adsorbed antigen. Moreover, the immune response was biased towards the Th1 pathway, as shown by an increased level of IgG2a, which is particularly important to confront tumour cells. In another study, OVA-loaded mannan-decorated PLGA nanoparticles were also found to elicit higher T-cell responses when compared with OVA-loaded non-decorated PLGA nanoparticles [159], after subcutaneous administration to mice. Finally, *in vitro* macrophage uptake studies performed using poly(2-hydroxiethyl methacrylate-co-methacrylic acid) (P(HEMA-co-MAA)) nanogels, loaded with OVA, revealed a high internalization that was accompanied of a high production of co-stimulatory molecules, such as CD86, CD40 and CD80 [160].

Glucomannan, a polymer of β-1,4 linked D-mannose and D-glucose, has also been studied for the preparation of nanoparticles [161–165]. Bovine serum albumin (BSA)-loaded chitosan nanoparticles were functionalized with glucomannan with the aim of achieving an improved interaction with macrophages [163]. Upon oral administration of these decorated nanoparticles to mice, the immune response, in terms of IgG and IgA titers and cytokine production, was significantly higher with respect to non-functionalized nanoparticles. A novel approach recently explored comprises the modification of bilosomes (non-ionic surfactant-based nanovesicles containing bile salts) with glucomannan for oral antigen delivery [164,165]. BSA and TT were used as model antigens and *in vitro* studies showed that glucomannan-modified bilosomes had an increased uptake by macrophages in comparison with the unmodified ones. This translated into stronger systemic and mucosal immune responses following oral administration to mice.

3.3.2. Nucleic acid-based nanovaccines

Nanoengineering of nucleic acids with mannan has been mainly achieved through the synthesis of cationic derivatives of mannan [166–169]. For example, a cationic mannan-spermine copolymer was engineered for complexation of a model pDNA (pGL3), and the transfection efficiency was tested in a panel of immortalised cell lines, including macrophage and dendritic cells. The results showed high transfection levels in

macrophages, which were associated to the receptor-mediated internalization of the mannan complexes [166].

Polymer conjugates of mannan (in the oxidized or reduced form) and poly-L-lysine (PLL) have also been used for the complexation of pDNA encoding MUC1, a tumour-associated antigen, for anticancer immunotherapy. Upon intradermal immunization, these nanovaccines lead to increased T cell activity and antibody production, which protected animals against a tumour challenge with MUC-1 expressing B16 melanoma cells [167]. Additional data also showed the ability of these nanovaccines to induce DC maturation, through a TLR-2 dependent pathway, and to mediate the cross-presentation of antigens *in vivo* [168].

Mannan-modified cholesterol has also been synthesized and used for the preparation of functionalized cationic liposomes that associate a plasmid encoding a melanoma-associated antigen (pUb-M) [169]. Mice were immunized through intraperitoneal route with this prototype, using liposomes prepared with unmodified cholesterol and naked plasmid DNA as controls. Results evidenced increased gene delivery in splenic macrophages and DCs, achieved via mannose receptor, and a higher specificity of CTL activity with the functionalized liposomes. More importantly, after challenging the animals through the injection of tumour cells, the group treated with the mannan-targeted nanosystems showed rejection of the injected cells, suppression of their growth and increasing survival.

In general, this work highlights the potential interest of mannan as a targeting agent that may improve the ability of nanosystems to interact with the immunocompetent cells. Further studies are necessary in order to assess the specific benefit of this strategy.

3.4. Beta glucans as biomaterials for nanoengineering antigens

β-glucans are a group of very different polymers of glucose, varying in chain length as well as in number and position of branches. These polysaccharides can be found in the cell walls of many organisms, from bacteria to yeast and even some species of seaweed [170]. Some of them, such as β-1,3(D)-glucan (known as baker's yeast, from *Saccharomyces cerevisae*) and β-glucans from *Ganoderma lucidum* mycelium and from *Aureobasidium pullulans,* have GRAS status given by the FDA and are used as food ingredients [141]. In terms of immunomodulating properties, most of the research has been done with non-cellulosic β-(1,3) or β-(1,6)-glucans such as curdlan (from *Alcaligenes faecalis*), laminarin (from *Laminaria digitata*), lentinan (from Lentinus

edodes), pleuran (from *Pleurotus ostreatus*) and zymosan (from *Saccharomyces* spp.) [89,170].

The role of beta glucans in the immune response has already been explored for several decades. Their presence in the structure of different bacteria and fungi makes them easily recognizable by the PRRs, acting as natural PAMPs [171]. However, the adjuvant properties of one specific beta glucan cannot be generalized to others, due to differences in parameters such as size, branching and molecular structure [171–174].

The intervention of beta glucans in immunity is mainly due to their interaction with specific cell receptors, as pictured in figure 4. Though both types of recognition lead to similar biological effects, it is worth describing in further detail each one of them. On one hand, CR3 (complement receptor 3), widely present in myeloid cells such as macrophages, DCs and natural killer (NK) cells, was the first receptor described for the recognition of beta glucans. Though the biological effects of this specific interaction are not yet fully understood, it has already been shown that it leads to complement activation and subsequent opsonisation, followed by internalization of the structures [170,171,175]. On the other hand, Dectin-1, a type II transmembrane protein present in myeloid cells, is the most studied β-glucan receptor. The interaction of beta glucan-containing microorganisms or synthetic particles with this receptor mediates several processes such as reactive oxygen species (ROS) and cytokines production, being also a trigger for the internalization of pathogens via phagocytosis [170,176,177].

This interaction has led to the use of beta glucans also as targeting moieties for the enhancement of specific immune responses. For example, in the work of Dube *et al* [178], chitosan-coated PLGA nanoparticles were functionalized with 1,3-β-glucan from Euglena gracilis for Dectin-1 targeting. As a result, it was reported an increased intracellular delivery of the encapsulated anti-tuberculosis drug, as well as an enhanced pro-inflammatory reaction with relevant production of reactive oxygen and nitrogen species.

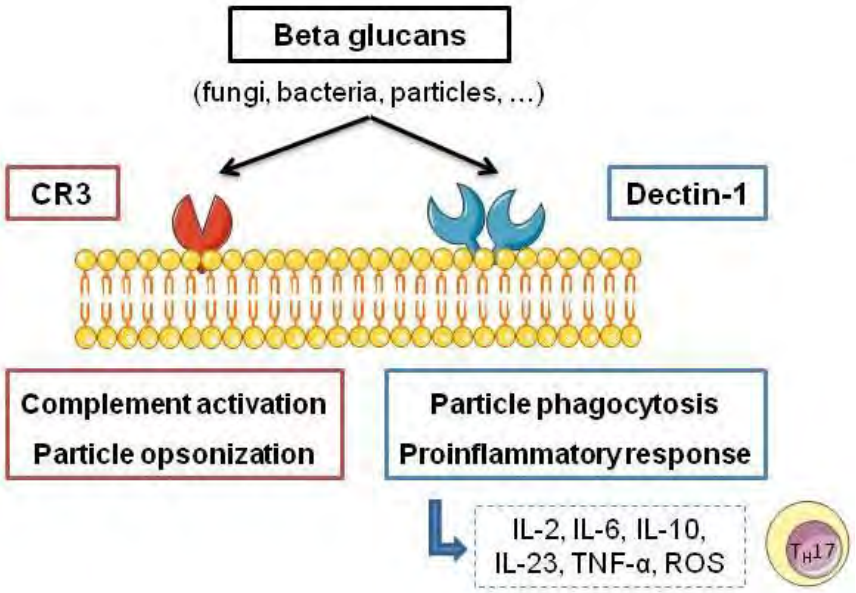


Figure 4. *Schematic representation of beta glucan recognition by immune cell receptors. The two main beta glucan receptors in APCs such as macrophages and dendritic cells are CR3 (complement receptor 3) and Dectin-1. The interaction of beta glucan-containing fungi and bacteria species, as well as synthetic glucan nanoparticles, with these cells may lead, on one hand, to the complement activation and particle opsonisation for phagocytosis (CR3 recognition), or, on the other hand, to the secretion of proinflammatory cytokines and ROS, together with the enhancement of particle phagocytosis by other pathways (Dectin-1 recognition). Moreover, in this last case, the ability to trigger the action T helper (Th17) cells in the adaptive immune response process may also be a relevant feature of beta glucan-containing structures once recognized by Dectin-1.*

3.4.1. Protein nanovaccines

The above indicated properties of the beta glucans have attracted the attention of a few researchers who have explored their use as antigen carriers. A conjugate of β-mannan, tetanus toxoid and laminarin (from *Laminaria digitata*) was evaluated for the development of a vaccine against *Candida albicans* [176]. This new conjugated antigen was able to promote receptor-based DC uptake mediated by the interaction of laminarin with Dectin-1, altogether resulting in an enhanced cytokine production (IL-4, IL-6 and TGF-β) and strong IgG antibody responses after intraperitoneal or subcutaneous administration to mice, with IFA as adjuvant [176].

Given the potential exhibited by beta glucans for immunomodulation, it is expected an increasing interest in their use for the development of nanovaccines. However, to the best of our knowledge, only one example relates to the use of the polysaccharide PS4 (isolated from *G. lucidum* mushrooms), with strong immunomodulating properties, based on the regulation of TNF-α, nitric oxide (NO) and cytokine production through interaction with TLR 2, for the preparation of nanosized complexes with poly(I:C) [179]. IgG responses observed after intraperitoneal administration of OVA-loaded PS4 nanoparticles to mice were higher than those elicited by the controls (i.e. physical mixtures of PS4 and OVA, or poly(I:C) and OVA). This positive in vivo response was correlated to an increased cytokine production after incubation of these nanoparticles with macrophages.

3.4.2. Nucleic acid-based nanovaccines

Some studies have also highlighted the potential of beta glucans for the delivery of nucleic acid antigens. Synthetic cationic glucans obtained from *G. lucidum* have been reported to efficiently associate a model pDNA (pGL-3 encoding luciferase), and to achieve an adequate transfection in human embryonic kidney transformed cells (HEK293T) [180]. Another example is the one making use of schizophyllan (SPG) complexed with TNF-α oligonucleotides (ODN) for delivery to macrophages, which are their therapeutic target in induced hepatic damage. Upon intraperitoneal administration of either the SPG-ODN complex or both components individually to mice bearing LPS-induced hepatic damage, the ODN were efficiently delivered to APCs through Dectin-1 targeting and exhibited an encouraging therapeutic effect [177].

Overall, beta glucans present intrinsic properties that increase the interest in their use for vaccine development, as demonstrated in this review. Nevertheless, the results achieved with these polymers are still preliminary, particularly in the case of the development of antigen nanocarriers, and the real potential of beta glucans in this field is yet to be demonstrated.

4. Concluding remarks

Throughout the past decades, several innovative biological techniques have been applied to the design and development of safer and more effective vaccines. However, the need for potent adjuvants that can enhance the immune response elicited by modern antigens has also grown and is nowadays an important research field. Within this frame, nanotechnology has been playing an increasingly important role, given the appropriate

features offered by nanocarriers to the administration of antigens. These include their particulate nature, controllable particle size, ability to protect antigens from degradation and to deliver them in a controlled manner, possibility to overcome mucosal routes, and ability to include co-stimulatory molecules that may enhance the global adjuvant effect of the nanocarrier.

In this context, the use of polysaccharides in the development of novel nanovaccines has represented a crucial step. Due to the interesting intrinsic properties of chitosan, such as its mucoadhesiveness and effect in the activation of macrophages, both particularly relevant for vaccine delivery, this polysaccharide has been the most studied one in the development of nanovaccines. Nevertheless, the use of other polysaccharides in this field is slowly growing and even though many of them are barely known as nanovaccine components, their recognized immunomodulating properties open room for further research and progress in this particular area.

Globally, recent work in nanovaccine development has demonstrated the potential of this strategy to confront current barriers in vaccination. Nevertheless, it is important to take into consideration that the combination of the biomaterials used in a specific formulation, the chosen antigen and the route of administration of the nanovaccines will be critical factors influencing the final outcome of the vaccination strategy.

5. Future perspectives

The use of biomaterials, especially natural polymers, for the preparation of antigen delivery systems, is one of the main tendencies on the field of vaccination. However, the natural origin of these materials is often responsible for high variability in the characteristics of each specific polymer and, therefore, may lead to variable and even controversial results in terms of their adjuvant activity. This is also a challenge in terms of the progress of these innovative vaccine formulations towards clinical development and commercialization. Therefore, it is expected that the next few years bring deeper knowledge in terms of the mechanism of action of these polymers, which might drive the path for a more rational design of polysaccharide-based nanoparticle adjuvants.

Other particular issues, such as the achievement of thermally stable formulations that can avoid the cold chain of vaccine storage, might be one of the challenges nanovaccines are likely to face in the next steps of the way. Finally, the optimization of the preparation methods used in nanovaccine development in order to adequately manufacture them at the industrial level is a major topic that should be addressed in the

near future of this field. The combination of this accumulated knowledge could be the necessary driving force to take polysaccharide-based and other nanovaccines towards the next level and ultimately to achieve clinical use.

6. Acknowledgments

The authors acknowledge financial support given by the Ministry of Economy and Competitiveness-MINECO (SAF2011-30337-C02-01), the Carlos III Health Institute-ISCIII (CP12/03150), and the Xunta de Galicia (Consellería de Educación-GRC2013-043, FEDER Funds). The first author acknowledges a predoctoral grant from the FPI program from the MINECO.

7. References

[1] Delany I, Rappuoli R, De Gregorio E. Vaccines for the 21st century. EMBO Mol Med 2014;6:708–20. doi:10.1002/emmm.201403876.

[2] Nabel GJ. Designing tomorrow's vaccines. N Engl J Med 2013;368:551–60. doi:10.1056/NEJMra1204186.

[3] Ohtake S, Arakawa T. Recombinant therapeutic protein vaccines. Protein Pept Lett 2013;20:1324–44.

[4] Donati C, Rappuoli R. Reverse vaccinology in the 21st century: improvements over the original design. Ann N Y Acad Sci 2013;1285:115–32. doi:10.1111/nyas.12046.

[5] Batzloff MR, Sriprakash K, Good MF. Vaccine development for group A streptococcus infections and associated diseases. Curr Drug Targets 2004;5:57–69.

[6] Skwarczynski M, Toth I. Peptide-Based Subunit Nanovaccines. Curr Drug Deliv 2011;8:282–9.

[7] Gori A, Longhi R, Peri C, Colombo G. Peptides for immunological purposes: design, strategies and applications. Amino Acids 2013;45:257268. doi:10.1007/s00726-013-1526-9.

[8] Purcell AW, McCluskey J, Rossjohn J. More than one reason to rethink the use of peptides in vaccine design. Nat Rev Drug Discov 2007;6:404–14.

doi:10.1038/nrd2224.

[9] Rosendahl Huber S, van Beek J, de Jonge J, Luytjes W, van Baarle D. T cell responses to viral infections - opportunities for Peptide vaccination. Front Immunol 2014;5:171. doi:10.3389/fimmu.2014.00171.

[10] Girard MP, Osmanov SK, Kieny MP. A review of vaccine research and development: the human immunodeficiency virus (HIV). Vaccine 2006;24:4062–81. doi:10.1016/j.vaccine.2006.02.031.

[11] Mohit E, Hashemi A, Allahyari M. Breast cancer immunotherapy: monoclonal antibodies and peptide-based vaccines. Expert Rev Clin Immunol 2014;10:927–61. doi:10.1586/1744666X.2014.916211.

[12] Ott PA, Fritsch EF, Wu CJ, Dranoff G. Vaccines and melanoma. Hematol Oncol Clin North Am 2014;28:559–69. doi:10.1016/j.hoc.2014.02.008.

[13] Fox CB, Kramer RM, Barnes V L, Dowling QM, Vedvick TS. Working together: interactions between vaccine antigens and adjuvants. Ther Adv Vaccines 2013;1:7–20. doi:10.1177/2051013613480144.

[14] Deering RP, Kommareddy S, Ulmer JB, Brito LA, Geall AJ. Nucleic acid vaccines: prospects for non-viral delivery of mRNA vaccines. Expert Opin Drug Deliv 2014;11:885–99. doi:10.1517/17425247.2014.901308.

[15] Xiang SD, Selomulya C, Ho J, Apostolopoulos V, Plebanski M. Delivery of DNA vaccines: an overview on the use of biodegradable polymeric and magnetic nanoparticles. WIREs Nanomedicine and Nanobiotechnology 2010;2:205–18. doi:10.1002/wnan.88.

[16] Alpar HO, Papanicolaou I, Bramwell VW. Strategies for DNA vaccine delivery. Expert Opin Drug Deliv 2005;2:829–42. doi:10.1517/17425247.2.5.829.

[17] Kramps T, Probst J. Messenger RNA-based vaccines: progress, challenges, applications. Wiley Interdiscip Rev RNA 2013;4:737–49. doi:10.1002/wrna.1189.

[18] Mazid R, Tan MX, Danquah MK. Molecular delivery of plasmids for genetic vaccination. Curr Pharm Biotechnol 2013;14:615–22.

[19] Liu M. DNA vaccines: a review. J Intern Med 2003;253:402410. doi:10.1046/j.1365-2796.2003.01140.x.

[20] Schalk JA, Mooi FR, Berbers GA, van Aerts LA, Ovelgönne H, Kimman T. Preclinical and clinical safety studies on DNA vaccines. Hum Vaccin 2006;2:45–

53.

[21] Azmi F, Fuaad AAHA, Skwarczynski M, Toth I. Recent progress in adjuvant discovery for peptide-based subunit vaccines. Hum Vaccin Immunother 2014;10. doi:10.4161/hv.27332.

[22] Lindblad EB. Aluminium adjuvants—in retrospect and prospect. Vaccine 2004;22:3658–68. doi:10.1016/j.vaccine.2004.03.032.

[23] Bomford R. Aluminium Salts: Perspectives in their Use as Adjuvants. In: Gregoriadis G, Allison AC, Poste G, editors. Immunol. Adjuv. Vaccines, Boston, MA: Springer US; 1989, p. 35–42.

[24] Demento SL, Siefert AL, Bandyopadhyay A, Sharp FA, Fahmy TM. Pathogen-associated molecular patterns on biomaterials: a paradigm for engineering new vaccines. Trends Biotechnol 2011;29:294–306.

[25] Steinhagen F, Kinjo T, Bode C, Klinman DM. TLR-based immune adjuvants. Vaccine 2011;29:3341–55.

[26] Hill DR, Ford L, Lalloo DG. Oral cholera vaccines : use in clinical practice. Lancet Infect Dis 2006;6:361–73.

[27] Frech SA, Dupont HL, Bourgeois AL, McKenzie R, Belkind-Gerson J, Figueroa JF, et al. Use of a patch containing heat-labile toxin from Escherichia coli against travellers' diarrhoea: a phase II, randomised, double-blind, placebo-controlled field trial. Lancet 2008;371:2019–25. doi:10.1016/S0140-6736(08)60839-9.

[28] Glenn GM, Thomas DN, Poffenberger KL, Flyer DC, Ellingsworth LR, Andersen BH, et al. Safety and immunogenicity of an influenza vaccine A/H5N1 (A/Vietnam/1194/2004) when coadministered with a heat-labile enterotoxin (LT) adjuvant patch. Vaccine 2009;27S:G60-66. doi:10.1016/j.vaccine.2009.10.031.

[29] Behrens RH, Cramer JP, Jelinek T, Shaw H, von Sonnenburg F, Wilbraham D, et al. Efficacy and safety of a patch vaccine containing heat-labile toxin from Escherichia coli against travellers' diarrhoea: A phase 3, randomised, double-blind, placebo-controlled field trial in travellers from Europe to Mexico and Guatemala. Lancet Infect Dis 2014;14:197–204. doi:10.1016/S1473-3099(13)70297-4.

[30] Mallapragada SK, Narasimhan B. Immunomodulatory biomaterials. Int J Pharm 2008;364:265–71. doi:10.1016/j.ijpharm.2008.06.030.

[31] Reed SG, Bertholet S, Coler RN, Friede M. New horizons in adjuvants for vaccine development. Trends Immunol 2009;30:23–32. doi:10.1016/j.it.2008.09.006.

[32] Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK, Wannemuehler MJ, Narasimhan B. Vaccine adjuvants: current challenges and future approaches. J Pharm Sci 2009;98:1278–316. doi:10.1002/jps.21523.

[33] The RTS.S Clinical Trials Partnership. Efficacy and Safety of the RTS,S/AS01 Malaria Vaccine during 18 Months after Vaccination: A Phase 3 Randomized, Controlled Trial in Children and Young Infants at 11 African Sites. PLoS Med 2014;11:e1001685. doi:10.1371/journal.pmed.1001685.

[34] Kester KE, Gray Heppner D, Moris P, Ofori-Anyinam O, Krzych U, Tornieporth N, et al. Sequential Phase 1 and Phase 2 randomized, controlled trials of the safety, immunogenicity and efficacy of combined pre-erythrocytic vaccine antigens RTS,S and TRAP formulated with AS02 Adjuvant System in healthy, malaria naïve adults. Vaccine 2014;32:6683–91. doi:10.1016/j.vaccine.2014.06.033.

[35] Barr I, Sjölander A, Cox JC. ISCOMs and other saponin based adjuvants. Adv Drug Deliv Rev 1998;32:247–71. doi:10.1016/s0169-409x(98)00013-1.

[36] Sun H-X, Xie Y, Ye Y-P. ISCOMs and ISCOMATRIX. Vaccine 2009;27:4388–401. doi:10.1016/j.vaccine.2009.05.032.

[37] Hughes HP. Cytokine adjuvants: lessons from the past - guidelines for the future? Vet Immunol Immunopathol 1998;63:131–8.

[38] Lynch JM, Briles DE, Metzger DW. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. Infect Immun 2003;71:4780–8. doi:10.1128/iai.71.8.4780-4788.2003.

[39] Hedlund J, Langer B, Konradsen H, Örtqvist Å. Negligible adjuvant effect for antibody responses and frequent adverse events associated with IL-12 treatment in humans vaccinated with pneumococcal polysaccharide. Vaccine 2002;20:164–9.

[40] Fox CB. Squalene Emulsions for Parenteral Vaccine and Drug Delivery. Molecules 2009;14:3286–312.

[41] Perrin-Cocon L, Agaugué S, Coutant F, Saint-Mézard P, Guironnet-Paquet A, Nicolas J-F, et al. Lysophosphatidylcholine is a natural adjuvant that initiates

cellular immune responses. Vaccine 2006;24:1254–63. doi:http://dx.doi.org/10.1016/j.vaccine.2005.09.036.

[42] Mora-Solano C, Collier JH. Engaging adaptive immunity with biomaterials. J Mater Chem B 2014;2:2409. doi:10.1039/c3tb21549k.

[43] Rappuoli R, Mandl CW, Black S, De Gregorio E. Vaccines for the twenty-first century society. Nat Rev Immunol 2011;11:865–72. doi:10.1038/nri3085.

[44] Sahdev P, Ochyl LJ, Moon JJ. Biomaterials for nanoparticle vaccine delivery systems. Pharm Res 2014;31:2563–82. doi:10.1007/s11095-014-1419-y.

[45] González-Aramundiz JV, Cordeiro AS, Csaba N, de la Fuente M, Alonso MJ. Nanovaccines: nanocarriers for antigen delivery. Biol Aujourdhui 2012;206:249–61. doi:10.1051/jbio/2012027.

[46] Correia-Pinto JF, Csaba N, Alonso MJ. Vaccine delivery carriers: Insights and future perspectives. Int J Pharm 2013;440:27–38. doi:10.1016/j.ijpharm.2012.04.047.

[47] Aguilar J, Rodríguez E. Vaccine adjuvants revisited. Vaccine 2007;25:3752–62. doi:10.1016/j.vaccine.2007.01.111.

[48] Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol 2010;10:787–96. doi:10.1038/nri2868.

[49] De Temmerman M-L, Rejman J, Demeester J, Irvine DJ, Gander B, De Smedt SC. Particulate vaccines: on the quest for optimal delivery and immune response. Drug Discov Today 2011;16:569–82. doi:10.1016/j.drudis.2011.04.006.

[50] Duncan R, Gaspar R. Nanomedicine(s) under the microscope. Mol Pharm 2011;8:2101–41. doi:10.1021/mp200394t.

[51] Etheridge ML, Campbell SA, Erdman AG, Haynes CL, Wolf SM, McCullough J. The big picture on nanomedicine: the state of investigational and approved nanomedicine products. Nanomedicine Nanotechnology, Biol Med 2013;9:1–14. doi:http://dx.doi.org/10.1016/j.nano.2012.05.013.

[52] Reddy ST, Swartz MA, Hubbell JA. Targeting dendritic cells with biomaterials: developing the next generation of vaccines. Trends Immunol 2006;27:573–9. doi:10.1016/j.it.2006.10.005.

[53] Zolnik BS, González-Fernández Á, Sadrieh N, Dobrovolskaia MA. Nanoparticles and the immune system. Endocrinology 2010;151:458–65. doi:10.1210/en.2009-1082.

[54] Storni T, Kündig TM, Senti G, Johansen P. Immunity in response to particulate antigen-delivery systems. Adv Drug Deliv Rev 2005;57:333–55. doi:10.1016/j.addr.2004.09.008.

[55] Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat Biotechnol 2007;25:1159–64. doi:10.1038/nbt1332.

[56] Joshi VB, Geary SM, Salem AK. Biodegradable particles as vaccine delivery systems: size matters. AAPS J 2013;15:85–94. doi:10.1208/s12248-012-9418-6.

[57] Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol 2004;173:3148–54. doi:10.4049/jimmunol.173.5.3148.

[58] Vila A, Sánchez A, Evora C, Soriano I, McCallion O, Alonso MJ. PLA-PEG particles as nasal protein carriers: the influence of the particle size. Int J Pharm 2005;292:43–52. doi:10.1016/j.ijpharm.2004.09.002.

[59] Kanchan V, Panda AK. Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response. Biomaterials 2007;28:53445357. doi:10.1016/j.biomaterials.2007.08.015.

[60] Gutierro I, Hernández RM, Igartua M, Gascón AR, Pedraz JL. Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine 2002;21:67–77.

[61] Oyewumi MO, Kumar A, Cui Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev Vaccines 2010;9:1095–107. doi:10.1586/erv.10.89.

[62] Reddy ST, Rehor A, Schmoekel HG, Hubbell JA, Swartz MA. In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. J Control Release 2006;112:26–34. doi:10.1016/j.jconrel.2006.01.006.

[63] des Rieux A, Fiévez V, Garinot M, Schneider Y-J, Préat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. J Control Release 2006;116:1–27. doi:10.1016/j.jconrel.2006.08.013.

[64] Csaba N, García-Fuentes M, Alonso MJ. Nanoparticles for nasal vaccination. Adv Drug Deliv Rev 2009;61:140–57. doi:10.1016/j.addr.2008.09.005.

[65] Jani PU, Halbert GW, Langridge J, Florence AT. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J Pharm Pharmacol 1990;42:821–6.

[66] Desai MP, Labhasetwar V, Amidon GL, Levy RJ. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharm Res 1996;13:1838–45.

[67] Grabovac V, Guggi D, Bernkop-Schnürch A. Comparison of the mucoadhesive properties of various polymers. Adv Drug Deliv Rev 2005;57:1713–23. doi:10.1016/j.addr.2005.07.006.

[68] Tobío M, Gref R, Sánchez A, Langer RS, Alonso MJ. Stealth PLA-PEG nanoparticles as protein carriers for nasal administration. Pharm Res 1998;15:270–5. doi:10.1023/A:1011922819926.

[69] Chadwick S, Kriegel C, Amiji MM. Nanotechnology solutions for mucosal immunization. Adv Drug Deliv Rev 2010;62:394–407. doi:10.1016/j.addr.2009.11.012.

[70] Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev 2006;58:68–89. doi:10.1016/j.addr.2005.12.003.

[71] Peine KJ, Bachelder EM, Vangundy Z, Papenfuss T, Brackman DJ, Gallovic MD, et al. Efficient delivery of the toll-like receptor agonists polyinosinic:polycytidylic acid and CpG to macrophages by acetalated dextran microparticles. Mol Pharm 2013;10:2849–57. doi:10.1021/mp300643d.

[72] Vicente S, Peleteiro M, Díaz-Freitas B, Sánchez A, González-Fernández Á, Alonso MJ, et al. Co-delivery of viral proteins and a tlr7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy. J Control Release 2013;172:773–81. doi:http://dx.doi.org/10.1016/j.jconrel.2013.09.012.

[73] Roman F, Vaman T, Gerlach B, Markendorf A, Gillard P, Devaster J-MM. 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;28:1740–5. doi:10.1016/j.vaccine.2009.12.014.

[74] Esposito S, Tagliaferri L, Daleno C, Valzano A, Picciolli I, Tel F, et al. Pandemic

influenza A/H1N1 vaccine administered sequentially or simultaneously with seasonal influenza vaccine to HIV-infected children and adolescents. Vaccine 2011;29:1677–82. doi:10.1016/j.vaccine.2010.12.047.

[75] Usonis V, Bakasénas V, Valentelis R, Katiliene G, Vidzeniene D, Herzog C. Antibody titres after primary and booster vaccination of infants and young children with a virosomal hepatitis A vaccine (Epaxal®). Vaccine 2003;21:45884592. doi:10.1016/s0264-410x(03)00509-7.

[76] Leroux-Roels I, Koutsoukos M, Clement F, Steyaert S, Janssens M, Bourguignon P, 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;28:7016–24. doi:10.1016/j.vaccine.2010.08.035.

[77] Aide P, Dobaño C, Sacarlal J, Aponte JJ, Mandomando I, Guinovart C, et al. Four year immunogenicity of the RTS,S/AS02(A) malaria vaccine in Mozambican children during a phase IIb trial. Vaccine 2011;29:6059–67. doi:10.1016/j.vaccine.2011.03.041.

[78] Kreuter J, Speiser PP. New adjuvants on a polymethylmethacrylate base. Infect Immun 1976;13:204–10.

[79] Rice-Ficht AC, Arenas-Gamboa AM, Kahl-McDonagh MM, Ficht TA. Polymeric particles in vaccine delivery. Curr Opin Microbiol 2010;13:106–12. doi:10.1016/j.mib.2009.12.001.

[80] Vicente S, Prego C, Csaba N, Alonso MJ. From single-dose vaccine delivery systems to nanovaccines. J Drug Deliv Sci Technol 2010;20:267–76. doi:10.1016/S1773-2247(10)50044-3.

[81] Blanco M, Alonso MJ. Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres. Eur J Pharm Biopharm 1997;43:287–94.

[82] Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. J Control Release 2012;161:505–22. doi:10.1016/j.jconrel.2012.01.043.

[83] www.clinicaltrials.gov. Last visited in August 2014. n.d.

[84] Alonso MJ, Gupta RK, Min C, Siber GR, Langer RS. Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. Vaccine 1994;12:299–306. doi:10.1016/0264-410x(94)90092-2.

[85] Tobío M, Alonso MJ. Study of the inactivation process of the tetanus toxoid in contact with poly (lactic/glycolic acid) degrading microspheres. STP Pharma Sci 1998;8:303–10.

[86] Schwendeman SP, Costantino H, Gupta RK, Tobío M, Chang A, Alonso MJ, et al. Strategies for stabilising tetanus toxoid towards the development of a single-dose tetanus vaccine. Dev Biol Stand 1996;87:293–306.

[87] Sánchez A, Villamayor B, Guo Y, McIver J, Alonso MJ. Formulation strategies for the stabilization of tetanus toxoid in poly(lactide-co-glycolide) microspheres. Int J Pharm 1999;185:255–66.

[88] Calvo P, Remuñán-López C, Vila-Jato JL, Alonso MJ. Chitosan and Chitosan/Ethylene Oxide-Propylene Oxide Block Copolymer Nanoparticles as Novel Carriers for Proteins and Vaccines. Pharm Res 1997;14:1431–6. doi:10.1023/a:1012128907225.

[89] Petrovsky N, Cooper PD. Carbohydrate-based immune adjuvants. Expert Rev Vaccines 2011;10:523–37. doi:10.1586/erv.11.30.Carbohydrate-based.

[90] Dykstra T, Utermoehlen O, Haas A. Defined particle ligands trigger specific defense mechanisms of macrophages. Innate Immun 2011;17:388–402. doi:10.1177/1753425910374889.

[91] Calvo P, Remuñán-López C, Vila-Jato JL, Alonso MJ. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. J Appl Polym Sci 1997;63:125–32. doi:10.1002/(SICI)1097-4628(19970103)63:1<125::AID-APP13>3.0.CO;2-4.

[92] Kean T, Roth S, Thanou M. Trimethylated chitosans as non-viral gene delivery vectors: cytotoxicity and transfection efficiency. J Control Release 2005;103:643–53. doi:10.1016/j.jconrel.2005.01.001.

[93] Calvo P, Remuñán-López C, Vila-Jato JL, Alonso MJ. Development of positively charged colloidal drug carriers: chitosan-coated polyester nanocapsules and submicron-emulsions. Colloid Polym Sci 1997;275:46–53.

[94] Vauthier C, Bouchemal K. Processing and scale-up of polymeric nanoparticles. In: Prokop A, editor. Intracell. Deliv. Fundam. Appl., vol. 5, Springer Science+Business Media; 2011, p. 433–56. doi:10.1007/978-94-007-1248-5.

[95] Amorij J-P, Kersten GF, Saluja V, Tonnis WF, Hinrichs WL, Slütter B, et al. Towards tailored vaccine delivery: Needs, challenges and perspectives. J

Control Release 2012;161:363–7. doi:10.1016/j.jconrel.2011.12.039.

[96] Chen D, Zehrung D. Desirable attributes of vaccines for deployment in low-resource settings. J Pharm Sci 2013;102:29–33. doi:10.1002/jps.23352.

[97] García-Fuentes M, Alonso MJ. Chitosan-based drug nanocarriers: Where do we stand? J Control Release 2012;161:496–504. doi:http://dx.doi.org/10.1016/j.jconrel.2012.03.017.

[98] Gomes CP, Ferreira Lopes CD, Duarte Moreno PM, Varela-Moreira A, Alonso MJ, Pêgo AP. Translating chitosan to clinical delivery of nucleic acid-based drugs. MRS Bull 2014;39:60–70. doi:10.1557/mrs.2013.314.

[99] Sogias IA, Williams AC, Khutoryanskiy V V. Why is chitosan mucoadhesive? Biomacromolecules 2008;9:1837–42. doi:10.1021/bm800276d.

[100] Islam MA, Firdous J, Choi Y-J, Yun C-H, Cho C-S. Design and application of chitosan microspheres as oral and nasal vaccine carriers: an updated review. Int J Nanomedicine 2012;7:6077–93. doi:10.2147/ijn.s38330.

[101] Peluso G, Petillo O, Ranieri M, Santin M, Ambrosio L, Calabró D, et al. Chitosan-mediated stimulation of macrophage function. Biomaterials 1994;15:1215–20.

[102] Porporatto C, Bianco ID, Correa SG. Local and systemic activity of the polysaccharide chitosan at lymphoid tissues after oral administration. J Leukoc Biol 2005;78:62–9. doi:10.1189/jlb.0904541.

[103] Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine 2007;25:2085–94. doi:10.1016/j.vaccine.2006.11.034.

[104] Boateng JS, Matthews KH, Stevens HN, Eccleston GM. Wound healing dressings and drug delivery systems: a review. J Pharm Sci 2008;97:2892–923. doi:10.1002/jps.21210.

[105] Atmar RL, Bernstein DI, Harro CD, Al-Ibrahim MS, Chen WH, Ferreira J, et al. Norovirus Vaccine against Experimental Human Norwalk Virus Illness. N Engl J Med 2011;365:2178–87. doi:10.1056/NEJMoa1101245.

[106] Huo Z, Sinha R, McNeela EA, Borrow R, Giemza R, Cosgrove C, et al. Induction of protective serum meningococcal bactericidal and diphtheria-neutralizing antibodies and mucosal immunoglobulin A in volunteers by nasal insufflations of the Neisseria meningitidis serogroup C polysaccharide-CRM197 conjugate

vaccine mixed with. Infect Immun 2005;73:8256–65. doi:10.1128/IAI.73.12.8256-8265.2005.

[107] Neimert-Andersson T, Binnmyr J, Enoksson M, Langebäck J, Zettergren L, Hällgren A-C, et al. Evaluation of safety and efficacy as an adjuvant for the chitosan-based vaccine delivery vehicle ViscoGel in a single-blind randomised Phase I/IIa clinical trial. Vaccine 2014;32:5967–74. doi:10.1016/j.vaccine.2014.08.057.

[108] Prego C, Torres D, Alonso MJ. The potential of chitosan for the oral administration of peptides. Expert Opin Drug Deliv 2005;2:843–54. doi:doi:10.1517/17425247.2.5.843.

[109] Arca HC, Günbeyaz M, Senel S. Chitosan-based systems for the delivery of vaccine antigens. Expert Rev Vaccines 2009;8:937–53. doi:10.1586/erv.09.47.

[110] Prego C, Paolicelli P, Díaz B, Vicente S, Sánchez A, González-Fernández Á, et al. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. Vaccine 2010;28:2607–14. doi:10.1016/j.vaccine.2010.01.011.

[111] Borges OM, Silva M, de Sousa A, Borchard G, Junginger HE, Cordeiro-da-Silva A. Alginate coated chitosan nanoparticles are an effective subcutaneous adjuvant for hepatitis B surface antigen. Int Immunopharmacol 2008;8:1773–80. doi:10.1016/j.intimp.2008.08.013.

[112] Lugade AA, Bharali DJ, Pradhan V, Elkin G, Mousa SA, Thanavala Y. Single low-dose un-adjuvanted HBsAg nanoparticle vaccine elicits robust, durable immunity. Nanomedicine 2013;9:923–34. doi:10.1016/j.nano.2013.03.008.

[113] Prego C, Torres D, Alonso MJ. Chitosan nanocapsules: a new carrier for nasal peptide delivery. J Drug Deliv Sci Technol 2006;16:331–7.

[114] Vicente S, Díaz-Freitas B, Peleteiro M, Sanchez A, Pascual DW, González-Fernández Á, et al. A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach. PLoS One 2013;8:e62500. doi:10.1371/journal.pone.0062500.

[115] Vicente S, Goins BA, Sánchez A, Alonso MJ, Phillips WT. Biodistribution and lymph node retention of polysaccharide-based immunostimulating nanocapsules. Vaccine 2014;32:1685–92. doi:10.1016/j.vaccine.2014.01.059.

[116] Vila A, Sánchez A, Janes KA, Behrens I, Kissel T, Vila-Jato JL, et al. Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine

delivery in mice. Eur J Pharm Biopharm 2004;57:123–31. doi:10.1016/j.ejpb.2003.09.006.

[117] Sawaengsak C, Mori Y, Yamanishi K, Mitrevej A, Sinchaipanid N. Chitosan nanoparticle encapsulated hemagglutinin-split influenza virus mucosal vaccine. AAPS PharmSciTech 2014;15:317–25. doi:10.1208/s12249-013-0058-7.

[118] Figueiredo L, Cadete A, Gonçalves LM, Corvo M, Almeida AJ. Intranasal immunisation of mice against Streptococcus equi using positively charged nanoparticulate carrier systems. Vaccine 2012;30:6551–8. doi:10.1016/j.vaccine.2012.08.050.

[119] Slütter B, Plapied L, Fiévez V, Alonso-Sande M, des Rieux A, Schneider Y-J, et al. Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination. J Control Release 2009;138:113–21. doi:10.1016/j.jconrel.2009.05.011.

[120] Slütter B, Bal SM, Keijzer C, Mallants R, Hagenaars N, Que I, 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;28:6282–91. doi:10.1016/j.vaccine.2010.06.121.

[121] Slütter B, Soema PC, Ding Z, Verheul RJ, Hennink WE, Jiskoot W. Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen. J Control Release 2010;143:207–14. doi:10.1016/j.jconrel.2010.01.007.

[122] Slütter B, Bal SM, Que I, Kaijzel E, Löwik C, Bouwstra JA, et al. Antigen−Adjuvant Nanoconjugates for Nasal Vaccination: An Improvement over the Use of Nanoparticles? Mol Pharm 2010;7:2207–15. doi:10.1016/j.jconrel.2010.06.009.(15).

[123] Sayın B, Somavarapu S, Li X, Thanou M, Sesardic D, Alpar HO, et al. Mono-N-carboxymethyl chitosan (MCC) and N-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery. Int J Pharm 2008;363:139–48. doi:10.1016/j.ijpharm.2008.06.029.

[124] Verheul RJ, Slütter B, Bal SM, Bouwstra JA, Jiskoot W, Hennink WE. Covalently stabilized trimethyl chitosan-hyaluronic acid nanoparticles for nasal and intradermal vaccination. J Control Release 2011;156:46–52. doi:10.1016/j.jconrel.2011.07.014.

[125] Bal SM, Slütter B, Verheul RJ, Bouwstra JA, Jiskoot W. Adjuvanted, antigen loaded N-trimethyl chitosan nanoparticles for nasal and intradermal vaccination: Adjuvant- and site-dependent immunogenicity in mice. Eur J Pharm Sci 2012;45:475–81. doi:10.1016/j.ejps.2011.10.003.

[126] Cambridge CD, Singh SR, Waffo AB, Fairley SJ, Dennis VA. Formulation, characterization, and expression of a recombinant MOMP Chlamydia trachomatis DNA vaccine encapsulated in chitosan nanoparticles. Int J Nanomedicine 2013;8:1759–71. doi:10.2147/ijn.s42723.

[127] Feng G, Jiang Q, Xia M, Lu Y, Qiu W, Zhao D, et al. Enhanced immune response and protective effects of nano-chitosan-based DNA vaccine encoding T cell epitopes of Esat-6 and FL against Mycobacterium tuberculosis infection. PLoS One 2013;8. doi:10.1371/journal.pone.0061135.

[128] Ai W, Yue Y, Xiong S, Xu W. Enhanced protection against pulmonary mycobacterial challenge by chitosan-formulated polyepitope gene vaccine is associated with increased pulmonary secretory IgA and gamma-interferon + T cell responses. Microbiol Immunol 2013;57:224–35. doi:10.1111/1348-0421.12027.

[129] Meerak J, Wanichwecharungruang SP, Palaga T. Enhancement of immune response to a DNA vaccine against Mycobacterium tuberculosis Ag85B by incorporation of an autophagy inducing system. Vaccine 2013;31:784–90. doi:10.1016/j.vaccine.2012.11.075.

[130] Khatri K, Goyal AK, Gupta PN, Mishra N, Vyas SP. Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. Int J Pharm 2008;354:235–41. doi:10.1016/j.ijpharm.2007.11.027.

[131] Raghuwanshi D, Mishra V, Das D, Kaur K, Suresh MR. Dendritic cell targeted chitosan nanoparticles for nasal DNA immunization against SARS CoV nucleocapsid protein. Mol Pharm 2012;9:946–56. doi:10.1021/mp200553x.

[132] Heuking S, Adam-Malpel S, Sublet E, Iannitelli A, di Stefano A, Borchard G. Stimulation of human macrophages (THP-1) using Toll-like receptor-2 (TLR-2) agonist decorated nanocarriers. J Drug Target 2009;17:662–70. doi:10.1080/10611860903106034.

[133] Heuking S, Borchard G. Toll-like receptor-7 agonist decoration enhances the adjuvanticity of chitosan-DNA nanoparticles. J Pharm Sci 2012;101:1166–77.

doi:10.1002/jps.23017.

[134] Li G, Liu Z, Liao B, Zhong N. Induction of Th1-type immune response by chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen Der p 2 for oral vaccination in mice. Cell Mol Immunol 2009;6:45–50. doi:10.1038/cmi.2009.6.

[135] Oliveira CR, Rezende CM, Silva MR, Borges OM, Pêgo AP, Goes AM. Oral vaccination based on DNA-chitosan nanoparticles against Schistosoma mansoni infection. ScientificWorldJournal 2012;2012:938457. doi:10.1100/2012/938457.

[136] Chen L, Zhu J, Li Y, Lu J, Gao L, Xu H, et al. Enhanced nasal mucosal delivery and immunogenicity of anti-caries DNA vaccine through incorporation of anionic liposomes in chitosan/DNA complexes. PLoS One 2013;8. doi:10.1371/journal.pone.0071953.

[137] Khatri K, Goyal AK, Gupta PN, Mishra N, Mehta A, Vyas SP. Surface modified liposomes for nasal delivery of DNA vaccine. Vaccine 2008;26:2225–33. doi:10.1016/j.vaccine.2008.02.058.

[138] Raemdonck K, Martens TF, Braeckmans K, Demeester J, De Smedt SC. Polysaccharide-based nucleic acid nanoformulations. Adv Drug Deliv Rev 2013;65:1123–47. doi:10.1016/j.addr.2013.05.002.

[139] Baldwin AD, Kiick KL. Polysaccharide-modified synthetic polymeric biomaterials. Biopolymers 2010;94:128–40. doi:10.1002/bip.21334.

[140] de Belder AN. Medical applications of dextran and its derivatives. In: Dumitriu S, editor. Polysaccharides Med. Appl., New York, USA: CRC Press; 1996, p. 505–24.

[141] www.fda.gov. Last visited in August 2014 n.d.

[142] McCarthy R, Arnold L, Babcock G. Dextran sulphate: an adjuvant for cell-mediated immune responses. Immunology 1977;32:963.

[143] Kerlin R, Watson D. Effect of dextran sulphate on IgG subclass of antibody in efferent popliteal lymph of sheep. Immunol Cell Biol 1987;65 Pt 5:411–7. doi:10.1038/icb.1987.47.

[144] Laroui H, Ingersoll SA, Liu HC, Baker MT, Ayyadurai S, Charania MA, et al. Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon. PLoS One

2012;7. doi:10.1371/journal.pone.0032084.

[145] Finnerty M, Enright W, Morrison C, Roche J. Immunization of bull calves with a GnRH analogue–human serum albumin conjugate: effect of conjugate dose, type of adjuvant and booster interval on immune, endocrine, testicular and growth responses. J Reprod Fertil 1994;101:333–43.

[146] Cox JC, Coulter AR. Adjuvants—a classification and review of their modes of action. Vaccine 1997;15:248–56. doi:10.1016/s0264-410x(96)00183-1.

[147] Shen L, Higuchi T, Tubbe I, Voltz N, Krummen M, Pektor S, et al. A trifunctional dextran-based nanovaccine targets and activates murine dendritic cells, and induces potent cellular and humoral immune responses in vivo. PLoS One 2013;8:e80904. doi:10.1371/journal.pone.0080904.

[148] Sharma S, Mukkur TK, Benson HA, Chen Y. Enhanced immune response against pertussis toxoid by IgA-loaded chitosan-dextran sulfate nanoparticles. J Pharm Sci 2012;101:233–44. doi:10.1002/jps.22763.

[149] Drogoz A, Munier S, Verrier B, David L, Domard A, Delair T. Towards biocompatible vaccine delivery systems: interactions of colloidal PECs based on polysaccharides with HIV-1 p24 antigen. Biomacromolecules 2008;9:583–91. doi:10.1021/bm701154h.

[150] Schully KL, Sharma S, Peine KJ, Pesce JT, Elberson MA, Fonseca ME, et al. Rapid vaccination using an acetalated dextran microparticulate subunit vaccine confers protection against triplicate challenge by bacillus anthracis. Pharm Res 2013;30:1349–61. doi:10.1007/s11095-013-0975-x.

[151] Sharma S, Benson HA, Mukkur TK, Rigby P, Chen Y. Preliminary studies on the development of IgA-loaded chitosan-dextran sulphate nanoparticles as a potential nasal delivery system for protein antigens. J Microencapsul 2013;30:283–94. doi:10.3109/02652048.2012.726279.

[152] Apostolopoulos V, Thalhammer T, Tzakos AG, Stojanovska L. Targeting antigens to dendritic cell receptors for vaccine development. J Drug Deliv 2013;2013:869718. doi:10.1155/2013/869718.

[153] Petersen SV, Thiel S, Jensenius JC. The mannan-binding lectin pathway of complement activation: biology and disease association. Mol Immunol 2001;38:133–49. doi:10.1016/s0161-5890(01)00038-4.

[154] Joshi MD, Unger WJ, Storm G, van Kooyk Y, Mastrobattista E. Targeting tumor

antigens to dendritic cells using particulate carriers. J Control Release 2012;161:25–37. doi:10.1016/j.jconrel.2012.05.010.

[155] Al-Barwani F, Young SL, Baird MA, Larsen DS, Ward VK. Mannosylation of virus-like particles enhances internalization by antigen presenting cells. PLoS One 2014;9:e104523. doi:10.1371/journal.pone.0104523.

[156] Ghotbi Z, Haddadi A, Hamdy S, Hung RW, Samuel J, Lavasanifar A. Active targeting of dendritic cells with mannan-decorated PLGA nanoparticles. J Drug Target 2011;19:281–92. doi:10.3109/1061186X.2010.499463.

[157] Thomann-Harwood L, Kaeuper P, Rossi N, Milona P, Herrmann B, McCullough KC. Nanogel vaccines targeting dendritic cells: contributions of the surface decoration and vaccine cargo on cell targeting and activation. J Control Release 2013;166:95–105. doi:10.1016/j.jconrel.2012.11.015.

[158] Gou M, Dai M, Li X, Yang L, Huang M, Wang Y, et al. Preparation of mannan modified anionic PCL-PEG-PCL nanoparticles at one-step for bFGF antigen delivery to improve humoral immunity. Colloids Surfaces B Biointerfaces 2008;64:135–9. doi:10.1016/j.colsurfb.2007.12.014.

[159] Hamdy S, Haddadi A, Shayeganpour A, Samuel J. Activation of antigen-specific T cell-responses by mannan-decorated PLGA nanoparticles. Pharm Res 2011;28:2288–301. doi:10.1007/s11095-011-0459-9.

[160] Durán-Lobato M, Carrillo-Conde B, Khairandish Y, Peppas NA. Surface-Modified P (HEMA-co-MAA) Nanogel Carriers for Oral Vaccine Delivery: Design, Characterization, and In Vitro Targeting Evaluation. Biomacromolecules 2014;15:2725–34. doi:10.1021/bm500588x.

[161] Alonso-Sande M, Teijeiro-Osorio D, Remuñán-López C, Alonso MJ. Glucomannan, a promising polysaccharide for biopharmaceutical purposes. Eur J Pharm Biopharm 2009;72:453–62. doi:10.1016/j.ejpb.2008.02.005.

[162] Alonso-Sande M, Cuña M, Remuñán-López C, Teijeiro-Osorio D, Alonso-Lebrero JL, Alonso MJ. Formation of New Glucomannan−Chitosan Nanoparticles and Study of Their Ability To Associate and Deliver Proteins. Macromolecules 2006;39:4152–8. doi:10.1021/ma060230j.

[163] Harde H, Agrawal AK, Jain S. Development of stabilized glucomannosylated chitosan nanoparticles using tandem crosslinking method for oral vaccine delivery. Nanomedicine 2014. doi:10.2217/nnm.13.225.

[164] Jain S, Harde H, Indulkar A, Agrawal AK. Improved stability and immunological potential of tetanus toxoid containing surface engineered bilosomes following oral administration. Nanomedicine Nanotechnology, Biol Med 2014;10:431–40. doi:10.1016/j.nano.2013.08.012.

[165] Jain S, Indulkar A, Harde H, Agrawal AK. Oral Mucosal Immunization Using Glucomannosylated Bilosomes. J Biomed Nanotechnol 2014;10:932947. doi:10.1166/jbn.2014.1800.

[166] Ruan G-X, Chen Y-Z, Yao X-L, Du A, Tang G-P, Shen Y-Q, et al. Macrophage mannose receptor-specific gene delivery vehicle for macrophage engineering. Acta Biomater 2014;10:1847–55. doi:10.1016/j.actbio.2014.01.012.

[167] Tang CK, Sheng K-C, Pouniotis D, Esparon S, Son H-Y, Kim C-W, et al. Oxidized and reduced mannan mediated MUC1 DNA immunization induce effective anti-tumor responses. Vaccine 2008;26:3827–34. doi:10.1016/j.vaccine.2008.05.008.

[168] Tang CK, Sheng K-C, Esparon S, Proudfoot O, Apostolopoulos V, Pietersz G a. Molecular basis of improved immunogenicity in DNA vaccination mediated by a mannan based carrier. Biomaterials 2009;30:1389–400. doi:10.1016/j.biomaterials.2008.11.010.

[169] Lu Y, Kawakami S, Yamashita F, Hashida M. Development of an antigen-presenting cell-targeted DNA vaccine against melanoma by mannosylated liposomes. Biomaterials 2007;28:3255–62. doi:10.1016/j.biomaterials.2007.03.028.

[170] Goodridge HS, Wolf AJ, Underhill DM. β-glucan recognition by the innate immune system. Immunol Rev 2009;230:38–50. doi:10.1111/j.1600-065X.2009.00793.x.

[171] Soltanian S, Stuyven E, Cox E, Sorgeloos P, Bossier P. Beta-glucans as immunostimulant in vertebrates and invertebrates. Crit Rev Microbiol 2009;35:109–38. doi:doi:10.1080/10408410902753746.

[172] Adams EL, Rice PJ, Graves B, Ensley HE, Yu H, Brown GD, et al. Differential High-Affinity Interaction of Dectin-1 with Natural or Synthetic Glucans Is Dependent upon Primary Structure and Is Influenced by Polymer Chain Length and Side-Chain Branching. J Pharmacol Exp Ther 2008;325:115–23. doi:10.1124/jpet.107.133124.

[173] Barsanti L, Passarelli V, Evangelista V, Frassanito AM, Gualtieri P. Chemistry, physico-chemistry and applications linked to biological activities of [small beta]-glucans. Nat Prod Rep 2011;28:457–66. doi:10.1039/c0np00018c.

[174] Sletmoen M, Stokke BT. Higher order structure of (1,3)-β-D-glucans and its influence on their biological activities and complexation abilities. Biopolymers 2008;89:310–21. doi:10.1002/bip.20920.

[175] Bose N, Chan A, Guerrero F, Maristany C, Qiu X, Walsh R, et al. Binding of Soluble Yeast β-Glucan to Human Neutrophils and Monocytes is Complement-Dependent. Front Immunol 2013;4:230. doi:10.3389/fimmu.2013.00230.

[176] Lipinski T, Fitieh A, St Pierre J, Ostergaard HL, Bundle DR, Touret N. Enhanced immunogenicity of a tricomponent mannan tetanus toxoid conjugate vaccine targeted to dendritic cells via Dectin-1 by incorporating β-glucan. J Immunol 2013;190:4116–28. doi:10.4049/jimmunol.1202937.

[177] Mochizuki S, Sakurai K. Dectin-1 targeting delivery of TNF-α antisense ODNs complexed with β-1,3-glucan protects mice from LPS-induced hepatitis. J Control Release 2011;151:155–61. doi:10.1016/j.jconrel.2011.01.026.

[178] Dube A, Reynolds JL, Law W-C, Maponga CC, Prasad PN, Morse GD. Multimodal nanoparticles that provide immunomodulation and intracellular drug delivery for infectious diseases. Nanomedicine Nanotechnology, Biol Med 2014;10:831–8. doi:http://dx.doi.org/10.1016/j.nano.2013.11.012.

[179] Tincer G, Yerlikaya S, Yagci FC, Kahraman T, Atanur OM, Erbatur O, et al. Immunostimulatory activity of polysaccharide–poly(I:C) nanoparticles. Biomaterials 2011;32:4275–82. doi:10.1016/j.biomaterials.2011.01.028.

[180] Wang J, Guo C, Yue T, Yuan Y, Liu X, Kennedy JF. Cationization of Ganoderma lucidum polysaccharides in concentrated alkaline solutions as gene carriers. Carbohydr Polym 2012;88:966–72. doi:10.1016/j.carbpol.2012.01.054.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Background, hypothesis and objectives

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Background

1. Nanotechnology has played a major role in the development of novel adjuvants for vaccines in the last few decades. Aiming at improving the immune response generated by modern antigens such as proteins and peptides, particulate delivery systems have been described as potential vaccine carriers for a variety of infectious diseases[1]. In particular, these vehicles may improve the stability of antigens against degradation, their targeting to antigen presenting cells (APCs) and/or lymphoid tissues, and their interaction with these cells, particularly in mucosal surfaces[2].

2. Particle size has been described as one of the major parameters influencing the transport of antigen nanocarriers from the administration site to the draining lymph nodes[3]. Other characteristics of the nanosystems, such as surface charge and composition[4,5] may also play a role in this process, and can therefore be tuned to achieve an optimal antigen nanocarrier.

3. Beta glucans are a group of polysaccharides with various length chains and branched forms, naturally occurring in the cell wall of different organisms such as bacteria and yeast. They present pathogen-associated molecular patterns (PAMPs) [6,7], a fact that facilitates their recognition by antigen presenting cells (APCs) such as macrophages and dendritic cells, through receptors at the surface of these cells, such as complement receptor 3 and Dectin-1[8].

[1] Cordeiro AS, Alonso MJ. Recent advances in vaccine delivery. Pharm Pat Anal 2016; 5:49–73.
[2] De Temmerman M-L, *et al*. Particulate vaccines: on the quest for optimal delivery and immune response. Drug Discov Today 2011; 16:569–82.
[3] Benne N, *et al*. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. J Control Release 2016; 234:124–34.
[4] Oussoren C, *et al*. Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposomal size, lipid composition and lipid dose. Biochim Biophys Acta 1997; 1328:261–72.
[5] Fromen CA, *et al*. Nanoparticle surface charge impacts distribution, uptake and lymph node trafficking by pulmonary antigen-presenting cells. Nanomedicine Nanotechnology, Biol Med 2016; 12:677–87.
[6] Goodridge HS, Wolf AJ, Underhill DM. β-glucan recognition by the innate immune system. Immunol Rev 2009; 230:38–50.
[7] Mahla RS, *et al*. Sweeten PAMPs: Role of sugar complexed PAMPs in innate immunity and vaccine biology. Front Immunol 2013; 4:1–16.
[8] Huang H, *et al*. Relative contributions of Dectin-1 and complement to immune responses to particulate beta-glucans. J Immunol 2012; 189:312–7.

4. Beta glucans have been studied in the modulation of immune responses in the last decades[9], particularly in the form of microparticles, usually obtained from emptying the cellular content of *Saccharomyces cerevisiae* obtaining hollow structures[10]. However, to the best of our knowledge, only one publication reports the use of beta glucans in nanoparticles for antigen delivery[11]. In this work, authors report the preparation of nanocomplexes with the immunostimulant poly(I:C) and their co-administration with ovalbumin intraperitoneally to mice, eliciting high antibody responses.

5. Chitosan was first studied in our laboratory in the form of nanoparticles as carriers for the transport of antigens, i.e. tetanus toxoid and hepatitis B surface antigen (rHBsAg), across the nasal mucosa[12,13,14]. Subsequently, we also developed chitosan nanocapsules for the delivery of rHBsAg[15] and nanocomplexes of chitosan with the immunostimulant poly(I:C) and a T-helper peptide (PADRE)[16]. These nanocarriers were able to be freeze-dried to a dry powder form which maintained the colloidal properties of the nanocapsules and the immunogenicity of the associated antigen. Therefore, chitosan is a versatile polymer with adjuvant properties[17] and a demonstrated capacity to form different types of nanostructures for antigen delivery.

[9] Novak *et al*. Beta-glucans, history, and the present: immunomodulatory aspects and mechanisms of action. Journal of Immunotoxicology 2008; 5(1):47-57.
[10] De Smet *et al*. β-Glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination. Journal of Controlled Release 2013; 172(3):671-8.
[11] Tincer *et al*. Immunostimulatory activity of polysaccharide–poly(I:C) nanoparticles. Biomaterials 2011; 32:4275-82.
[12] Vila A *et al*. Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice. European Journal of Pharmaceutics and Biopharmaceutics 2004; 57(1):123-31.
[13] Vila A, *et al*. Design of biodegradable particles for protein delivery. Journal of Controlled Release 2004; 78:15-24.
[14] Prego C *et al*. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. Vaccine 2010; 28:2607–14.
[15] Vicente S *et al*. A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach. PLoS ONE 2013; 8(4):e62500.
[16] Correia-Pinto JF *et al*. Chitosan-Poly (I:C)-PADRE Based Nanoparticles as Delivery Vehicles for Synthetic Peptide Vaccines. Vaccines 2015; 3(3):730-50.
[17] Zaharoff DA *et al*. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine 2007; 25:2085–94.

Hypothesis

1. Engineering of key features of antigen nanocarriers, such as their surface composition and charge, as well as their size, may lead to improved biodistribution of antigen nanocarriers to the lymphatic system upon subcutaneous or intramuscular administration, as well as enhanced interaction with immune cells and adjuvant action.

2. Based on its immunomodulatory properties, carboxymethyl-β-glucan is an attractive polymer for its use in the development of nanoparticles intended for the delivery of antigens to immune cells and for the triggering of appropriate immune responses.

3. Polysaccharide-based nanoparticles, and notably nanoparticles made of chitosan and carboxymethyl-β-glucan can be prepared as thermostable dry powder forms and adapted for potential administration by non-parenteral routes.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Objectives

Considering the background knowledge and the subsequent hypothesis, the main goal of this thesis was to investigate the interaction of different polymer-based nanocarriers of different composition and physicochemical properties with the immune cells, as well as their biodistribution following subcutaneous administration to mice. An additional goal was to generate a new prototype of nanoparticles consisting of chitosan and carboxymethyl-β-glucan and to evaluate their potential as thermally stable vaccine delivery nanocarriers. To achieve this goal, the following specific aims have been grouped in two blocks and are described as following:

1. Study of the influence of particle size, surface charge and polymer composition in the cellular uptake and lymphatic biodistribution of polymer-based nanocapsules.

1.1. Development of a panel of polymer-coated nanocapsules with different polymeric shell, particle size, and surface charge.

1.2. Study of the influence of different parameters in the in vitro uptake of the nanocapsules by macrophages.

1.3. Comparison of the lymphatic distribution of the different nanocapsule prototypes in an animal model, upon subcutaneous administration, using innovative ex vivo and in vivo fluorescence microscopy techniques.

The results corresponding to this block are described in chapter 2.

2. Development of a new polysaccharide-based nanoparticle prototype for antigen delivery.

2.1. Design and development of nanoparticles based on carboxymethyl-β-glucan and chitosan for the association and delivery of a model antigen.

2.2. Evaluation of the ability of the developed nanosystems to be transported to the lymphatic system following subcutaneous administration and to interact with immune cells, allowing antigen presentation.

2.3. Development of alternative forms of presentation of chitosan:carboxymethyl-β-glucan nanoparticles, which may allow storage at high temperature and administration by non-parenteral routes.

The results corresponding to this block are described in chapter 3.

Chapter 2

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter 2

Engineering polymeric nanocapsules for an efficient drainage to the lymphatic system

This work was done in collaboration with Belén López-Bouzo[1,2], Mercedes Peleteiro[3], África Fernández[3] and Santiago F. González[4]

[1] Translational Medical Oncology Group, Health Research Institute of Santiago de Compostela (IDIS), Clinical University Hospital of Santiago de Compostela (CHUS), 15706 Santiago de Compostela, Spain

[2] Center for Research in Molecular Medicine & Chronic Diseases (CIMUS), Health Research Institute of Santiago de Compostela (IDIS), School of Pharmacy, University of Santiago de Compostela, Campus Vida, 15706 Santiago de Compostela, Spain

[3] Immunology, Biomedical Research Center (CINBIO), Institute of Biomedical Research of Ourense, Pontevedra and Vigo (IBI), University of Vigo, Campus Lagoas Marcosende, 36310 Vigo, Pontevedra, Spain

[4] Institute for Research in Biomedicine, Università della Svizzera Italiana, via Vincenzo Vela 6, 6500 Bellinzona, Switzerland

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

ABSTRACT

Targeting the lymphatic system for the triggering of an effective immune response is one of the major challenges in vaccine development. Subcutaneous and intramuscular vaccines should cross the interstitial matrix and drain into the nearest lymph node, to encounter the adequate antigen presenting cells and initiate the proper response. It is now accepted that the use of nanotechnology will help reaching this goal. In this regard, it is known that particle size, surface charge and composition may strongly influence the distribution and interaction of nanosystems with immune cells. In this work, we developed chitosan nanocapsules with a size of 100 nm and evaluated their interaction with macrophages *in vitro*, by studying the internalization routes and the cytokine induction profile resulting from these interactions. We compared this *in vitro* mechanism of action with the one of classical 200 nm chitosan nanocapsules. Finally, we compared the *in vivo* lymphatic drainage of nanocapsules with different particle sizes (100 and 200 nm) and with different polymeric coatings (polyarginine, inulin and carboxymethyl-β-glucan). Results showed that both chitosan nanocapsules and control nanoemulsions were efficiently internalized by macrophages irrespective of size, through a combination of pathways including phagocytosis and receptor-mediated endocytosis. Additionally, 100 nm chitosan nanocapsules were able to induce higher MCP-1 levels than those with a size of 200 nm, showing a potentially more interesting profile for immunization. Concerning the biodistribution in mice, smaller cationic nanocapsules accumulated highly in the popliteal lymph node following subcutaneous injection. However, anionic control nanoemulsions and carboxymethyl-β-glucan nanocapsules also showed high accumulation levels, leading to the possibility of a multifactorial process guiding the lymphatic drainage of these systems. Further studies are required to understand the mechanistic pathways behind this differential behaviour.

1. INTRODUCTION

Progress in vaccination has brought significant improvement of the global health status in the last decades. This is mainly due to the potential of this healthcare intervention to control the spreading and prevent the morbidity and mortality associated to countless infectious diseases. Vaccines have also been evaluated as preventive and therapeutic tools for the control of other threatening diseases such as cancer, autoimmune and neurodegenerative pathologies [1–3]. However, vaccination success is still hindered by some limitations, such as the need for cold-chain storage conditions and the high number of doses that are usually required [4,5]. Moreover, scientific advances in vaccine development have led to the use of safer yet less immunogenic antigens, which demand the use of better adjuvants to compensate this low immunogenicity [6,7].

Nanotechnology has played an important role in this field, attempting to overcome some of the previously mentioned challenges. Due to their intrinsic physicochemical characteristics and versatility, nanosystems are interesting as platforms for antigen delivery and as potential vaccine adjuvants *per se* [8,9]. In particular, the use of natural and synthetic polymers, such as polysaccharides, poly lactic-co-glycolic acid (PLGA) or poly lactic acid (PLA), has led to the development of interesting prototypes, with relevant adjuvant abilities [10,11]. Nevertheless, a thorough understanding of the critical properties that drive the first steps of an immune response, such as trafficking, recognition, internalization and processing of antigens and antigen carriers, is still necessary to smooth the path towards the rational development of efficient nanovaccines.

Particularly in the case of subcutaneous vaccines, authors have reported the importance of targeting the lymphatic system for an efficient initiation of the immune response [12,13]. Conventionally, vaccines form a depot at the injection site following administration, slowly draining through the interstitium until they reach the lymphatic vessels and are transported to the draining lymph nodes. However, circulating immune cells may also capture particulate carriers at the administration region and transport them after internalization [14]. In any case, the physicochemical properties of the nanosystems are key factors that will determine the evolution and outcome of this process. To date, most efforts have been focussed on understanding the relevance of particle size and surface charge [15–18]. In terms of particle size, considering the physiology of the interstitium and the lymphatic vessels, 100 nm has been set as a boundary value. Studies showed as polymer-based nanosystems such as PLGA nanoparticles or polyaminoacid nanocapsules are able to freely drain to the lymph node if the particle size

is below 100 nm, while larger ones are more retained at the injection site [19,20]. In relation to surface charge, authors have reported contradictory results, with positive [21,22], negative [19,23] and even neutral [24] nanosystems showing efficient lymphatic drainage and retention, as well as adequate uptake by antigen presenting cells. Considering these reports, it is expected that a combination of factors, including particle size, surface charge and composition of the nanosystems is influencing the distribution process. Therefore, there is a need for systematic studies using nanosystems specifically developed for antigen delivery, in order to achieve possible conclusions that may lead the rationale behind future nanovaccine development.

Imaging techniques such as confocal or fluorescence microscopy and magnetic resonance imaging (MRI), among others, have drastically changed the paradigm of particle tracking and visualization of biological processes in the last decades [25,26]. Multiphoton intravital microscopy (MPM) is an innovative imaging technology that allows the observation of biological interactions at subcellular level, in real time, and with high resolution [27,28], of particular interest for tracking of drug delivery systems in biological microenvironments [29,30]. This technique relies on the use of a near-infrared laser source to excite fluorophores using two or three low-energy photons at the same time, minimizing the risk of tissue damage. Therefore, the use of MPM allows deeper penetration in the tissue (up to several hundred microns), with reduced photobleaching and phototoxicity [30].

Considering this background, the aim of the work included in this chapter was to analyse comparatively the lymphatic biodistribution and lymph node accumulation of a variety of nanocapsules with different particle sizes and polymeric coatings. For this, we performed different *in vitro* and *in vivo* assays and made use of two-photon microscopy and flow cytometry.

2. MATERIALS AND METHODS

2.1. Materials

Squalene and vitamin E (α-tocopherol, Calbiochem®) were acquired from Merck Millipore (Darmstadt, Germany). Deoiled phosphatidylcholine-enriched lecithin (Epikuron 145 V) was kindly donated by Cargill (Barcelona, Spain). Vitamin E TPGS was supplied by Antares (Frankfurt, Germany). Surfactants sodium cholate and glycocholate were purchased from Dextra Laboratories (Reading, UK). Chitosan, in the form of ultrapure hydrochloride salt (Protasan UP CL113), with a molecular weight of 125 kDa and a deacetylation degree of 86% was obtained from FMC Biopolymer (NovaMatrix, Sandvika, Norway). Polyarginine, in the form of n-Butyl-poly(arginine) hydrochloride and with a molecular weight of 28 kDa, was acquired from PTS Polypeptides (Valencia, Spain). Inutec SP1, an inulin-based surfactant, was obtained from Creachem (Leuvenselaan, Belgium). Carboxymethyl-β-glucan, obtained from *Saccharomyces cerevisae* and modified with carboxymethyl groups at an 85% substitution degree, was a kind donation from Mibelle AG Biochemistry (Buchs, Switzerland). Fluorescent marker DiI C18(5) oil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) (DiD) was purchased from Life Technologies (Carlsbad, USA). Nile Red, Tween® 80 and cetyl trimethylammonium bromide (CTAB) were obtained from Sigma-Aldrich (Saint Louis, USA). All solvents were HPLC grade, supplied by Thermo Fisher Scientific (Waltham, USA) or Scharlab SL (Barcelona, Spain).

Culture media DMEM and RPMI 1640, as well as the antibiotics penicillin-streptomycin were acquired from Thermo Fisher Scientific (Waltham, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories (GE Healthcare, Pasching, Austria). Lipopolysaccharide (LPS) was purchased from InvivoGen (San Diego, USA). Chlorpromazine hydrochloride, Filipin III, 5-(N,N-dimethyl)amiloride hydrochloride and Cytochalasin D were supplied by Sigma-Aldrich (Saint Louis, USA).

Goat anti-mouse SR-A (R&D systems; Minneapolis, USA), goat anti-β actin (Abcam; Cambridge, UK) and rabbit anti-goat antibodies conjugated to horseradish peroxidase (Immunostep; Salamanca, Spain) were used in Western blot assays.

2.2. Preparation of chitosan nanocapsules and control nanoemulsions

Chitosan nanocapsules were prepared as described previously by our group [31], with minimal modifications to the conventional solvent displacement technique. In summary, the organic phase was first prepared by dissolving 30 mg of lecithin and 62.5 µL of

squalene in 5 mL of ethanol. Then, this solution was poured over 10 mL of an aqueous chitosan solution (0.25 mg/mL) upon magnetic stirring, leading to the spontaneous formation of the nanocapsules. After 10 minutes of stirring, the organic solvents were eliminated by evaporation under vacuum (Heidolph Hei-VAP Advantage; Schwabach, Germany) and the nanocapsules were isolated and concentrated to a final theoretical chitosan concentration of 1 mg/mL by centrifugation (Hettich Universal 32 R; Tuttlingen, Germany) at 21250xg for 90 min at 15ºC. In parallel, control nanoemulsions were prepared according to the same method, using ultrapure water as the aqueous phase, without chitosan.

To achieve the reduction of particle size for these formulations, we used the combination of two strategies: reducing the amount of the components used (maintaining the proportions between them) and changing the preparation method. For the latter, we studied the options of sonication and injection of the organic phase into the aqueous one. The sonication method involved pouring the organic phase over the aqueous one, followed immediately by the homogenization of the mixture using a sonication probe (Branson Model 250 Sonifier; Danbury (CT), USA). In detail, squalene and lecithin were dissolved in propanol and this organic phase was further diluted with acetone up to 2 mL. Subsequently, the organic phase was poured over 4 mL of water or aqueous chitosan solution (0.25 mg/mL) and sonicated for one minute at 10% amplitude. The remaining steps of the preparation were done as described above. In addition to the standard composition, and in order to reduce their particle size, chitosan nanocapsules were also prepared using reduced amounts of the components (6.25, 12.5, 25 and 50% of the initial concentrations) and injecting the organic phase in the aqueous one with a syringe 0.60 × 60 mm needle (23G × 2 3/8", B. Braun Melsungen, Germany).

2.3. Preparation of polyarginine, inulin and carboxymethyl-β-glucan nanocapsules

For the preparation of polyarginine, inulin and carboxymethyl-β-glucan nanocapsules, the solvent displacement technique was selected, injecting the organic phase into the aqueous one. Polyarginine nanocapsules were prepared using vitamin E (60 mg), TPGS (25 mg) and sodium cholate (5 mg) in 5 mL of ethanol as organic phase, injected into 10 mL of an aqueous solution of polyarginine (5 mg). Similarly, in the case of inulin nanocapsules, the organic phase was composed of 2.5 mL of an ethanolic solution of vitamin E (30 mg) and sodium glycocholate (5 mg, dissolved in water), added to 10 mL of an aqueous solution of modified inulin (15 mg). Finally, for the preparation of carboxymethyl-β-glucan nanocapsules, an organic phase of squalene (26 mg), Tween®

80 (11 mg) and CTAB (0.1 mg) in 2 mL of ethanol was injected into 10 mL of an aqueous solution of the polymer (2.5 mg).

For the purification of the nanocapsules, different methods were used, following a selection and optimization process specific for each prototype. In the case of polyarginine and carboxymethyl-β-glucan nanocapsules, formulations were centrifuged for one hour at 84035xg and 61740xg, respectively (Optima™ L-90K Ultracentrifuge; Beckman Coulter, Brea (CA), USA). Polyarginine nanocapsules were previously mixed with a solution of sucrose at 20% (1:3 volume ratio) to facilitate the isolation and resuspension process. In the case of inulin nanocapsules, the isolation process selected was size-exclusion filtration using appropriate columns (CentriPure P10, emp BIOTECH GmbH; Berlin, Germany) and following manufacturer's instructions. Finally, all isolated nanosystems were resuspended to initial concentration in ultrapure water for further use. In the case of inulin nanocapsules, due to the specificities of the selected technique, the isolated systems were diluted 1.5x in comparison with the non-isolated ones.

2.4. Physicochemical characterization of polymeric nanocapsules

The particle size and polydispersity index of the nanocapsules were determined by photon correlation spectroscopy (PCS) (Zetasizer NanoZS®, Malvern Instruments; Malvern, United Kingdom). Samples were previously diluted in ultrapure water and the measurements were done at room temperature. In the case of surface charge, zeta potential measurements were done by laser Doppler anemometry (LDA) using the same equipment. For this purpose, samples were diluted in an aqueous solution of KCl (1 mM).

2.5. Fluorescent labelling of polymeric nanocapsules and characterization of the labelled nanosystems

The preparation of the fluorescent labelled chitosan nanocapsules was done as described in section 2.2, through the addition of DiD (at a final concentration of 20 or 50 μg/mL) or Nile Red (at a final concentration of 77 μg/mL) to the organic phase. In the case of the rest of the polymeric nanocapsules studied, the fluorescent labelling was done by the inclusion of DiD in the organic phase of each formulation, at a final concentration of 20 μg/mL.

Besides the physicochemical characterization, these prototypes were also characterized in terms of the fluorophore encapsulation efficiency, release profile and stability of the

labelling in storage and in experimental media, as described in the following sections. The labelling efficiency and the DiD release profile were evaluated by spectrofluorimetry (TECAN Infinite® M1000 PRO; Männedorf, Switzerland). For the encapsulation efficiency studies, DiD-loaded nanocapsules were isolated as described previously and we determined the fluorescence intensity present in the undernatants, containing the non-encapsulated fluorophore. In a similar manner, isolated nanocapsules were diluted (1:5) with complete RPMI medium, containing 10% fetal bovine serum and 1% penicillin-streptomycin and incubated at 37 ºC (Heidolph Titramax/Inkubator 1000; Schwabach, Germany). At defined time points (30, 60, 120 and 240 minutes), the samples were centrifuged as described previously and the undernatants containing free DiD were analysed by spectrofluorimetry (TECAN Infinite® M1000 PRO; Männedorf, Switzerland). Fluorescence measurements were performed at the excitation (644 nm) and emission (665 nm) wavelengths.

2.5.1. Stability studies

The stability of the chitosan nanocapsules was evaluated in the media used for *in vitro* studies in terms of the main colloidal properties of the nanosystems, particle size and polydispersity index. Nanosystems were incubated after an appropriate dilution (1:10 for small prototypes and 1:200 for medium ones, corresponding to 0.9 and 0.2 mg/mL respectively) with complete RPMI media, containing fetal bovine serum and antibiotics, and kept at 37 ºC for up to 4 hours. Particle size and polydispersity index were measured at 30, 60, 120 and 240 minutes post incubation. Stability in storage conditions (4 ºC), as well as DiD leakage, were also evaluated for all formulations tested. In this case, particle size, polydispersity index and DiD release were measured at 7, 14 and 21 days after preparation.

2.6. *In vitro* study of the internalization pathways for chitosan nanocapsules

2.6.1. Immortalized and bone-marrow derived macrophages

The model of phagocytic cells used was the Raw 264.7 cell line (American Type Culture Collection (ATCC); Middlesex, United Kingdom). Moreover, a derivative cell line transduced to silence the macrophage scavenger receptor A, generated as described previously [32], was also used for internalization assays. Both cell lines were cultured in complete RPMI medium, containing 2 mM glutamine, 100 U/mL penicillin-streptomycin and 10% (v/v) heated-inactivated fetal bovine serum, at 37 ºC in a 5% CO_2 atmosphere. To maintain 70-80% confluent cultures, cells were split every other day. Mycoplasma

contamination was controlled and discarded from all cultures using the MycoAlert™ PLUS assay (Lonza; Rockland, USA) according to the manufacturer's instructions.

On the other hand, bone-marrow derived macrophages (BMDM) were obtained either from wild type C57BL/6 mice (Charles River; Barcelona, Spain) or from TLR 4 knock-out mice (TLR 4-/-), with C57/BL6 background (Life and Health Sciences Research Institute (ICVS), Minho University; Braga, Portugal). The animals were housed under specific pathogen-free conditions and were given food and water *ad libitum*. All procedures were done according to the guidelines for care and handling of laboratory animals (European Union Directive 86/609/EEC) and the study was approved by the Portuguese National Authority for animal experimentation (Direcção Geral de Alimentação e Veterinária). BMDM cells were obtained by flushing the diaphysis of the femurs and tibias of both animal types (8-12 weeks old) with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, similarly to the method described by Appelberg *et al* [33]. Following, these cells were cultured for seven days in a selective medium containing 20% L929 cell-conditioned medium (LCCM) as a source of macrophage colony stimulating factor (M-CSF). Finally, the completely differentiated macrophages were recovered with non-pyrogenic PBS and counted before being used in the experiments.

2.6.2. Endotoxin study

To discard the presence of endotoxins in the nanosystems suspensions, the Gel clot Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod, Inc.; East Falmouth, USA) was used according to the manufacturer's instructions. To avoid any interference in the assay by (1→3)-β-D-glucans, LAL was dissolved in Glushield buffer prior to the experiment. As an inhibition/enhancement control, to discard possible interferences with the method, nanosystems were studied alone or spiked with endotoxin. Therefore, samples and endotoxin (positive control) in different dilutions were incubated with LAL for 1 hour at 37 ºC. Subsequently, all tubes were centrifuged and evaluated for the presence of a compact clot. All formulations showed levels of endotoxins below the detection limit of the assay (0.3 EU/mL).

2.6.3. Evaluation of cytokine induction

For the quantification of the cytokine induction by the different formulations, BMDM were plated ($2x10^5$ cells/well) with complete RPMI and incubated in the presence of nanocapsules or nanoemulsions (approximately 0.3 or 0.9 mg/mL of total components, corresponding to 10 or 25 µg/mL of chitosan in nanocapsules) for 24 hours at 37 ºC (5% CO_2 atmosphere). RPMI and lipopolysaccharide (LPS) at 1 µg/mL were used as negative and positive controls, respectively. The plates were centrifuged at 195xg for 5 min and

the supernatants were collected and stored at -20 ºC before use. Flow cytometry was used to determine the cytokine induction levels, using a Cytometric Bead Assay (BD Bioscience; San Diego, USA) that simultaneously detects six cytokines: IL-12, IL-6, IL-10, INF-γ, TNF-α and MCP-1. The assay was performed according to manufacturer's instructions and the samples were analysed on a BD Accuri™ C6 flow cytometer (Accuri Cytometers; Ann Arbor, USA). Data from three independent experiments, performed in duplicate with a pool of cells from two mice, was analysed using FCAP Array™ software (BD Bioscience; San Diego, USA).

2.6.4. Expression of scavenger receptor A (SR-A)

To assess the efficiency of the SR-A silencing in the Raw 267.4 clone cell line, the expression of the receptor was evaluated by Western blot. First, cell lysates were prepared with lysis buffer (Tris.HCl 10 mM, pH 8, NaCl 150 mM, EDTA 2.5 mM and 1% NP-40) supplemented with a protease and phosphatase inhibitor (Complete Mini and PhosphoStop, Roche; Basel, Switzerland). These extracts were incubated with sample buffer (containing dithiothreitol) at 95 ºC for 5 min. Following, proteins were separated by electrophoresis in a 10% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad; Hercules, USA). Membranes were further blocked for one hour at room temperature using a 5% solution of non-fat dried milk in Tween-supplemented Tris-buffered saline (TBS-T) and incubated overnight at 4 ºC with specific goat anti-mouse SR-A antibodies. As a control of the protein loading, goat anti-β actin antibodies were used, with an incubation time of one hour at room temperature. Finally, the secondary antibodies used were rabbit anti-goat IgG conjugated to horseradish peroxidase (HRP). The detection was done using the Clarity Western ECL Substrate (Bio-Rad; Hercules, USA) and a ChemiDoc XRS imaging system (Bio-Rad; Hercules, USA).

2.6.5. Internalization studies

Uptake studies with chitosan nanocapsules and the corresponding nanoemulsiones were done using the Raw 264.7 cell lines previously described (section 2.2). For this purpose, both cell lines were plated ($5x10^6$ cells/well) and incubated with fluorescent nanocapsules or nanoemulsions (approximately 0.3 or 0.9 mg/mL of total components, corresponding to 10 or 25 µg/mL of chitosan in nanocapsules) for 1 hour at 37 ºC (5% CO_2 atmosphere). After thorough washing of the cells, they were detached using Accutase® (PAA laboratories, GE Healthcare; Pasching, Austria) and incubated with 0.4% Trypan Blue (Sigma-Aldrich; Saint Louis, USA) in PBS for one minute, to quench the fluorescence of the nanocapsules that could be attached to the cell membrane. Finally, the cellular suspension obtained was also analysed by flow cytometry, measuring

the signal detected by the FL3 channel. In total, three independent experiments were performed in duplicate.

In parallel, the internalization of the formulations was also studied by confocal microscopy, to assure the real uptake rather than the mere attachment of the nanosystems to the cell membrane. This assay was done as mentioned above, however the cells were seeded on a glass coverslip and fixed with formalin (Sigma-Aldrich; Saint Louis, USA) after incubation with the nanosystems. Furthermore, the macrophages cytoskeleton was stained with Alexa Fluor 488-phalloidin and the coverslips were mounted on slides with ProLong® Gold Antifade mounting medium (Invitrogen; Eugene, USA) containing DAPI (4',6-diamidino-2-phenylindole). The slides were then analysed under a confocal microscope (Leica SP5; Leica Microsystems, Mannheim, Germany).

Aiming at the evaluation of the potential uptake routes used by the macrophages when incubated with the formulations, four inhibitors were used: cytochalasin D (5 µM) for phagocytosis, chlorpromazine hydrochloride (20 µM) for clathrin-mediated endocytosis, Filipin III (1 µg/mL) for caveolae-mediated endocytosis and 5-(N,N-dimethyl)amiloride hydrochloride (DMA, 10 µg/mL) for macropinocytosis. In this experiment, both cell types were pretreated with the inhibitors for one hour and the subsequent steps were performed as described at the beginning of this section. Finally, the median fluorescence intensity (MFI) of each cellular suspension was measured by flow cytometry. Cells incubated with the formulations in the absence of inhibitors were used as positive control (100% uptake) and the results were normalized to this control, providing data represented as "Normalized uptake (%)". As in the other *in vitro* experiments, three independent assays were done in duplicate.

2.7. Evaluation of the biodistribution of nanocapsules in the lymphatic system

2.7.1. Animals and administration schedule

All *in vivo* studies were performed using C57/BL6 female 6-8 weeks old mice, bred in-house or acquired from Janvier labs (Le Genest-Saint-Isle, France). Animals were maintained in pathogen-free facilities at the Institute for Research in Biomedicine (Bellinzona, Switzerland) and given food and water *ad libitum*. All experiments were done in accordance with the Swiss Federal Veterinary Office guidelines and animal protocols approved by the veterinarian local authorities.

For lymph node accumulation studies, 4 µL of each formulation (approximately 35 µg of total components) were administered to mice at the same DiD dose, through a single

injection in each footpad. Following, 20 minutes after this injection, 0.5 µg of two cell-labelling antibodies [αCD169 and αCD21/35 (BioLegend; San Diego (CA), USA)], in a final volume of 5 µL of PBS, were also administered in both footpads.

In the case of *in vivo* dynamics of the nanosystems distribution, a catheter for administration of the nanocapsule formulations was placed in the mice right footpad. A mixture of chitosan nanocapsules of both particle sizes, each labelled with a different fluorescent marker at the same dose, was previously prepared and set for administration through the catheter. The formulation mixture was injected three minutes after initiating the image acquisition.

2.7.2. Ex vivo lymph node imaging

For the 3D reconstruction imaging studies experiments, the popliteal and lumbar lymph nodes of the studied mice were harvested at 12 hours post-injection and kept in PBS at 4 ºC. One axillary lymph node was also collected at the same time for control purposes. Lymph node imaging was done using a customised two-photon platform (TrimScope, LaVision BioTec GmbH; Bielefeld, Germany). 2-photon excitation of the fluorescent probes was achieved using two tunable Ti:Sapphire lasers with an output wavelength in the range of 690-1080 nm (Chamaleon Ultra I, Chamaleon Ultra II, Coherent Inc.; Santa Clara (CA), USA), and an optical parametric oscillator emitting in the range of 1010 to 1340 nm (Chamaleon Compact OPO, Coherent Inc.; Santa Clara (CA), USA). The objective used to obtain 3D whole lymph node reconstructions was a Nikon Plan Apo λ 10X/0.45, with a mosaic of up to 4x3 adjacent field-of-view image acquisitions.

2.7.3. Real-time in vivo lymph node imaging

In the case of real-time *in vivo* video images of the popliteal lymph node, the protocol followed was similar to the one described by González *et al* [34]. Mice were anaesthetized with a mixture of ketamine and xylazine (100 and 10 mg/kg bodyweight, respectively) and positioned in a custom-made surgical board. Subsequently, the right popliteal lymph node was exteriorized through surgical techniques aimed at maintaining the anatomical structure of both the lymph node and the surrounding lymphatic and blood vessels. A catheter for administration of anaesthetics was inserted in the peritoneal cavity and another one for administration of the nanocapsules was inserted subcutaneously in the right footpad.

Images were acquired every 15 seconds for 30 minutes, and consecutive videos were recorded up to 4 hours of image acquisition. The objective used was a Nikon Plan Apo λ 4X/0.20 and images were taken to a depth of 30 µm into the lymph node.

2.7.4. Image analysis

Mosaic images obtained in the two-photon microscopy experiments were reconstructed by stitching on FIJI software [35], using an automated image processing custom-developed script. Furthermore, the hyperstacks were loaded on Imaris 7.7.2 (Bitplane; Zurich, Switzerland) software to obtain the 3D rendering of the lymph nodes. The intensity of the signal obtained for each channel was fixed in a range throughout each experiment and quantified using the Imaris software to create individualized surfaces corresponding roughly to particles or agglomerates. In the case of the real-time *in vivo* videos, the image sequences acquired were rendered into four-dimensional videos and FIJI software was used to analyse the data. These sequences were then analysed by measuring the mean fluorescence intensity (MFI) of a region of interest (ROI size: 74 µm x 56 µm), localised in the medullary area.

2.7.5. Flow cytometry

The lymph nodes collected for imaging, as described previously, were disrupted with tweezers and digested for 10 min at 37 °C in an enzyme mix - DNase I [0.28 mg/ml] (Amresco; Solon, USA), Dispase [1 U/ml] (Corning; New York, USA) and Collagenase P [0.5 mg/ml] (Roche; Basel, Switzerland) - in calcium- and magnesium-free PBS (PBS-) (Sigma-Aldrich; Saint Louis, USA). Subsequently, the reaction was stopped using a solution of 2 mM EDTA (Sigma-Aldrich; Saint Louis, USA) and 2% heat-inactivated filter-sterilised Fetal Bovine Serum (Thermo Fisher Scientific; Waltham (MA), USA) in PBS. Different cell populations were stained using fluorescently labelled antibodies [αLy-6G(1A8), αMHCII (M5/114.15.2), αCD11c (N418), αCD11b (M1/70), αF4/80 (BM80); BioLegend, San Diego (CA), USA] and the samples were analysed by flow cytometry on LSRFortessa™ (BD Biosciences; San Jose, USA). Data were analysed with FlowJo® software (FlowJo, LLC; Ashland, USA).

2.8. Statistical analysis

Statistical analysis and data representation were performed with Prism 7 (GraphPad Software; La Jolla, USA). In general, unpaired two-tailed t tests with Welch's correction were applied to the different datasets and statistical significance was defined as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ****($p<0.0001$).

3. RESULTS AND DISCUSSION

The evaluation of antigen nanocarriers properties, and the way to modulate them to achieve more effective immune responses, has been a matter of discussion throughout the years. Published studies have shown contradictory results regarding the effect of the physicochemical characteristics of the antigen carriers in the efficacy of the vaccination strategy [15–18]. In addition, the strict comparison of these studies is difficult because the selected antigen and the route of administration are, in some cases, different. For this reason, it is particularly important to perform *in vitro* and *in vivo* screening studies with carefully designed prototypes that may allow an accurate comparison and deeper understanding of these complex processes. Ideally, this detailed evaluation would help in a more rational design and development of antigen nanocarriers that are potentially more efficient.

To perform these studies, we selected chitosan nanocapsules as starting prototype, given the promising results achieved with this vehicle in antigen delivery, as shown in several articles published by our group [31,36–38]. This prototype was tested with antigens for hepatitis B and influenza immunization [37], and showed versatility to incorporate additional immunostimulant molecules such as imiquimod [31]. Moreover, chitosan solutions parenterally administered to mice have shown immunostimulant properties both with model antigens such as β-galactosidase [39] and with inactivated influenza virus [40,41]. Spray-dried chitosan particles were also mixed with ovalbumin and used for the subcutaneous immunization of mice with positive adjuvant effect [42]. Therefore, this prototype was optimized for the achievement of two nanocarriers with the same composition but different particle size.

3.1. Development and characterization of the nanocapsules panel

In general, the influence of particle size, shape and surface charge of nanosystems in their interaction with immune cells has been a controversial subject of discussion in the literature [15,43,44]. Unfortunately, most of these studies have used model nanoparticles, such as polystyrene beads or metallic nanoparticles. To the best of our knowledge, comparison studies using nanocarriers composed of biodegradable biopolymers are very limited [18,20,45].

Standard chitosan nanocapsules, composed of a squalene core stabilized by lecithin and an external chitosan shell, exhibited an average size of 220-250 nm and a positive zeta potential of +30 mV [31]. In addition, non-coated nanoemulsions with the same

composition were prepared as control formulations, with similar particle size and markedly negative zeta potential. Subsequently, our aim was to reduce the particle size of chitosan nanocapsules based on previous information from our group [20]. As depicted in **figure 1**, chitosan nanocapsules and control nanoemulsions were prepared by two methodologies (sonication and solvent displacement). Three different concentrations of components were tested, maintaining the mass ratio between them (squalene:lecithin:chitosan 1:0.6:0.05 (w/w)), and using the concentration of squalene as reference (21.4, 5.4 and 1.35 mg/mL). Both techniques rendered reproducible prototypes with nanometric size and adequate surface charge, despite the concentration of the components tested. A reduction in particle size was observed, as the concentration of components decreased, being this reduction more effective in the case of the nanosystems prepared by solvent displacement.

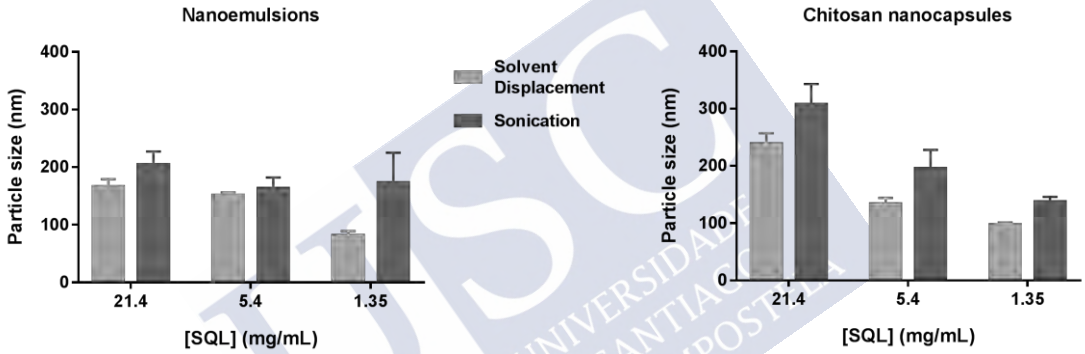


Figure 1. *Particle size of the control nanoemulsions (NE) and chitosan nanocapsules (NC) prepared through sonication and solvent displacement techniques, with different squalene concentrations. The concentration of the other components was reduced maintaining the initial mass ratio (squalene:lecithin:chitosan 1:0.6:0.05 (w/w)). Results are shown as mean ± SD of at least 3 replicates.*

For this reason, we decided to proceed with this technique for the preparation of chitosan nanocapsules and control nanoemulsions with particle size below 100 nm. This value was selected given the anatomical features of the lymphatic system, which is the main target tissue for our nanosystems. The interstitium surrounding the lymphatic vessels is a matrix of proteins and other components with pores that limit the penetration of particles according to their size and flexibility, usually allowing the drainage of 10-100 nm particles [46]. To achieve these low values, we had to slightly modify the method of addition of the organic phase over the aqueous one, directly injecting it with a needle.

The results showed that we were able to obtain the smaller nanosystems using 5.4 mg/mL of squalene (25% of the initial concentration).

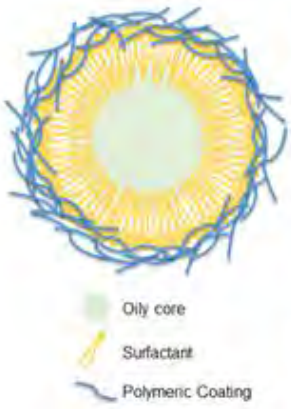
Table 1. *Summary of the physicochemical characteristics of the nanoemulsions (NE) and nanocapsules (NC) prepared through sonication and solvent displacement techniques (with or without organic phase injection). Results are shown as mean ± SD of 3 replicates. n.d., not determined.*

	[SQL] (mg/mL)	Sonication			Solvent Displacement			Organic phase injection		
		Size (nm)	PdI	Z Pot (mV)	Size (nm)	PdI	Z Pot (mV)	Size (nm)	PdI	Z Pot (mV)
NE	21.4	204 ± 23	0.3	-91 ± 7	166 ± 13	0.1	-66 ± 3	n.d.	n.d.	n.d.
NC		307 ± 36	0.4	+67 ± 2	238 ± 19	0.2	+32 ± 1	n.d.	n.d.	n.d.
NE	5.4	163 ± 19	0.2	-77 ± 11	151 ± 5	0.1	-56 ± 3	87 ± 12	0.2	-45 ± 9
NC		195 ± 33	0.2	+54 ± 8	133 ± 11	0.2	+58 ± 4	93 ± 9	0.2	+22 ± 2
NE	1.35	173 ± 52	0.3	-58 ± 9	81 ± 8	0.3	-45 ± 6	n.d.	n.d.	n.d.
NC		137 ± 9	0.2	+38 ± 6	97 ± 4	0.2	+42 ± 4	n.d.	n.d.	n.d.

Subsequently, with the aim of comparing distinct polymeric shells of the nanocapsules (and, consequently, different surface charges), we selected four polymers with different properties, the cationic polymers chitosan and polyarginine, the neutral polymer inulin and the negatively charged polymer carboxymethyl-β-glucan. Our group has developed polyarginine-coated nanocapsules for several applications [47–49], and reports have shown its activity in the generation of efficient immune responses against tumor antigens [50] and its adjuvant potential in a therapeutic HCV vaccine that reached phase II clinical trials [51]. On the other hand, neutral and negatively charged natural polymers such as modified inulin or beta glucans have also been gaining attention in this field, with some studies indicating their potential as vaccine adjuvants. In particular, an inulin-based microparticulate adjuvant (Advax™) has been studied with a variety of antigens, including influenza [52], Japanese encephalitis virus [53] and HIV-1 gp120 protein [54], showing significant effects in the immune responses elicited in different animal models. This adjuvant is currently in phase I/II clinical trials for a variety of infectious diseases (trial identifiers NCT02335164, NCT03066986, NCT01951677 and NCT03038776) [55]. In the case of beta glucans, they are present in the cell wall of several pathogenic agents such as bacteria and fungi, acting as pathogen-associated molecular patterns (PAMPs) that allow recognition by immune cells. In particular, these polysaccharides are known

to be recognized by the Dectin-1 receptor present in dendritic cells and macrophages, leading to the activation of these cells [56,57]. Studies with beta glucan particles obtained from the cell wall of *Saccharomices cerevisae* and loaded with model antigens such as ovalbumin have shown the potential of these vehicles to induce robust T-cell immune responses and high antibody levels in mice [58,59].

Considering this, a panel of four different nanocapsule prototypes with similar particle size (below 100 nm) but different surface charge was successfully developed and characterized, as summarized in **figure 2**. All systems have an oil previously included in adjuvant formulations (squalene or vitamin E) [60,61], in a core stabilized by different surfactants depending on the specificities of each polymeric coating. This formulation strategy led to nanocapsules with particle size between 80 and 100 nm, low polydispersity and surface charge between +30 and +55 mV for the cationic ones and between -20 and -40 mV in the case of the anionic ones.



	Core	Surfactant(s)	Coating	Size (nm)	PdI	Zeta Potential (mV)
SQL NE S	Squalene	Lecithin	---	87 ± 12	0.2	-45 ± 9
CS NC S			Chitosan	93 ± 9	0.2	+22 ± 2
SQL NE M			---	166 ± 13	0.1	-66 ± 3
CS NC M			Chitosan	238 ± 19	0.2	+32 ± 5
PArg NC	Vitamin E	TPGS Sodium cholate	Polyarginine	90 ± 11	0.2	+55 ± 4
Inulin NC	Vitamin E	Sodium glycocholate	Inulin	80 ± 5	0.2	-23 ± 5
CMβG NC	Squalene	Tween 80 CTAB	Carboxymethyl-beta-glucan	96 ± 8	0.2	-38 ± 3

Figure 2. *Schematic representation of the developed set of nanosystems. Mean particle size, polydispersity index (PdI) and zeta potential (measured by DLS) are indicated for each prototype. SQL, squalene; NE, nanoemulsions; CS, chitosan; S, small; M, medium; PArg, polyarginine; CMβG, carboxymethyl-beta-glucan; NC, nanocapsules.*

All prototypes were labelled with a fluorescent dye, DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate), encapsulated within the core of the nanosystems. The efficiency of this encapsulation was high in all cases as shown in **table 2**, and the physicochemical characteristics of the nanosystems were not significantly modified by this process. Moreover, the leakage of the dye during storage, measured after centrifugation of the nanosystems, was very low or negligible.

Table 2. *Encapsulation efficiency and leakage upon storage of DiD-loaded nanocapsules and control nanoemulsions. Results are shown as mean ± SD of 3 replicates. SQL, squalene; NE, nanoemulsion; CS NC, chitosan nanocapsules; S, small; M, medium; PArg, polyarginine; CMβG, carboxymethyl-β-glucan; n.d., not determined.*

	Theoretical DiD concentration	Encapsulation efficiency (%)	Leakage (day 7) (%)	Leakage (day 14) (%)	Leakage (day 21) (%)
SQL NE S	50 µg/mL	95.7 ± 0.9	4.2 ± 1.0	3.7 ± 1.3	4.6 ± 0.2
CS NC S		94.7 ± 0.3	6.5 ± 1.4	5.0 ± 0.9	7.8 ± 0.4
SQL NE M		93.2 ± 0.2	4.1 ± 0.5	4.9 ± 0.5	4.7 ± 1.4
CS NC M		97.2 ± 0.3	1.6 ± 0.5	2.7 ± 0.5	4.1 ± 1.5
PArg NC	20 µg/mL	93.8 ± 0.7	0.7 ± 0.1	n.d.	n.d.
Inulin NC		100	n.d.	n.d.	n.d.
CMβG NC		84.6 ± 1.9	8.7 ± 1.0	4.6 ± 1.9	2.1 ± 1.3

Finally, the stability of the fluorescent nanocapsules in storage conditions was also assessed and considered appropriate for the type of studies to be performed, as can be seen in **figure 3**. In addition, a similar study was also done for the formulations used in cell studies, analyzing the colloidal stability of the nanosystems in contact with the cell culture media, as well as the DiD released in these conditions (**figure 4**).

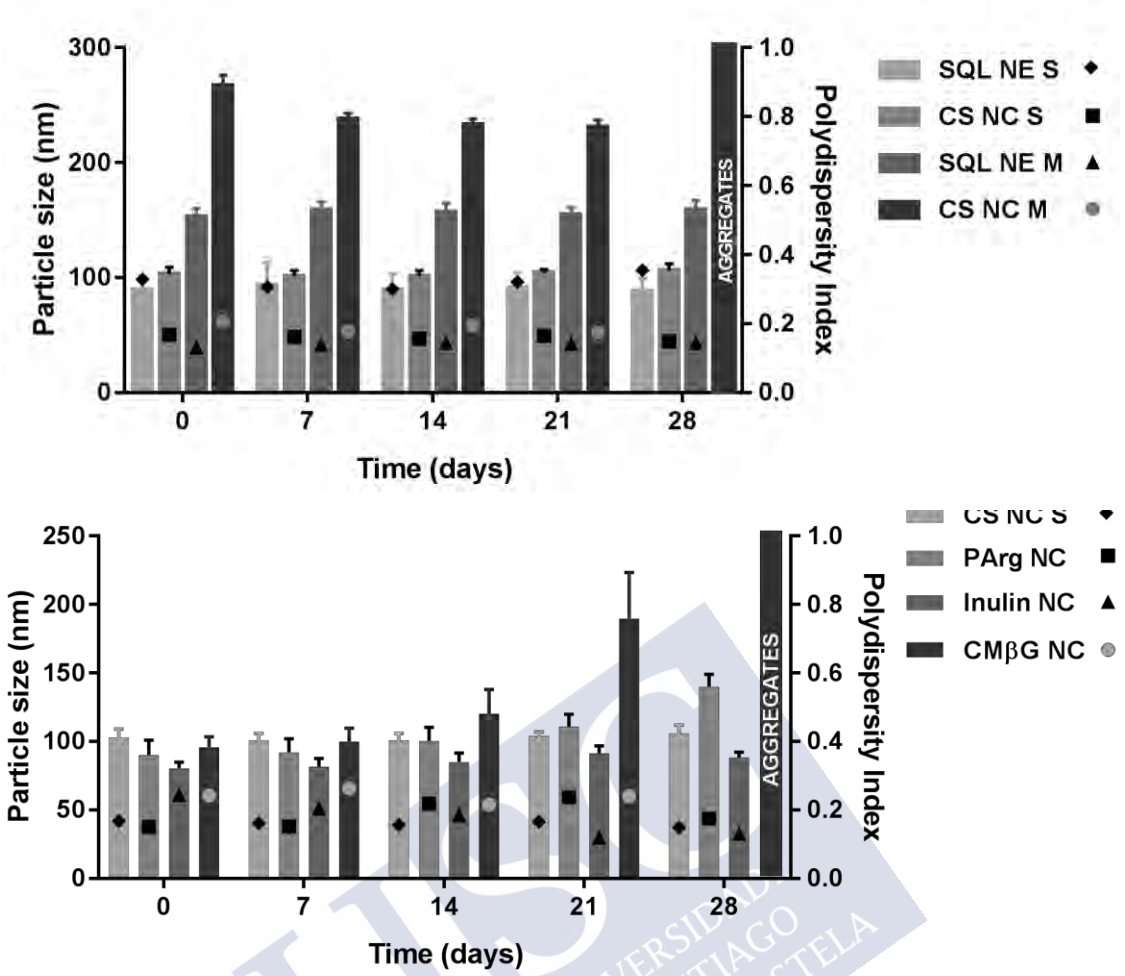


Figure 3. *Colloidal stability of the fluorescent-labelled nanosystems in storage conditions (4ºC). Results are shown as mean ± SD of at least three replicates.*

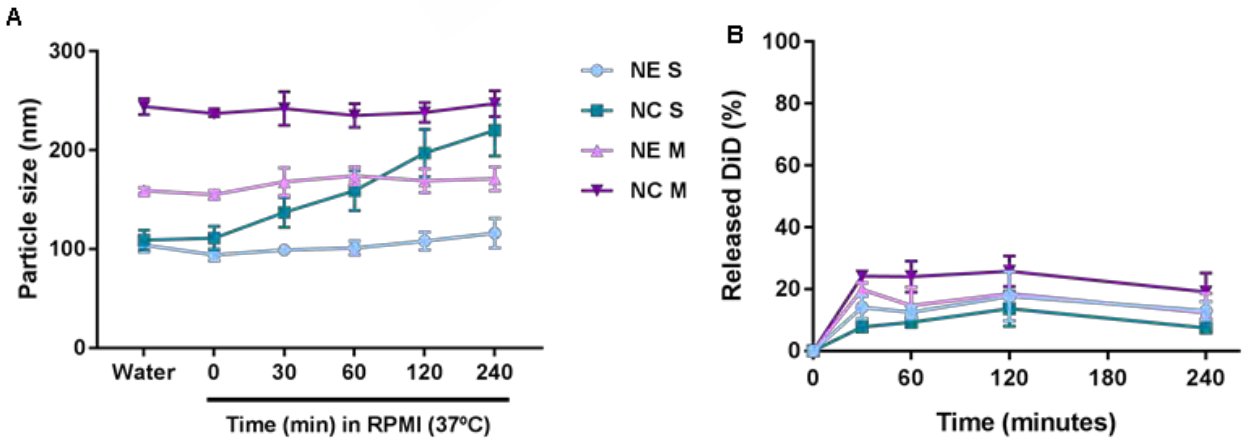


Figure 4. *Stability of the nanosystems (A) and DiD release (B) upon incubation with cell culture media (37 ºC). Results are shown as mean ± SD of at least three replicates.*

3.2. Interaction of chitosan nanocapsules with macrophages: routes of internalization and induction of cytokine secretion

The first necessary step for the efficacy of any delivery system is its interaction with the target cells. In the case of antigen nanocarriers, in order to achieve a robust immune response against the transported antigen, it is critical to achieve an effective interaction with immune cells. In general, the main antigen presenting cells (APCs), macrophages and dendritic cells, should capture nanocarriers at the administration site or in the draining lymph nodes. This process should then lead to an adequate processing of the antigen and to its presentation to effector cells such as T or B lymphocytes. Furthermore, the pathway used by the cells to internalize the carriers may strongly influence their intracellular fate and the outcome of their biological effect. In fact, the influence of different uptake pathways on the type of immune response generated upon internalization of antigen carriers has been previously shown [62,63].

As highlighted by several authors, by adjusting specific characteristics of the nanocarriers with the immune cells it might be possible to modulate their interaction with these cells and, possibly, to improve the elicited immune response [44,64]. Within this frame, the affinity of specific pattern-recognition receptors such as Toll-like receptors (TLR) for the nanosystems may also be of interest for their use as antigen carriers [65]. Considering this, in our work we focused first on studying the effect of the particle size (below 100 nm and above 200 nm) on the interaction of chitosan nanocapsules with macrophages and on the importance of Toll-like receptor 4 (TLR 4) and scavenger receptor A (SR-A) in their recognition. The routes of internalization used by the cells to take up the nanocapsules were evaluated using specific inhibitors for phagocytosis, macropinocytosis, clathrin-mediated and caveolae-mediated endocytosis. The cytokine profile elicited upon recognition of both types of nanocapsules was also studied in order to further characterize the influence of this parameter in their adjuvant potential. The concentrations selected for their studies were based on previous studies by our group [31].

As shown in **figure 5,** a clear internalization was observed for all formulations in wild-type macrophages. Confocal microscopy images show a homogenous distribution of nanoemulsions and nanocapsules in the cytoplasm. This result was not surprising as it has already been shown that nanosystems, independently of their nature, size, shape or charge can easily enter macrophages in culture [66,67].

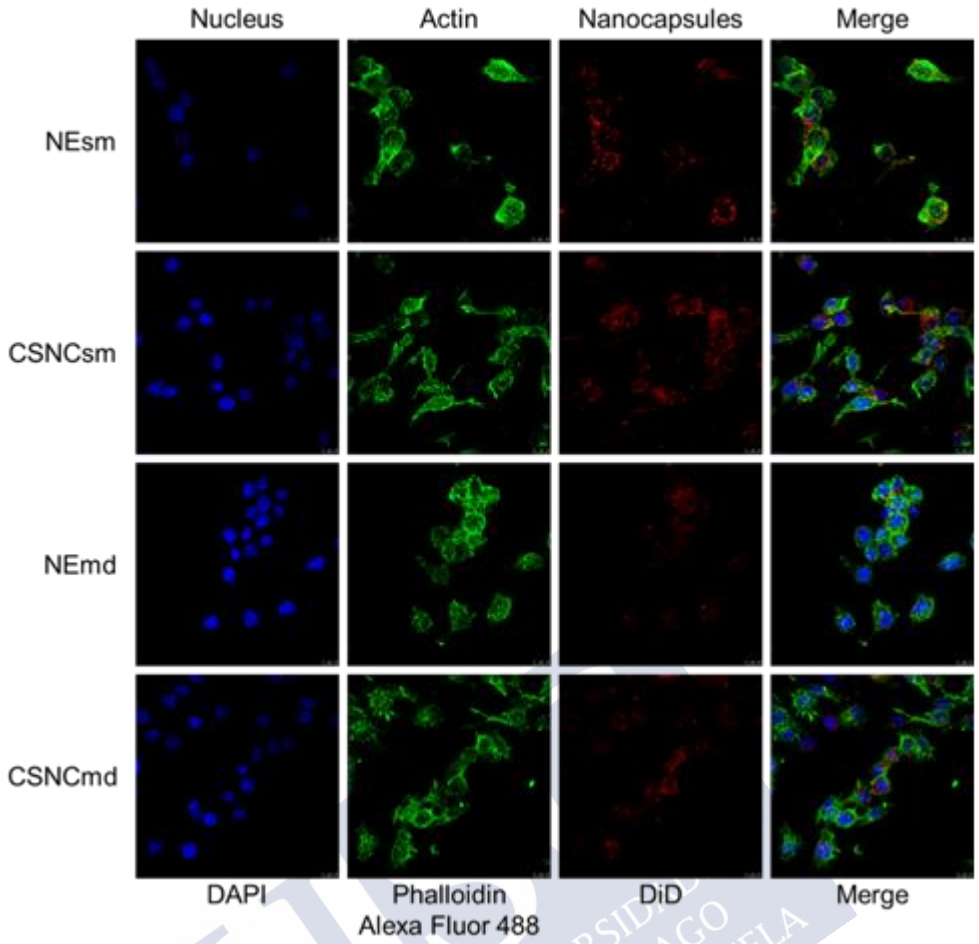


Figure 5. *Confocal microscopy images of the uptake of fluorescent nanosystems by Raw 264.7 cells after 1 hour incubation (0.3 mg/mL of nanoemulsions or nanocapsules). Nuclei are labelled with DAPI (blue), actin filaments with phalloidin Alexa Fluor 488 (green) and nanocapsules with DiD (red). Images display the maximum projections from the z-stack series for each channel and for the merged image.*

To evaluate the role of TLR 4 in the recognition and uptake of chitosan nanocapsules, we used wild-type and TLR 4 knockout bone-marrow derived macrophages. Upon incubation of these cells with the four prototypes, we measured the concentration of two cytokines (TNF-α and MCP-1) secreted by the cells, as an indication of cell activation. The results in **Figure 6** indicate that both nanoemulsions and nanocapsules generate very low responses in terms of TNF-α levels. However, in the case of MCP-1, statistically significant differences were observed in the levels achieved for small and medium size nanocapsules. Indeed, small chitosan nanocapsules (100 nm) elicited significantly higher MCP-1 levels than the medium-sized nanocapsules, particularly in TLR 4 (-/-) cells. The effect of chitosan *per se* on the TLR 4 receptor has been previously described

[68,69], however, in these previous studies a marked increase in the TNF-α levels was also observed. The difference between these previous studies and our study may rely on the type, purity and concentration of chitosan. Nevertheless, from our knowledge, the influence of the particle size in the specific recognition of chitosan nanocapsules by the TLR 4 receptor has not been described yet and may provide new insights on the mechanisms of immunostimulation of this type of nanocarriers.

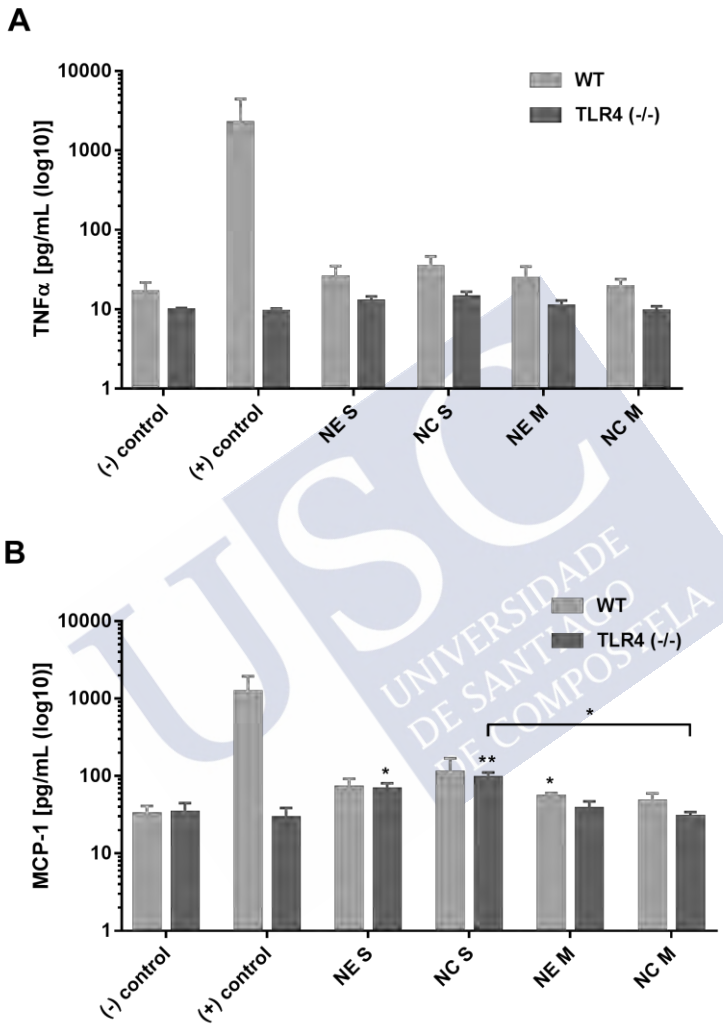


Figure 6. *Production of TNF-α (A) and MCP-1 (B) by bone marrow-derived macrophages from wild type (dark blue) and TLR 4 (light blue) knockout mice, upon 24 hours of incubation with the studied prototypes at 25 μg/mL (chitosan concentration in nanocapsules and equivalent in nanoemulsions). Negative control represents untreated cells and positive control represents cells treated with lipopolysaccharide. Results show mean ± SD of 3 replicates. Statistically significant differences correspond to nanostructures vs. negative control unless otherwise indicated (* p<0.05, ** p<0.01).*

Scavenger receptor A is commonly expressed in macrophages and generally responsible for the phagocytosis of microorganisms and apoptotic cells, as well as for the recognition of modified lipids, proteins and polyanions [70]. To study the influence of this receptor in the recognition of the developed nanocarriers, we generated a clone of Raw 267.4 macrophages (clone 1/2) with partially silenced expression of SR-A. This silencing was achieved through transduction with a virus, carrying siRNA that targeted this receptor, and the efficiency of the process was assessed by Western blot (data not shown). In agreement with previous studies [32], the silencing was efficient and stable, and we used the cloned cell line to compare with wild-type Raw 264.7 macrophages. Since SR-mediated phagocytosis is usually not accompanied by inflammatory response, we were interested in evaluating the influence of this receptor on the uptake of chitosan nanocapsules and control nanoemulsions. As depicted in **figure 7**, the uptake of all formulations was generally reduced in cells lacking SR-A expression, although the difference in uptake based on the presence or absence of this receptor was more significant for the small nanosystems. Moreover, the internalization of nanocapsules was, in all cases, significantly higher than that of nanoemulsions. Given the previously described preference of SR receptors for polyanionic ligands [70], we can hypothesize that the cationic nanocapsules may be covered by a protein corona when in contact with cell media, providing these systems with more appropriate conditions for SR-mediated uptake. In fact, Yan *et al* have described the involvement of these receptors in the internalization of poly(methacrylic acid) nanoparticles coated with bovine serum albumin but not in the absence of this coating [71]. Other authors have also reported the role played by this type of receptors in the uptake of different nanoparticles, including polymeric [72], metallic [32] and nucleic acid-based systems [73].

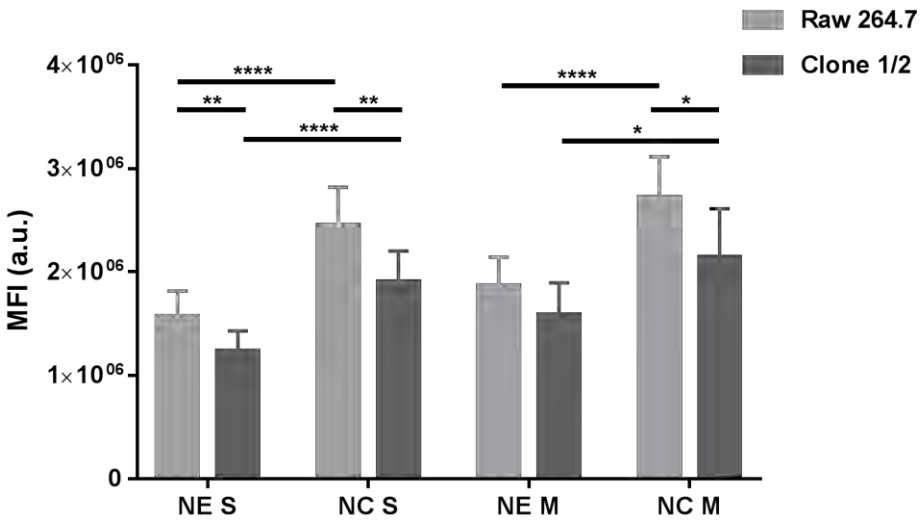


Figure 7. *Evaluation of the role of scavenger receptor (SR-A) in the recognition and internalization of nanosystems. Graph represents the median fluorescence intensity (MFI) emitted by the DiD-labelled nanosystems upon internalization by wild-type macrophages (Raw 264.7) and by macrophages with partially silenced SR-A expression (clone 1/2). Results show mean ± SD of 9 replicates; NE, nanoemulsions; NC, nanocapsules; * p<0.05, ** p<0.01, **** p<0.0001.*

Subsequently, we used both cell lines to evaluate the different internalization routes of the nanocapsules. For this purpose, we treated the cells with one of four inhibitors corresponding to the main routes chosen by APCs to uptake any kind of particulate material. In general, particles larger than 500 nm are internalized by phagocytosis or macropinocytosis, while those below that value usually enter the cells via other endocytic pathways. Among these, clathrin- and caveolin-mediated endocytosis are the most studied, being usually responsible for the internalization of particles up to 200 nm in size [74]. Considering this, we preincubated wild-type and clone 1/2 macrophages with cytochalasin D (phagocytosis inhibitor), 5-(N,N-dimethyl)amiloride hydrochloride (DMA, macropinocytosis inhibitor), chlorpromazine hydrochloride (clathrin-mediated endocytosis inhibitor) or filipin III (caveolae-mediated endocytosis inhibitor) before treatment with nanocapsules and control nanoemulsions.

The results in **figure 8** indicate that most inhibitors led to a reduction of the internalization rate, as depicted. This was particularly evident in the case of cytochalasin D, reaching 50-60% of inhibition for most formulations. This inhibitor acts on the polymerization of actin, which is essential for both phagocytic and macropinocytic uptake routes, not providing specific information about only one of them. However, using DMA, which is

specific to the membrane sodium/hydrogen ATPase and therefore specific to macropinocytosis, no significant inhibition was observed. This leads to the conclusion that this pathway probably does not have much influence on the uptake of chitosan nanocapsules, while phagocytosis appears to be one of the main routes used by macrophages for the internalization of these nanosystems. Considering size comparison, the only statistically significant influence observed was with the phagocytosis inhibitor cytochalasin D. In this case, smaller chitosan nanocapsules were less internalized than medium-sized ones after the treatment with the inhibitor, corroborating the important influence of phagocytosis in the uptake of the smaller prototypes. Though, as previously mentioned, this uptake route is more common for larger particles, other authors have also reported receptor-mediated phagocytosis as the main pathway for the internalization of 30 nm metallic [32] and 110 nm polystyrene particles [75].

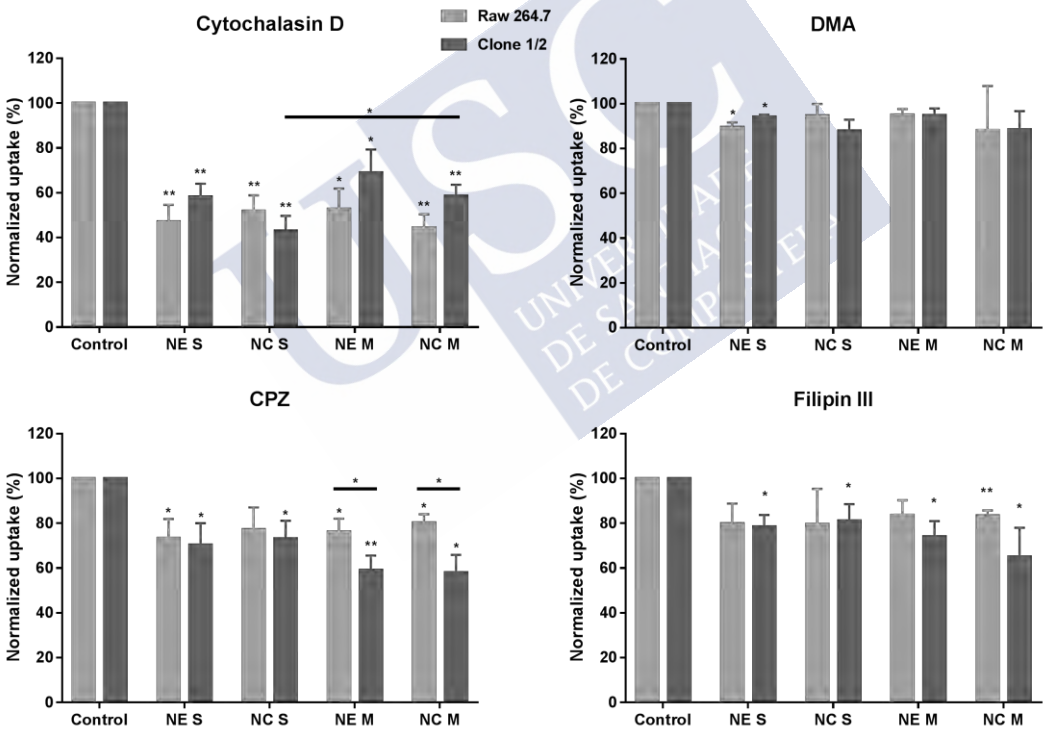


Figure 8. *Evaluation of the internalization routes used by macrophages (Raw 264.7 and clone 1/2) for the uptake of the studied nanosystems. Studies were performed in the presence of* ***Cytochalasin D*** *(phagocytosis inhibitor),* ***5-(N,N-dimethyl)amiloride hydrochloride (DMA)*** *(macropinocytosis inhibitor),* ***chlorpromazine hydrochloride (CPZ)*** *(inhibitor of clathrin-mediated endocytosis) and* ***Filipin III*** *(inhibitor of caveolin-mediated endocytosis). Median fluorescence intensity (MFI) observed in cells treated with inhibitors was normalized to the one from untreated cells (100% uptake values).*

*Results show mean ± SD (n=3) of normalized uptake (%). Statistically significant differences indicated above the columns correspond to nanosystems vs. positive control. NE, nanoemulsions; NC, nanocapsules; * p<0.05, ** p<0.01.*

Despite this observation, the same tendency in size-dependent internalization was not seen when using other inhibitors, leading to the hypothesis that a combination of factors, in addition to particle size, may be responsible for the different uptake pathways chosen by the cells. In fact, as previously described by other authors, endocytosis of nanoparticles depends on several parameters including particle size and surface charge but also cell type and cell status at the moment of particle recognition [67,76]. Moreover, it is worth considering the possibility of a different chitosan density at the surface of smaller and medium-size prototypes, given the higher surface area of the smaller nanocapsules. This difference may influence the interaction of chitosan with surface receptors and collaborate in directing the endocytosis of the particles. Therefore, further studies are necessary to validate this hypothesis, evaluating the amount of chitosan attached to the nanocapsules surface in each case.

Considering this, it is understandable that our results evidence a combination of multiple internalization routes used for the uptake of the formulations, somehow irrespective of their size. In fact, a comprehensive study performed by Firdessa *et al* described a similar behaviour using polystyrene nanoparticles [76]. Bone-marrow derived macrophages internalized the 100 nm non-functionalized nanoparticles through a combination of pathways that were visualized using electron microscopy and confirmed using inhibitors. In the case of microparticle uptake in cancer cells, several pathways were also described for this process, including macropinocytosis, phagocytosis and lipid raft-mediated endocytosis [77]. Similar results were observed with other polymeric carriers, such as hydrophobically-modified chitosan nanoparticles [78], mannan nanogels [79] or heparin-based functionalized nanoparticles [80], and with lipid-based nanosystems [81], showing that the use of multiple pathways for cellular internalization of particles is a common feature of this type of structures. Additionally, it is also possible that some compensation effect occurs over the endocytic pathways, with some routes being more used when others are blocked.

Finally, it is worth noting that, particularly in the case of clathrin-mediated endocytosis, the uptake inhibition was more significant in the cells lacking of SR-A expression, suggesting an involvement of this receptor in the recognition of particles for subsequent clathrin coating. This observation was particularly relevant in the case of medium-sized

nanocarriers, which could be interpreted as a combination of factors, namely particle size and SR-A involvement, leading to a higher inhibition of this pathway. These results are in accordance with previous reports, which mention the combined role of scavenger receptors and clathrin-mediated pathways in the uptake of different types of nanoparticles by macrophages [82–84].

Overall, our *in vitro* results show that macrophages use a combination of several uptake routes for the internalization of chitosan nanocapsules and control nanoemulsions of both sizes. However, it is worth noticing that the smaller nanocapsules appear to be preferentially internalized by phagocytosis, particularly mediated by scavenger receptors. Moreover, despite the generally low levels of cytokines elicited by all formulations, the smaller chitosan nanocapsules were able to elicit higher MCP-1 secretion than the medium-sized ones, possibly in a TLR 4-mediated process. Several authors have reported a size-dependent efficiency in nanoparticle internalization by macrophages, with smaller particles usually being better internalized than bigger ones [76,85]. However, contradictory results can also be found in this matter, with larger particles outperforming smaller ones in terms of cell uptake [86,87]. Additionally, we should not discard the possibility of a direct influence of the chitosan shell and density in these processes, as previously mentioned. In general, these results provide further insights into the potential of particles with size below 100 nm to efficiently interact with immune cells.

3.3. Biodistribution of nanocapsules with distinct physicochemical characteristics in the lymphatic system

A complete understanding of the fate of antigen nanocarriers after administration includes not only the knowledge about the mechanism of their interaction with the immune cells, as described in the previous section, but also their biodistribution. Most vaccines are currently administered through subcutaneous or intramuscular injections, leading to a deposition of the antigen-adjuvant mix in the interstitial matrix surrounding the injection site. However, when the antigen is associated to a nanocarrier there is the possibility for them to drain freely to the closest lymph node, and this possibility depends on the characteristics of the nanocarrier, such as particle size and surface charge. Authors have shown that particles smaller than 100 nm are more efficiently drained to the lymphatic vessels, while larger particles seem to be transported after uptake by APCs [12,88]. Moreover, hydrophilic coatings on different nanosystems have also been described as facilitating their free drainage from the injection site [24,89]. There is also

the possibility for the antigen-loaded carrier to be captured at the injection site by phagocytic immune cells, such as circulating macrophages and dendritic cells and, then, be transported by these cells to the lymph nodes.

In this study, we analysed the effect of the physicochemical properties and composition of the nanocapsules on their access to the lymphatic system, following subcutaneous administration to mice. The prototypes analysed were chitosan nanocapsules with two different particle sizes (below 100 nm and above 200 nm), as well as nanocapsules with three different coatings (polyarginine, inulin and carboxymethyl-β-glucan). The physicochemical properties of these nanocapsules are presented in **figure 2**. For the biodistribution study, we combined imaging and cytometry techniques, using both 2-photon microscopy and fluorescence-activated cell sorting (FACS).

3.3.1. Influence of the particle size of chitosan nanocapsules

First, we evaluated the distribution of chitosan nanocapsules with two different particle sizes (S, below 100 nm and M, above 200 nm) upon subcutaneous administration to mice. Formulations and cell-labelling antibodies were injected in the footpads of the animals and draining lymph nodes, popliteal and lumbar, were excised 12 hours after injection. This time point was selected since it is the average time required for the innate branch of the immune response to act as a first line of defence. Subsequently, 2-photon imaging of these organs allowed the observation of a distinct accumulation of nanocapsules mostly in the medullary region of both lymph nodes. As depicted in **figure 9**, it was possible to quantify the fluorescence intensity observed for each marker by image analysis. Despite the high variability observed, common in imaging techniques, small nanocapsules showed a clear tendency to accumulate in the lymph nodes more significantly than medium-sized ones. This is particularly evident in the popliteal lymph node, which is the first node draining from the injection site.

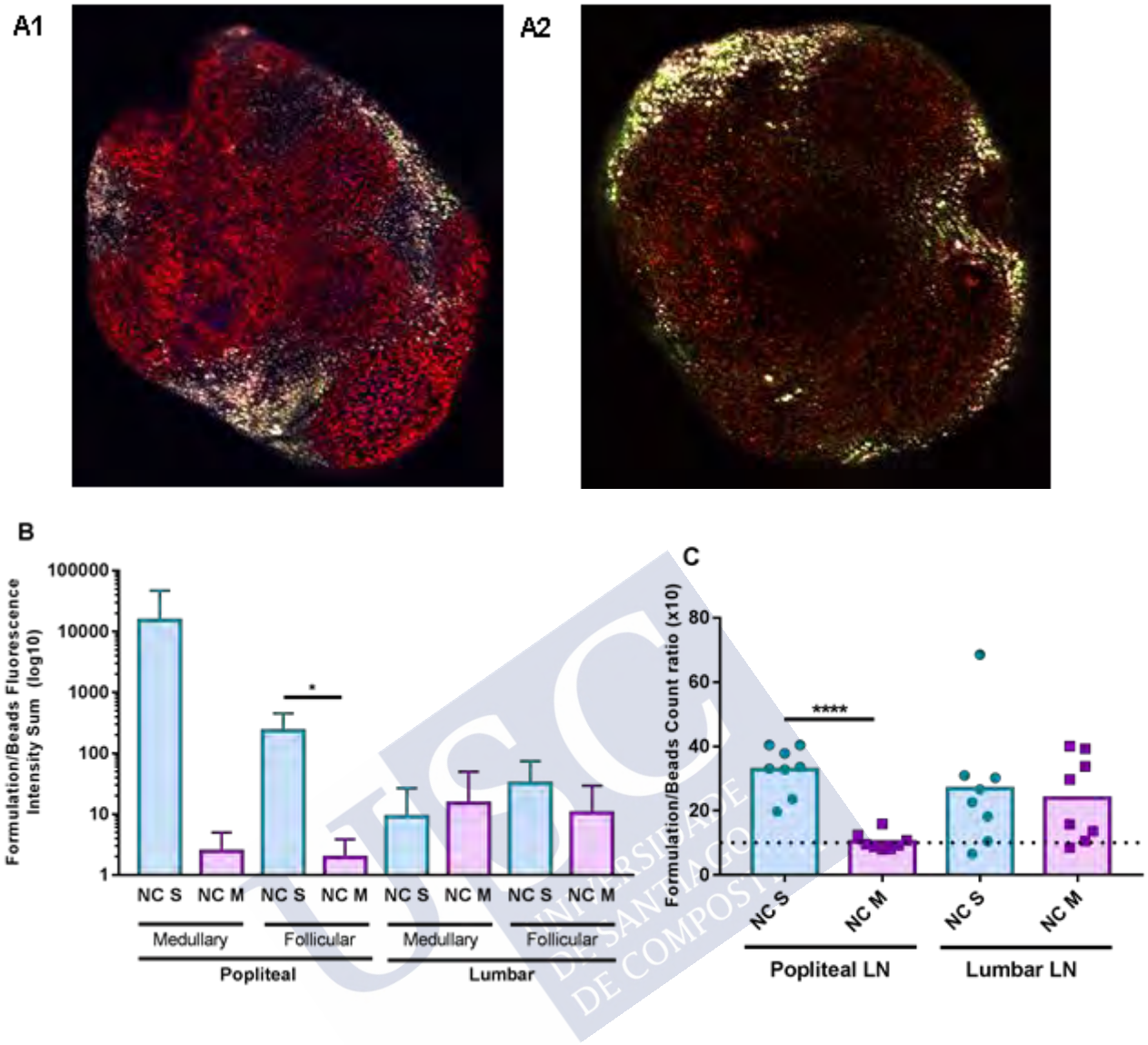


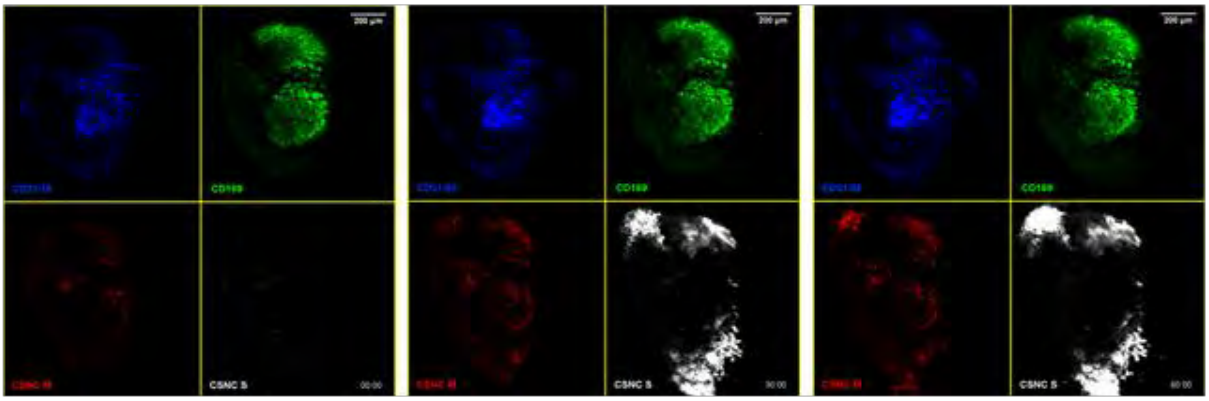
Figure 9. *Distribution of DiD-labelled CSNC of two particle sizes (NC S, below 100 nm and NC M, above 200 nm) in popliteal and lumbar lymph nodes, after subcutaneous injection. Ex vivo, 2-photon microscopy images show the accumulation of the nanosystems (A1, NC S and A2, NC M) in the lymph node. Macrophages are shown in red (CD169-PE), nanocapsules in white (DiD) and control polystyrene beads in green (Firefli™ Fluorescent Green). Fluorescence intensity was quantified in the medullary and follicular regions of the lymph nodes by image analysis (B) and in total by flow cytometry (C). Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanocapsules and beads). * p<0.05, **** p<0.0001*

The flow cytometry results allowed us to confirm those obtained with the imaging techniques used. In fact, normalizing the number of fluorescent particles quantified by

FACS with the number obtained for co-injected control polystyrene beads, we could observe a statistically significant greater accumulation of the small nanocapsules, as compare to the larger ones, in the popliteal lymph node. This result agrees with those reported for model polystyrene particles [88] and with those recently reported by our group for polyaminoacid nanocapsules [20].

To further elucidate the dynamics of particle transport from the injection site to the draining lymph nodes, we used intravital microscopy to achieve *in vivo* real-time videos of the particle arrival to the popliteal lymph node. Through this technique, it is possible to monitor the trafficking of the fluorescent-labelled nanocapsules with different sizes in real time, particularly when co-injected at the same moment. In **figure 10**, we can see two still-frames of a representative video obtained, taken at 15 and 45 minutes post-injection. All acquired videos clearly showed the arrival of the smaller nanocapsules at earlier time points (around 8-10 minutes post-injection), while medium-sized nanocapsules only began accumulating at the lymph node around 30 minutes post-injection and to a much lower extent. Analysing the images obtained, we could also translate these results as graphical representations of the mean fluorescence intensity recorded for the nanocapsules as a function of time, as displayed also in **figure 10**. The graphs show the tendency observed in the videos, with the smaller nanocapsules arriving before and accumulating more in the lymph node than their medium-sized counterparts. These results also allow us to assume a minimal influence of the injection pressure on the arrival of the nanosystems to the draining lymph node, since the videos do not show an immediate appearance of the nanocapsules. Thus, the faster and stronger accumulation of smaller nanocapsules in the popliteal lymph node upon subcutaneous administration in the footpad must be influenced by intrinsic parameters such as particle size, surface charge and composition.

A



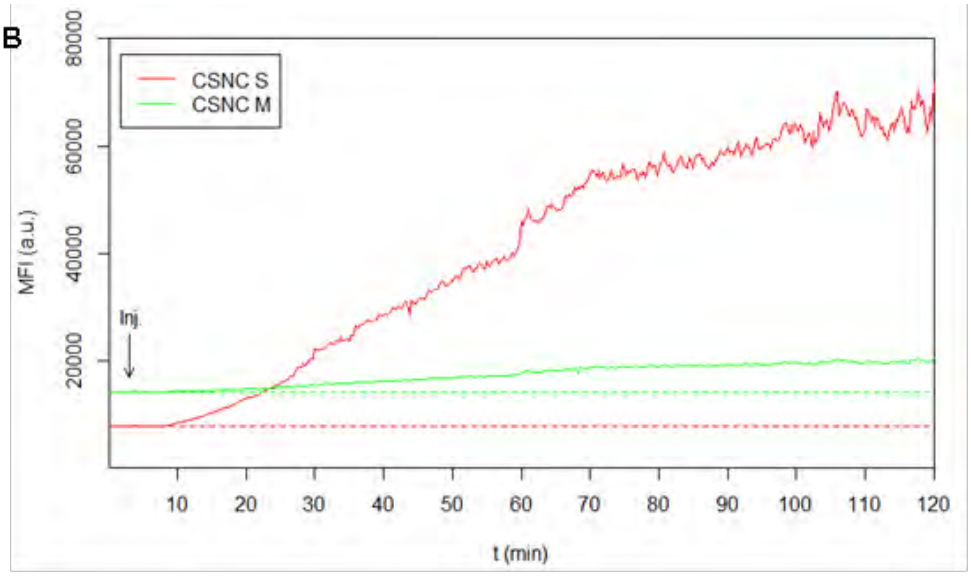


Figure 10. *Dynamics of the arrival of chitosan nanocapsules (CSNC S, below 100 nm and M, above 200 nm) to the popliteal lymph node after subcutaneous injection. (**A**) Representative still-frames of one of the video sequences acquired at 0, 30 and 60 minutes (as indicated in the image), with macrophages labelled in green (CD169-FITC), CSNC S in white (DiD) and CSNC M in red (Nile Red). (**B**) Graphical representation of the median fluorescence intensity acquired for DiD- (CSNC S) and Nile Red- (CSNC M) labelled nanocapsules as a function of time (injection time is indicated with an arrow).*

Upon arrival at the lymph node, it is essential that antigen nanocarriers interact with the main APCs, namely macrophages and dendritic cells. The subcapsular sinus is the region of the lymph node where the freely drained antigens and particulate materials first arrive, encountering mostly macrophages. These cells sample the flowing lymph to internalize the foreign materials and make them available to B cells in the cortical region of the lymph node. On the other hand, the medullary region is rich not only in macrophages but also in dendritic cells, which are major players in the triggering of immune responses [28]. Bearing this in mind, we were interested in studying the trafficking of the different nanocapsule prototypes within the lymph nodes and their possible co-localization with different cell types. For this purpose, we quantified the number of particles co-localized with three types of APCs by flow cytometry, in particular subcapsular sinus macrophages, medullary macrophages and dendritic cells.

As depicted in **figure 11**, we could observe a significantly higher accumulation of the small size chitosan nanocapsules in the three cell types and, notably, co-localizing with medullary macrophages and dendritic cells. The explanation for this could be found in a

study by Manolova *et al,* who showed the early co-localization of small particles in the subcapsular sinus region (2h), and a subsequent distribution to other deeper areas of the lymph node [88]. In fact, at the time point selected in our study (12 hours post-injection), we observe that small chitosan nanocapsules had already passed through the subcapsular sinus and were accumulating in the more internal regions of the lymph node, probably internalized by medullary macrophages and DCs. These results allowed us to conclude that particle size is determinant in the drainage of chitosan nanocapsules administered subcutaneously. In particular, small-sized chitosan nanocapsules were able to arrive faster to the draining lymph node, accumulating more in that organ and co-localizing more with the main APCs present there. This profile could lead to a better outcome in terms of antigen presentation and immune response generated.

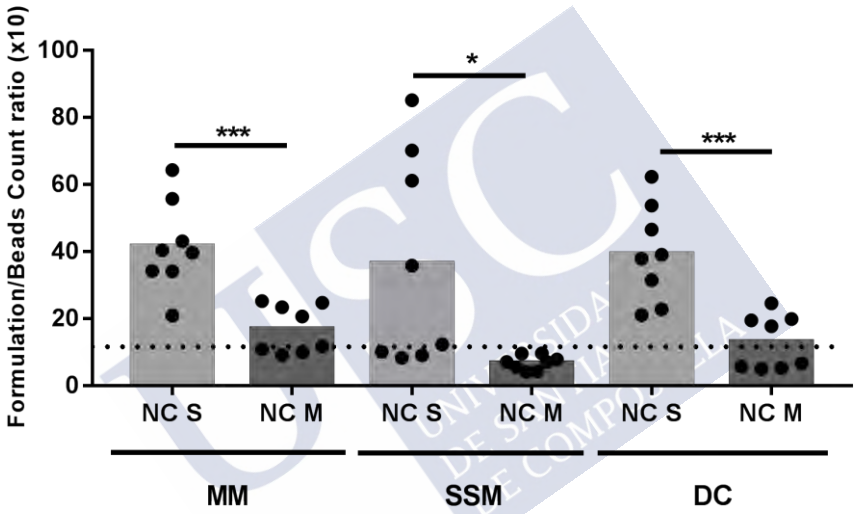


Figure 11. *Differential co-localization of DiD-labelled chitosan nanocapsules of two particle sizes (NC S, below 100 nm and NC M, above 200 nm) with immune cell populations in popliteal lymph node, after subcutaneous injection. Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanocapsules and beads). MM, medullary macrophages; SSM, subcapsular sinus macrophages; DC, dendritic cells; * p<0.05, *** p<0.001.*

3.3.2. Influence of the chitosan shell of the nanocapsules

In order to investigate the role of the chitosan shell in the biodistribution of chitosan nanocapsules in the popliteal and lumbar lymph nodes, we compared their biodistribution with that of control nanoemulsions, which have similar properties and composition, with the exception of the chitosan shell.

The results in **Figure 12** indicate that, as expected, the small nanocapsules accumulate in the popliteal lymph node in a higher extent than the medium-sized ones. Surprisingly, the accumulation of the small size NE was lower than that of the small nanocapsules. In contrast, the accumulation of medium size NE was superior to that of the nanocapsules. A similar trend was observed in the lumbar lymph nodes, although in this case, the accumulation was reduced for all formulations. The greater accumulation of the medium size NE in both lymph nodes, in comparison with all other formulations, could be related with their possible easier diffusion through the interstitial tissue and facilitated access to the lymph node chain, given their negative surface charge. Alternatively, they could also be captured by APCs at the injection site and quickly transported to the lymph node. In fact, similar results were reported by Makidon *et al*, according to which a soybean-based oil-in-water nanoemulsion was efficiently captured by mature dendritic cells and transported to the draining lymph node [90]. Nevertheless, and despite their use as adjuvants in marketed vaccines, only few reports approach the distribution of lipid-based nanoemulsions to the lymph nodes. In this regard, other nanoemulsions have been developed specifically for lymph node accumulation, with diagnostic or theranostic purposes [91,92].

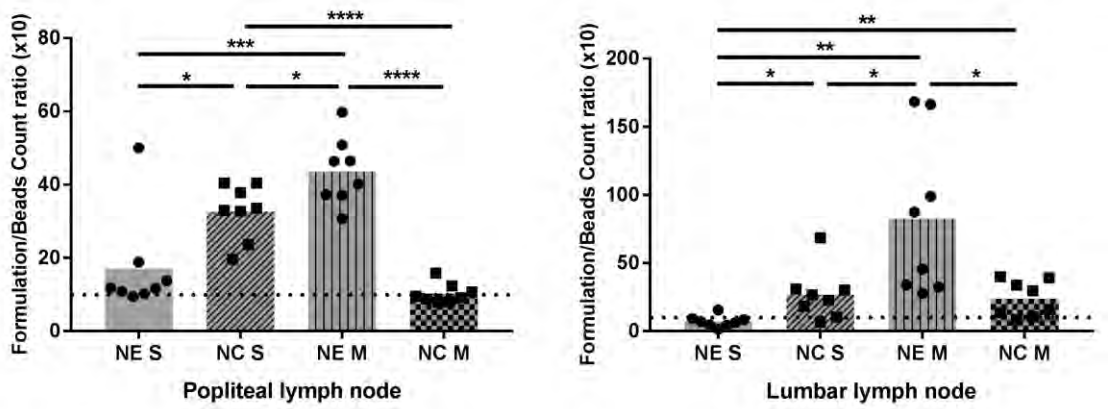


Figure 12. *Distribution of DiD-labelled chitosan nanocapsules (NC) and control nanoemulsions (NE) with two particle sizes (S, below 100 nm and M, above 200 nm) in popliteal and lumbar lymph nodes, after subcutaneous injection. Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanosystems and beads). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

However, smaller nanoemulsions seem to contradict this behaviour, with low accumulation numbers in both lymph nodes studied. In this case, we may consider the possibility that, given their small size and negative surface charge, they could readily diffuse and not remain retained in the proximal lymph nodes analysed. Further studies are necessary to confirm this hypothesis and evaluate the mechanism behind this behaviour.

3.3.3. Influence of the polymeric nature of the nanocapsules' shell

With the objective of analysing the relevance of the polymeric shell of the nanocapsules in their biodistribution, we performed lymph node accumulation studies with a new panel of nanocapsules. In this case, we compared four prototypes of nanocapsules with similar particle size (all below 100 nm) and different polymeric shells. In detail, we used chitosan and polyarginine nanocapsules as cationic prototypes and inulin and carboxymethyl-β-glucan nanocapsules as anionic ones. As previously described, these polymers were selected based on their adjuvant capacities when presented as such, or in the form of nanoparticulate carriers [59,93–95]. However, a comparison between them in terms of their ability to access the lymphatics when incorporated in a nanocarrier has never been reported.

Results showed different accumulation profiles for all prototypes, depending on their surface charge but also on the lymph node studied. As shown in **figure 13**, cationic nanocapsules accumulated more in the popliteal lymph node than their anionic counterparts do. This was particularly significant in the case of polyarginine nanocapsules (**figure 13D**), and appears to be strongly connected to the surface charge of the carriers. Likely, the cationic charge of the chitosan and polyarginine nanocapsules leads to a higher interaction with the immune cells in the popliteal lymph node, given the electrostatic interactions between cationic particles and anionic cell membranes [22,96]. On the contrary, anionic nanocapsules may experience electrostatic repulsion to cells [19,97], and drain along the lymphatics instead of remaining stuck in the first draining lymph node, therefore presenting reduced levels of accumulation in the popliteal node.

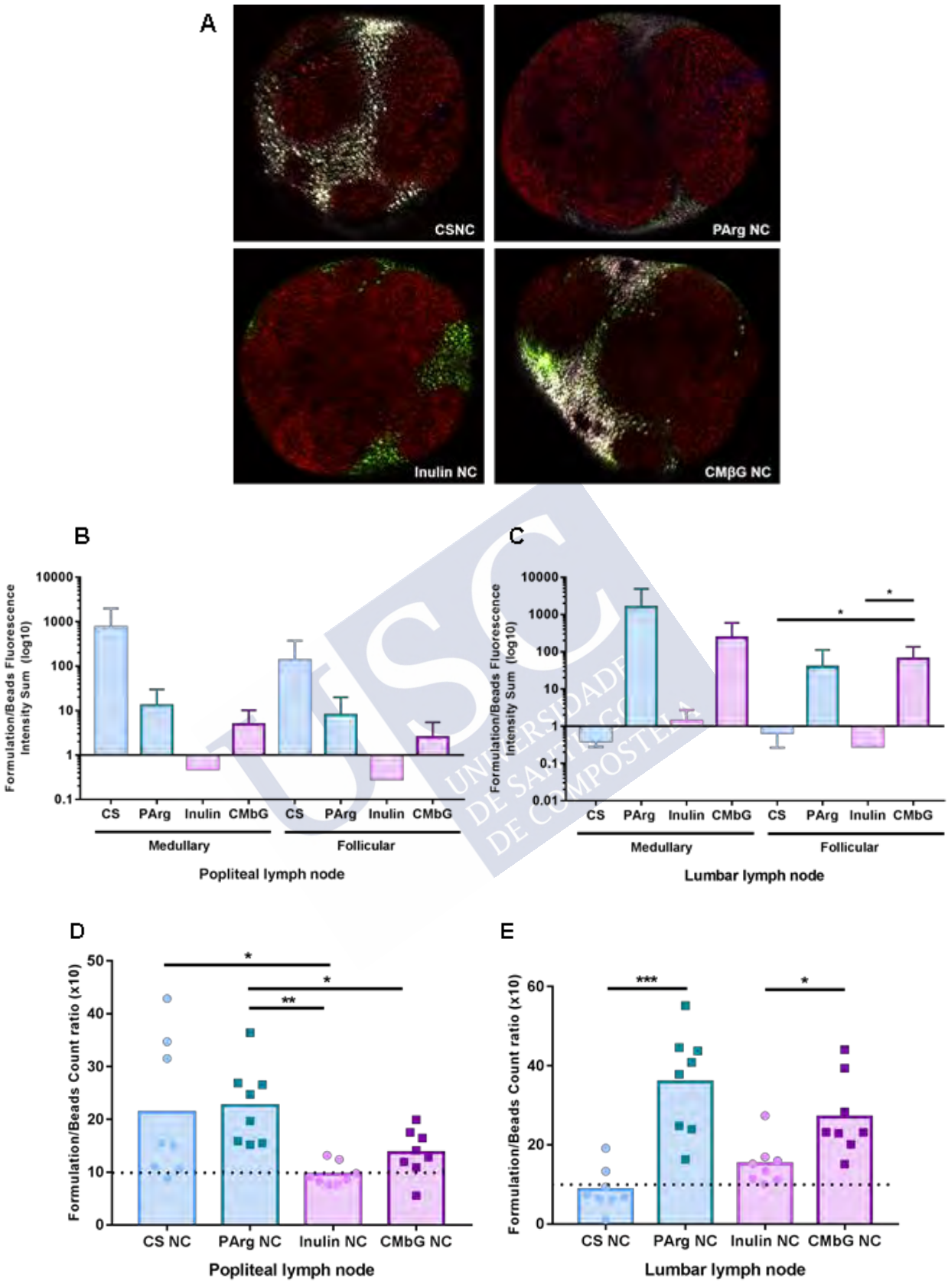


Figure 13. *Distribution of DiD-labelled chitosan (CS), polyarginine (PArg), inulin and carboxymethyl-beta-glucan (CMbG) coated nanocapsules (60-90 nm) in popliteal and*

*lumbar lymph nodes, after subcutaneous injection. Ex vivo, 2-photon microscopy images (**A**) show the accumulation of the nanosystems in the popliteal lymph node. Macrophages are shown in red (CD169-PE), nanocapsules in white (DiD) and control polystyrene beads in green (Firefli™ Fluorescent Green). Fluorescence intensity was quantified in the medullary and follicular regions of the lymph nodes by image analysis (**B, C**) and in total by flow cytometry (**D, E**). Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanocapsules and beads). * p<0.05, ** p<0.01, *** p<0.001*

When analysing the lumbar lymph nodes of the animals, we observed a statistically significantly higher accumulation of polyarginine and carboxymethyl-β-glucan nanocapsules in comparison with chitosan and inulin ones. These results evidence that not only the surface charge influences the distribution of the nanocapsules in the lymphatic system but also the intrinsic specificities of the polymer used may play a role in this process. On one hand, polyarginine nanocapsules seem to drain well enough through the first draining lymph nodes and to accumulate strongly even in the more distant lumbar lymph node, at 12 hours post-injection. On the other hand, carboxymethyl-β-glucan nanocapsules seem to bypass the popliteal lymph node, with lower levels of accumulation, however, they eventually accumulated in the following node, in levels that were similar to those observed for polyarginine nanocapsules. Comparative studies that may help explain this behaviour are still lacking in literature. However, in the absence of these reports, we can speculate that the affinity of some APCs receptors for these polymers may lead to their increased accumulation in lymph nodes more distant from the injection site. The efficacy of polyarginine to be internalized by immune cells has been previously described, and found to be higher than that of other polycationic polymers [98,99]. Similarly, other beta glucans in particulate forms have shown fast drainage to the lymph nodes upon injection in mice and specific co-localization with macrophages within those lymph nodes [100–102].

Overall, we could observe a strong influence of both particle size and surface charge in the biodistribution of polymeric nanocapsules. Moreover, the nature of the polymers used was found to play an important role in this process, as shown by the results obtained with polyarginine and carboxymethyl-β-glucan nanocapsules. Therefore, a careful selection of the physicochemical characteristics of each particular carrier developed for antigen delivery is necessary to achieve optimal accumulation in the draining lymph

nodes and general statements for specific parameters, like particle size, must be cautiously reviewed.

4. CONCLUSIONS

The results obtained in this study allow us to have a certain understanding about the biological fate of polymeric nanocapsules. Concerning *in vitro* studies, we observed an efficient internalization of chitosan nanocapsules and control nanoemulsions by macrophages, irrespective of particle size and through a combination of pathways. Moreover, 100 nm nanocapsules were more efficient than those with a size of 200 nm in eliciting MCP-1 secretion, possibly through a TLR 4-mediated process. We have also observed that small-sized cationic nanocapsules, especially those with polyarginine in the external shell, show high accumulation in the lymph nodes draining from the injection site. However, the unexpected results obtained with squalene nanoemulsions and with carboxymethyl-β-glucan nanocapsules open the possibility of a combined effect between particle size, surface charge and the specific materials used in the formulation. Further mechanistic studies should allow a better understanding of the outcome of this type of rational design of nanosystems in terms of the immune response elicited against the loaded antigen.

5. REFERENCES

[1] Deal CE, Balazs AB. Engineering humoral immunity as prophylaxis or therapy. Curr Opin Immunol 2015;35:113–22. doi:10.1016/j.coi.2015.06.014.

[2] Northrup L, Christopher MA, Sullivan BP, Berkland C. Combining antigen and immunomodulators: Emerging trends in antigen-specific immunotherapy for autoimmunity. Adv Drug Deliv Rev 2016;98:86–98. doi:10.1016/j.addr.2015.10.020.

[3] Sultan H, Fesenkova VI, Addis D, Fan AE, Kumai T, Wu J, et al. Designing therapeutic cancer vaccines by mimicking viral infections. Cancer Immunol Immunother 2017;66:203–13. doi:10.1007/s00262-016-1834-5.

[4] Delany I, Rappuoli R, De Gregorio E. Vaccines for the 21st century. EMBO Mol Med 2014;6:708–20. doi:10.1002/emmm.201403876.

[5] Nabel GJ. Designing tomorrow's vaccines. N Engl J Med 2013;368:551–60. doi:10.1056/NEJMra1204186.

[6] Gori A, Longhi R, Peri C, Colombo G. Peptides for immunological purposes: design, strategies and applications. Amino Acids 2013;45:257268. doi:10.1007/s00726-013-1526-9.

[7] Bobbala S, Hook S. Is There an Optimal Formulation and Delivery Strategy for Subunit Vaccines? Pharm Res 2016;33:2078–97. doi:10.1007/s11095-016-1979-0.

[8] Cordeiro AS, Alonso MJ. Recent advances in vaccine delivery. Pharm Pat Anal 2016;5:49–73. doi:10.4155/ppa.15.38.

[9] Irvine DJ, Swartz MA, Szeto GL. Engineering synthetic vaccines using cues from natural immunity. Nat Mater 2013;12:978–90. doi:10.1038/nmat3775.

[10] Cordeiro AS, Alonso MJ, de la Fuente M. Nanoengineering of vaccines using natural polysaccharides. Biotechnol Adv 2015;33:1279–93. doi:10.1016/j.biotechadv.2015.05.010.

[11] Gutjahr A, Phelip C, Coolen A-L, Monge C, Boisgard A-S, Paul S, et al. Biodegradable Polymeric Nanoparticles-Based Vaccine Adjuvants for Lymph Nodes Targeting. Vaccines 2016;4:34. doi:10.3390/VACCINES4040034.

[12] Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, et al. Exploiting lymphatic transport and complement activation in nanoparticle

vaccines. Nat Biotechnol 2007;25:1159–64. doi:10.1038/nbt1332.

[13] Johansen P, Mohanan D, Martínez-Gómez JM, Kündig TM, Gander B. Lympho-geographical concepts in vaccine delivery. J Control Release 2010;148:56–62. doi:10.1016/j.jconrel.2010.05.019.

[14] De Temmerman M-L, Rejman J, Demeester J, Irvine DJ, Gander B, De Smedt SC. Particulate vaccines: on the quest for optimal delivery and immune response. Drug Discov Today 2011;16:569–82. doi:10.1016/j.drudis.2011.04.006.

[15] Benne N, van Duijn J, Kuiper J, Jiskoot W, Slütter B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. J Control Release 2016;234:124–34. doi:10.1016/j.jconrel.2016.05.033.

[16] Lebre F, Hearnden CH, Lavelle EC. Modulation of Immune Responses by Particulate Materials. Adv Mater 2016:5525–41. doi:10.1002/adma.201505395.

[17] Kumar S, Anselmo AC, Banerjee A, Zakrewsky M, Mitragotri S. Shape and size-dependent immune response to antigen-carrying nanoparticles. J Control Release 2015;220:141–8. doi:10.1016/j.jconrel.2015.09.069.

[18] Shima F, Uto T, Akagi T, Baba M, Akashi M. Size effect of amphiphilic poly(γ-glutamic acid) nanoparticles on cellular uptake and maturation of dendritic cells in vivo. Acta Biomater 2013;9:8894–901. doi:10.1016/j.actbio.2013.06.010.

[19] Rao DA, Forrest ML, Alani AW, Kwon GS, Robinson JR. Biodegradable PLGA based nanoparticles for sustained regional lymphatic drug delivery. J Pharm Sci 2010;99:2018–31. doi:10.1002/jps.21970.

[20] Abellán-Pose R, Teijeiro-Valiño C, Santander-Ortega MJ, Borrajo E, Vidal A, García-Fuentes M, et al. Polyaminoacid Nanocapsules for Drug Delivery to the Lymphatic System: Effect of the Particle Size. Int J Pharm 2016;509:107–17. doi:10.1016/j.ijpharm.2016.05.034.

[21] Zeng Q, Jiang H, Wang T, Zhang Z, Gong T, Sun X. Cationic micelle delivery of Trp2 peptide for efficient lymphatic draining and enhanced cytotoxic T-lymphocyte responses. J Control Release 2015;200:1–12. doi:10.1016/j.jconrel.2014.12.024.

[22] Fromen CA, Rahhal TB, Robbins GR, Kai MP, Shen TW, Luft JC, et al. Nanoparticle surface charge impacts distribution, uptake and lymph node

trafficking by pulmonary antigen-presenting cells. Nanomedicine Nanotechnology, Biol Med 2016;12:677–87. doi:10.1016/j.nano.2015.11.002.

[23] Kaur CD, Nahar M, Jain NK. Lymphatic targeting of zidovudine using surface-engineered liposomes. J Drug Target 2008;16:798–805. doi:10.1080/10611860802475688.

[24] Kaminskas LM, Kota J, McLeod VM, Kelly BD, Karellas P, Porter CJ. PEGylation of polylysine dendrimers improves absorption and lymphatic targeting following SC administration in rats. J Control Release 2009;140:108–16. doi:10.1016/j.jconrel.2009.08.005.

[25] Nune S, Gunda P, Thallapally PK, Lin Y-Y, Forrest ML, Berkland CJ. Nanoparticles for biomedical imaging. Expert Opin Drug Deliv 2009;6:1175–94. doi:10.1517/17425240903229031.Nanoparticles.

[26] Pysz M, Gambhir S, Willmann J. Molecular imaging: current status and emerging strategies. Clin Radiol 2010;65:500–16. doi:10.1016/j.crad.2010.03.011.

[27] Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. Nat Biotechnol 2003;21:1369–77. doi:10.1038/nbt899.

[28] Stein J V, González SF. Dynamic intravital imaging of cell-cell interactions in the lymph node. J Allergy Clin Immunol 2017;139:12–20. doi:10.1016/j.jaci.2016.11.008.

[29] Kirui DK, Ferrari M. Intravital Microscopy Imaging Approaches for Image-Guided Drug Delivery Systems. Curr Drug Targets 2015;16:528–41.

[30] Amornphimoltham P, Masedunskas A, Weigert R. Intravital microscopy as a tool to study drug delivery in preclinical studies. Adv Drug Deliv Rev 2011;63:119–28. doi:10.1016/j.addr.2010.09.009.

[31] Vicente S, Peleteiro M, Díaz-Freitas B, Sánchez A, González-Fernández Á, Alonso MJ, et al. Co-delivery of viral proteins and a tlr7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy. J Control Release 2013;172:773–81. doi:http://dx.doi.org/10.1016/j.jconrel.2013.09.012.

[32] França A, Aggarwal P, Barsov E V, Kozlov S V, Dobrovolskaia MA, González-Fernández Á. Macrophage scavenger receptor A mediates the uptake of gold colloids by macrophages in vitro. Nanomedicine 2011;6:1175–88. doi:10.2217/nnm.11.41.

[33] Appelberg R, Orme I, Pinto de Sousa M, Silva M. In vitro effects of interleukin-4 on interferon-y-induced macrophage activation. Immunology 1992;76:553–9.

[34] González SF, Lukacs-Kornek V, Kuligowski MP, Pitcher L a, Degn SE, Kim Y-A, et al. Capture of influenza by medullary dendritic cells via SIGN-R1 is essential for humoral immunity in draining lymph nodes. Nat Immunol 2010;11:427–34. doi:10.1016/j.surg.2006.10.010.Use.

[35] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012;9:676–82. doi:10.1038/nmeth.2019.

[36] Vicente S, Díaz-Freitas B, Peleteiro M, Sanchez A, Pascual DW, González-Fernández Á, et al. A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach. PLoS One 2013;8:e62500. doi:10.1371/journal.pone.0062500.

[37] Vicente S, Peleteiro M, González-Aramundiz JV, Díaz-Freitas B, Martínez-Pulgarín S, Neissa JI, et al. Highly versatile immunostimulating nanocapsules for specific immune potentiation. Nanomedicine 2014;9:2273–89. doi:10.2217/nnm.14.10.

[38] Vicente S, Goins BA, Sánchez A, Alonso MJ, Phillips WT. Biodistribution and lymph node retention of polysaccharide-based immunostimulating nanocapsules. Vaccine 2014;32:1685–92. doi:10.1016/j.vaccine.2014.01.059.

[39] Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine 2007;25:2085–94. doi:10.1016/j.vaccine.2006.11.034.

[40] Ghendon Y, Markushin S, Krivtsov G, Akopova I. Chitosan as an adjuvant for parenterally administered inactivated influenza vaccines. Arch Virol 2008;153:831–7. doi:10.1007/s00705-008-0047-4.

[41] Ghendon Y, Markushin S, Vasiliev Y, Akopova I, Koptiaeva I, Krivtsov G, et al. Evaluation of Properties of Chitosan as an Adjuvant for Inactivated Influenza Vaccines Administered Parenterally. J Med Virol 2009;81:494–506. doi:10.1002/jmv.21415.

[42] Scherließ R, Buske S, Young K, Weber B, Rades T, Hook S. In vivo evaluation of chitosan as an adjuvant in subcutaneous vaccine formulations. Vaccine 2013;31:4812–9. doi:10.1016/j.vaccine.2013.07.081.

[43] Joshi VB, Geary SM, Salem AK. Biodegradable particles as vaccine delivery systems: size matters. AAPS J 2013;15:85–94. doi:10.1208/s12248-012-9418-6.

[44] Albanese A, Tang PS, Chan WC. The effect of nanoparticle size, shape, and surface chemistry on biological systems. Annu Rev Biomed Eng 2012;14:1–16. doi:10.1146/annurev-bioeng-071811-150124.

[45] Oussoren C, Zuidema J, Crommelin DJ, Storm G. Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposomal size, lipid composition and lipid dose. Biochim Biophys Acta 1997;1328:261–72.

[46] Thomas SN, Schudel A. Overcoming transport barriers for interstitial-, lymphatic-, and lymph node-targeted drug delivery. Curr Opin Chem Eng 2015;7:65–74. doi:10.1016/j.coche.2014.11.003.

[47] Lollo G, González-Paredes A, García-Fuentes M, Calvo P, Torres D, Alonso MJ. Polyarginine Nanocapsules as a Potential Oral Peptide Delivery Carrier. J Pharm Sci 2016;106:611–8. doi:10.1016/j.xphs.2016.09.029.

[48] Lozano MV, Lollo G, Alonso-Nocelo M, Brea J, Vidal A, Torres D, et al. Polyarginine nanocapsules: a new platform for intracellular drug delivery. J Nanoparticle Res 2013;15. doi:10.1007/s11051-013-1515-7.

[49] Reimóndez-Troitiño S, Alcalde I, Csaba N, Íñigo-Portugués A, de la Fuente M, Bech F, et al. Polymeric nanocapsules: a potential new therapy for corneal wound healing. Drug Deliv Transl Res 2016:708–21. doi:10.1007/s13346-016-0312-0.

[50] Mattner F, Fleitmann JK, Lingnau K, Schmidt W, Egyed A, Fritz J, 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 Res 2002;62:1477–80.

[51] Firbas C, Boehm T, Buerger V, Schuller E, Sabarth N, Jilma B, et al. Immunogenicity and safety of different injection routes and schedules of IC41, a Hepatitis C virus (HCV) peptide vaccine. Vaccine 2010;28:2397–407. doi:10.1016/j.vaccine.2009.12.072.

[52] Layton RC, Petrovsky N, Gigliotti AP, Pollock Z, Knight J, Donart N, et al. Delta inulin polysaccharide adjuvant enhances the ability of split-virion H5N1 vaccine to protect against lethal challenge in ferrets. Vaccine 2011;29:6242–51.

doi:10.1016/j.vaccine.2011.06.078.

[53] Lobigs M, Pavy M, Hall RA, Lobigs P, Cooper P, Komiya T, et al. An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. J Gen Virol 2010;91:1407–17. doi:10.1099/vir.0.019190-0.

[54] Cristillo AD, Ferrari MG, Hudacik L, Lewis B, Galmin L, Bowen B, et al. Induction of mucosal and systemic antibody and T-cell responses following prime-boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. J Gen Virol 2011;92:128–40. doi:10.1099/vir.0.023242-0.

[55] www.clinicaltrials.gov. Last visited in May 2017. n.d.

[56] Huang H, Ostroff GR, Lee CK, Wang JP, Specht CA, Levitz SM. Distinct patterns of dendritic cell cytokine release stimulated by fungal beta-glucans and toll-like receptor agonists. Infect Immun 2009;77:1774–81. doi:10.1128/IAI.00086-09.

[57] Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, et al. Dectin-1 is required for β-glucan recognition and control of fungal infection. Nat Immunol 2007;8:31–8. doi:10.1038/ni1408.

[58] Huang H, Ostroff GR, Lee CK, Specht CA, Levitz SM. Robust Stimulation of Humoral and Cellular Immune Responses following Vaccination with Antigen-Loaded B-Glucan Particles. MBio 2010;1:e00164-10-e00164-16. doi:10.1128/mBio.00164-10.

[59] De Smet R, Demoor T, Verschuere S, Dullaers M, Ostroff GR, Leclercq G, et al. β-Glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination. J Control Release 2013;172:671–8. doi:10.1016/j.jconrel.2013.09.007.

[60] Roman F, Vaman T, Gerlach B, Markendorf A, Gillard P, Devaster J-MM. 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;28:1740–5. doi:10.1016/j.vaccine.2009.12.014.

[61] Esposito S, Tagliaferri L, Daleno C, Valzano A, Picciolli I, Tel F, et al. Pandemic influenza A/H1N1 vaccine administered sequentially or simultaneously with

seasonal influenza vaccine to HIV-infected children and adolescents. Vaccine 2011;29:1677–82. doi:10.1016/j.vaccine.2010.12.047.

[62] Tonigold M, Mailänder V. Endocytosis and intracellular processing of nanoparticles in dendritic cells: routes to effective immunonanomedicines. Nanomedicine 2016;11:2625–30.

[63] Silva JM, Vandermeulen G, Oliveira VG, Pinto SN, Rodrigues C, Salgado A, et al. Development of functionalized nanoparticles for vaccine delivery to dendritic cells: a mechanistic approach. Nanomedicine 2014;9:2639–56. doi:10.2217/nnm.14.135.

[64] Shah RR, O'Hagan DT, Amiji MM, Brito LA. The impact of size on particulate vaccine adjuvants. Nanomedicine 2014;9:2671–81.

[65] Gause KT, Wheatley AK, Cui J, Yan Y, Kent SJ, Caruso F. Immunological Principles Guiding the Rational Design of Particles for Vaccine Delivery. ACS Nano 2017;11:54–68. doi:10.1021/acsnano.6b07343.

[66] Yue H, Wei W, Yue Z, Lv P, Wang L, Ma G, et al. Particle size affects the cellular response in macrophages. Eur J Pharm Sci 2010;41:650–7. doi:10.1016/j.ejps.2010.09.006.

[67] Dos Santos T, Varela J, Lynch I, Salvati A, Dawson KA. Quantitative assessment of the comparative nanoparticle-uptake efficiency of a range of cell lines. Small 2011;7:3341–9. doi:10.1002/smll.201101076.

[68] Dang Y, Li S, Wang W, Wang S, Zou M, Guo Y, et al. The effects of chitosan oligosaccharide on the activation of murine spleen CD11c+ dendritic cells via Toll-like receptor 4. Carbohydr Polym 2011;83:1075–81. doi:10.1016/j.carbpol.2010.08.071.

[69] Zhang P, Liu W, Peng Y, Han B, Yang Y. Toll like receptor 4 (TLR4) mediates the stimulating activities of chitosan oligosaccharide on macrophages. Int Immunopharmacol 2014;23:254–61. doi:10.1016/j.intimp.2014.09.007.

[70] Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. Nat Rev Immunol 2013;13:621–34. doi:10.1038/nri3515.

[71] Yan Y, Gause KT, Kamphuis MM, Ang C-S, O'Brien-Simpson NM, Lenzo JC, et al. Differential roles of the protein corona in the cellular uptake of nanoporous polymer particles by monocyte and macrophage cell lines. ACS Nano 2013;7:10960–70. doi:10.1021/nn404481f.

[72] Kanno S, Furuyama A, Hirano S. A murine scavenger receptor MARCO recognizes polystyrene nanoparticles. Toxicol Sci 2007;97:398–406. doi:10.1093/toxsci/kfm050.

[73] Ezzat K, Aoki Y, Koo T, McClorey G, Benner L, Coenen-Stass A, et al. Self-Assembly into Nanoparticles Is Essential for Receptor Mediated Uptake of Therapeutic Antisense Oligonucleotides. Nano Lett 2015;15:4364–73. doi:10.1021/acs.nanolett.5b00490.

[74] Kumari S, MG S, Mayor S. Endocytosis unplugged: multiple ways to enter the cell. Cell Res 2010;20:256–75. doi:10.1038/cr.2010.19.

[75] Lunov O, Syrovets T, Loos C, Beil J, Delacher M, Tron K, et al. Differential uptake of functionalized polystyrene nanoparticles by human macrophages and a monocytic cell line. ACS Nano 2011;5:1657–69. doi:10.1021/nn2000756.

[76] Firdessa R, Oelschlaeger TA, Moll H. Identification of multiple cellular uptake pathways of polystyrene nanoparticles and factors affecting the uptake: Relevance for drug delivery systems. Eur J Cell Biol 2014;93:323–37. doi:10.1016/j.ejcb.2014.08.001.

[77] Kastl L, Sasse D, Wulf V, Hartmann R, Mircheski J, Ranke C, et al. Multiple Internalization Pathways of Polyelectrolyte Multilayer Capsules into Mammalian Cells. ACS Nano 2013;7:6605–18. doi:10.1021/nn306032k.

[78] Nam HY, Kwon SM, Chung H, Lee S-Y, Kwon S-H, Jeon H, et al. Cellular uptake mechanism and intracellular fate of hydrophobically modified glycol chitosan nanoparticles. J Control Release 2009;135:259–67. doi:10.1016/j.jconrel.2009.01.018.

[79] Ferreira SA, Correia A, Madureira P, Vilanova M, Gama FM. Unraveling the uptake mechanisms of mannan nanogel in bone-marrow-derived macrophages. Macromol Biosci 2012;12:1172–80. doi:10.1002/mabi.201200075.

[80] Li Y, Wen G, Wang D, Zhang X, Lu Y, Wang J, et al. A complementary strategy for enhancement of nanoparticle intracellular uptake. Pharm Res 2014;31:2054–64. doi:10.1007/s11095-014-1307-5.

[81] Tiffany M, Szoka FC. Co-localization of Fluorescent Labeled Lipid Nanoparticles with Specifically Tagged Subcellular Compartments by Single Particle Tracking at Low Nanoparticle to Cell Ratios. J Drug Target 2016;2330:1–21. doi:10.1080/1061186X.2016.1233976.

[82] Lunov O, Zablotskii V, Syrovets T, Röcker C, Tron K, Nienhaus GU, et al. Modeling receptor-mediated endocytosis of polymer-functionalized iron oxide nanoparticles by human macrophages. Biomaterials 2011;32:547–55. doi:10.1016/j.biomaterials.2010.08.111.

[83] Herrmann M, Schäfer C, Heiss A, Gräber S, Kinkeldey A, Büscher A, et al. Clearance of fetuin-A-containing calciprotein particles is mediated by scavenger receptor-A. Circ Res 2012;111:575–84. doi:10.1161/CIRCRESAHA.111.261479.

[84] Orr GA, Chrisler WB, Cassens KJ, Tan R, Tarasevich BJ, Markillie LM, et al. Cellular recognition and trafficking of amorphous silica nanoparticles by macrophage scavenger receptor A. Nanotoxicology 2011;5:296–311. doi:10.3109/17435390.2010.513836.

[85] Reddy ST, Rehor A, Schmoekel HG, Hubbell JA, Swartz MA. In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. J Control Release 2006;112:26–34. doi:10.1016/j.jconrel.2006.01.006.

[86] Pacheco P, White D, Sulchek T. Effects of Microparticle Size and Fc Density on Macrophage Phagocytosis. PLoS One 2013;8:1–9. doi:10.1371/journal.pone.0060989.

[87] Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol 2004;173:3148–54. doi:10.4049/jimmunol.173.5.3148.

[88] Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol 2008;38:1404–13. doi:10.1002/eji.200737984.

[89] Zhuang Y, Ma Y, Wang C, Hai L, Yan C, Zhang Y, et al. PEGylated cationic liposomes robustly augment vaccine-induced immune responses: Role of lymphatic trafficking and biodistribution. J Control Release 2012;159:135–42. doi:10.1016/j.jconrel.2011.12.017.

[90] Makidon PE, Belyakov IM, Blanco LP, Janczak KW, Landers JJ, Bielinska AU, et al. Nanoemulsion mucosal adjuvant uniquely activates cytokine production by nasal ciliated epithelium and induces dendritic cell trafficking. Eur J Immunol 2012;42:2073–86. doi:10.1002/eji.201142346.

[91] Bae PK, Jung J, Chung BH. Highly enhanced optical properties of indocyanine

green/perfluorocarbon nanoemulsions for efficient lymph node mapping using near-infrared and magnetic resonance imaging. Nano Converg 2014;1:6. doi:10.1186/s40580-014-0006-6.

[92] Kim H, Lee SK, Kim YM, Lee E-H, Lim S-J, Kim SH, et al. Fluorescent Iodized Emulsion for Pre- and Intraoperative Sentinel Lymph Node Imaging: Validation in a Preclinical Model. Radiology 2015;275:196–204. doi:10.1148/radiol.14141159.

[93] Kitaoka M, Imamura K, Hirakawa Y, Tahara Y, Kamiya N, Goto M. Needle-free immunization using a solid-in-oil nanodispersion enhanced by a skin-permeable oligoarginine peptide. Int J Pharm 2013;458:334–9. doi:10.1016/j.ijpharm.2013.10.006.

[94] Amidi M, Mastrobattista E, Jiskoot W, Hennink WE. Chitosan-based delivery systems for protein therapeutics and antigens. Adv Drug Deliv Rev 2010;62:59–82. doi:10.1016/j.addr.2009.11.009.

[95] Saade F, Honda-Okubo Y, Trec S, Petrovsky N. A novel hepatitis B vaccine containing Advax^{TM}, a polysaccharide adjuvant derived from delta inulin, induces robust humoral and cellular immunity with minimal reactogenicity in preclinical testing. Vaccine 2013;31:1999–2007. doi:10.1016/j.vaccine.2012.12.077.

[96] Verma A, Stellacci F. Effect of Surface Properties on Nanoparticle-Cell Interactions. Small 2010;6:12–21. doi:10.1002/smll.200901158.

[97] Reddy ST, Berk DA, Jain RK, Swartz MA. A sensitive in vivo model for quantifying interstitial convective transport of injected macromolecules and nanoparticles. J Appl Physiol 2006;101:1162–9. doi:10.1152/japplphysiol.00389.2006.

[98] Mitchell D, Kim D, Steinman L, Fathman C, Rothbard J. Polyarginine enters cells more efficiently than other polycationic homopolymers. J Pept Res 2000;56:318–25. doi:10.1034/j.1399-3011.2000.00723.x.

[99] Maubant S, Banissi C, Beck S, Chauvat A, Carpentier AF. Adjuvant properties of Cytosine-phosphate-guanosine oligodeoxynucleotide in combination with various polycations in an ovalbumin-vaccine model. Nucleic Acid Ther 2011;21:231–40. doi:10.1089/nat.2011.0291.

[100] Mochizuki S, Morishita H, Kobiyama K, Aoshi T, Ishii KJ, Sakurai K. Immunization with antigenic peptides complexed with B-glucan induces potent cytotoxic T-lymphocyte activity in combination with CpG-ODNs. J Control

Release 2015;220:495–502. doi:10.1016/j.jconrel.2015.11.008.

[101] Kobiyama K, Aoshi T, Narita H, Kuroda E, Hayashi M, Tetsutani K, et al. Nonagonistic Dectin-1 ligand transforms CpG into a multitask nanoparticulate TLR9 agonist. Proc Natl Acad Sci 2014;111:3086–91. doi:10.1073/pnas.1319268111.

[102] Vu-Quang H, Muthiah M, Lee HJ, Kim Y-K, Rhee JH, Lee J-H, et al. Immune cell-specific delivery of beta-glucan-coated iron oxide nanoparticles for diagnosing liver metastasis by MR imaging. Carbohydr Polym 2012;87:1159–68. doi:10.1016/j.carbpol.2011.08.091.

Chapter 3

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter 3

Chitosan:carboxymethyl-β-glucan nanoparticles: new thermostable and efficient carriers for antigen delivery

This work was done in collaboration with Yagmur Farsakoglu[1] and Santiago F. González[1]

[1] Institute for Research in Biomedicine, Università della Svizzera Italiana, via Vincenzo Vela 6, 6500 Bellinzona, Switzerland

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

ABSTRACT

In the last few decades, adjuvant research has been in the core of the advances in immunization, aiming at the development of novel compound or formulations that may enhance the immune response generated against modern antigens. Nanotechnology has emerged as an important tool in immunization approaches, providing protection, stability and controlled release properties to those proteins or peptides. In fact, polymer-based nanocarriers have been widely studied in this field, with promising results. Among the biomaterials used in these prototypes, polysaccharides are particularly interesting given their wide distribution in the structure of pathogens, which facilitates their recognition by antigen presenting cells (APCs). In this work, we focused on a beta glucan derivative, carboxymethyl-β-glucan, and combined it with chitosan for the preparation of nanoparticles. These carriers were able to efficiently associate a model antigen (ovalbumin) and presented adequate colloidal stability in storage conditions for up to 9 weeks. Moreover, thermostability of this formulation was achieved through the optimization of a lyophilized form of the antigen-loaded nanoparticles, which remained stable for two months at 40 ºC. *In vivo* biodistribution studies in mice showed efficient drainage of the nanoparticles to the nearest lymph node following subcutaneous injection and significant co-localization with dendritic cells. Additionally, immunization of mice with the developed particles led to promising T cell proliferation levels, comparable to alum-adsorbed ovalbumin control. These results illustrate the potential of these nanocarriers, based on a novel immunostimulant polysaccharide, to be used as antigen delivery systems and to act as adjuvants in a potential vaccine formulation.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. INTRODUCTION

In the last decades, vaccine research has been mainly focused in two areas, developing antigens with better safety profiles and designing more efficient adjuvants. Safer antigens, usually proteins or peptides, are considered to have less risk of unwanted effects, but are also less immunogenic [1,2]. For this reason, the development and optimization of more potent adjuvants has been one of the leading research areas for the development of efficient vaccines.

Currently, marketed vaccine formulations include either aluminium salts (also known as alum) or lipid-based adjuvants, such as squalene emulsions (e.g. AS03™ and MF59™). Particulate-based adjuvants are gaining increasing importance in the field, with several formulations containing polymeric and lipid-based micro and nanoparticles under clinical development [3,4]. The specific characteristics of particulate antigen carriers make them very suitable for enhancing the immunogenic response of antigens. First, the fact that the antigen is entrapped in nanoparticles, helps protecting it against degradation, and allows its controlled delivery to the immune system. Moreover, nanoparticles might also incorporate within their structure molecular adjuvants to enhance the generated immune response [5,6].

Polymeric particles to be used as antigen adjuvants were already developed in the 70s, by Kreuter and Speiser [7]. Following this pioneering work based on polyacrylic nanoparticles, other authors have contributed to the design of polymer-based nanoparticles, with a more promising safety profile. For example, we pioneered the development of PLGA and PLA-PEG nanoparticles for protein/antigen delivery [8–12]. Following this early work and being conscious of the complexity of the PLGA-related technologies, as well as of the potential damage of this polymer towards the encapsulated antigens, we developed nanoparticles made of hydrosoluble polysaccharides, i.e. chitosan and glucomannan [13,14]. These biomaterials are particularly attractive given the presence of recognizable patterns in their structure, which are similar to those present in pathogens such as bacteria or virus and, hence, may lead to a better recognition by the immune cells [15–17]. For example, chitosan is a cationic polysaccharide that we selected for the design of nasal antigen delivery carriers based on its cell penetrating properties [17,18]. The results of the *in vivo* evaluation of these prototypes evidenced a high and long-lasting immune response against antigens such as tetanus toxoid or rHBsAg [19–21].

On the other hand, beta glucans, polysaccharides usually found in the cell wall of several microorganisms such as bacteria and yeast, have emerged as promising

immunostimulants [22,23]. Beta glucans can also interact with immune cells through the complement receptor 3 (CR3) and a type II transmembrane protein, Dectin-1 receptor [24,25]. These polymers have been used in oriental medicine as oral supplements for immune activity, particularly in cancer patients [26–28]. They have also been proposed for antifungal treatment [29], since beta glucans are attributed activity against fungal infections [30,31]. Recently, several authors reported the use of beta glucan microparticles in antigen delivery, in a process that empties the cell content of *Saccharomyces cerevisae*, rendering porous and hollow structures that can be loaded with antigens [32–34]. Despite their interest as immunostimulants, there is still only one report on the development of beta glucan nanoparticles for antigen delivery [35]. In this article, authors describe the preparation of nanocomplexes using a beta glucan from *Ganoderma lucidum* and the immunostimulant poly(I:C). Upon intraperitoneal immunization of mice, the antibody titres elicited by the co-administration of the nanocomplexes and ovalbumin were higher than those obtained with each component separately co-administered with the antigen.

Based on this information, we propose here the development of carboxymethyl-β-glucan nanoparticles in combination with chitosan. Moreover, we describe the association of ovalbumin as a model antigen and the evaluation of the potential of these antigen-loaded nanocarriers in vaccination. *In vivo* studies were performed to study the biodistribution of these nanoparticles upon subcutaneous administration and to determine their interaction with immune cells. Finally, solid-in-oil dispersion is presented as an innovative pharmaceutical form for the developed nanovaccines, interesting for alternative administration routes such as transdermal and oral.

2. MATERIALS AND METHODS

2.1. Materials

Pharmaceutical grade chitosan hydrochloride, with a molecular weight of 47 kDa and an 80-95% deacetylation degree, was acquired from Heppe Medical Chitosan GmbH (Halle, Germany). Carboxymethyl-β-glucan, obtained from *Saccharomyces cerevisae* and modified with carboxymethyl groups at an 85% substitution degree, was a kind donation from Mibelle AG Biochemistry (Buchs, Switzerland). Ovalbumin, in the form of complete protein with a molecular weight of 45 kDa, was provided by Invivogen (Toulouse, France). 5-carboxytetramethyl-rhodamine succinimidyl ester (5-TAMRA SE) was acquired from emp Biotech GmbH (Berlin, Germany). Sucrose dilaureate (Surfhope® D1216) was obtained from Mitsubishi-Chemical Foods Corporation (Tokyo, Japan). Sucrose was bought from Acofarma (Madrid, Spain) and trehalose (pharmaceutical grade) from Pfanstiehl (Waukegan (IL), USA). Polyvinyl pyrrolidone (PVP, Kollidon® 25) was purchased from BASF (Ludwigshafen, Germany). Squalene was acquired from Merck Millipore (Darmstadt, Germany). Sorbitan monooleate (Span® 80) and glycine were obtained from Sigma-Aldrich (Saint Louis, USA). All solvents were HPLC grade, supplied by Thermo Fisher Scientific (Waltham, USA) or Scharlab SL (Barcelona, Spain).

2.2. Preparation and characterization of chitosan:carboxymethyl-β-glucan (CS:CMβG) nanoparticles

Nanoparticles at three different CS:CMβG mass ratios (2:1, 4:1 and 1:2), were prepared by adding an aqueous solution of CMβG (2 mg/mL, 1mL) over an aqueous solution of CS (4 mg/mL, 1 mL, pH 5.5), under magnetic stirring for 5 minutes at room temperature.

2.2.1. Particle size, surface charge, and polydispersity index

The main physicochemical characteristics of the nanoparticles, particle size, surface charge and polydispersity index (PdI), were determined at room temperature, after proper dilution in ultrapure water or aqueous solution of KCl (1 mM) (for zeta potential). Particle size and PdI were determined by photon correlation spectroscopy (PCS), and zeta potential was measured by laser Doppler anemometry (LDA) (Zetasizer NanoZS®, Malvern Instruments; Malvern, United Kingdom).

2.2.2. Yield of production and elemental analysis

Nanoparticles were isolated by centrifugation at 15000xg for 30 minutes at 15 ºC (Hettich Universal 32 R; Tuttlingen, Germany). The pellets obtained after separation were freeze-

dried (Genesis SQ freeze-drier; Virtis, USA) and weighed after this process. Production yield was determined according to the following equation (Equation 1):

Eq. 1 $$Yield\ (\%) = \frac{Freeze{-}dried\ nanoparticle\ weight}{Theoretical\ total\ solids\ weight} \times 100$$

For elemental analysis, nanoparticles before and after isolation by centrifugation were freeze-dried and analysed for their content in carbon, oxygen and nitrogen (Finnigan FLASH EA 1112; Thermo Scientific, Waltham (MA), USA), with the nitrogen being specific for chitosan and therefore allowing differentiation between both polymers. Solutions of the starting polymers, chitosan and carboxymethyl-β-glucan, were also freeze-dried and analysed as controls.

2.3. Preparation of fluorescent CS:CMβG nanoparticles

For the preparation of fluorescently-labelled nanoparticles, chitosan was previously conjugated with the marker 5-Carboxytetramethylrhodamine NHS ester, single isomer (5-TAMRA-SE). For this purpose, 100 µL of a 10 mg/mL solution of TAMRA in DMSO was added to 1 mL of a solution of chitosan at 10 mg/mL in acetate buffer 10 mM (pH 5.5), under magnetic stirring. The reaction was kept at RT in dark for one hour. The resultant solution was dialysed for 24 hours against sodium chloride (50 mM) and then for 24 hours against ultrapure water. Finally, the labelled chitosan solution was recovered from the dialysis membrane and set to a final chitosan concentration of 5 mg/mL. Fluorescent particles were prepared as described in section 2.2, using for this purpose a physical mixture of plain chitosan and TAMRA-labelled chitosan (3:1 mass ratio).

2.4. Association of ovalbumin (OVA)

Encapsulation of ovalbumin (OVA) was achieved by mixing 40 µL of an aqueous solution of the protein (10 mg/mL) with 1 mL of the anionic solution of CMβG (2 mg/mL), and subsequent addition of this phase to 1 mL of CS solution (4 mg/mL) under magnetic stirring for 5 minutes. Nanoparticles were isolated and characterized as described previously (section 2.3.1). Fluorescent OVA-loaded nanoparticles were also prepared using this procedure.

To determine the association efficiency of the model antigen, OVA-loaded CS:CMβG nanoparticles were centrifuged for 30 min at 15000xg (Hettich Universal 32 R; Tuttlingen,

Germany) at 15 ºC on a glycerol bed. Supernatants were collected and free ovalbumin was quantified through linearized Bradford protein assay [36], against a calibration curve prepared with the supernatant of blank nanoparticles. Protein absorbance was measured at 620 nm and 450 nm (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific; Waltham (MA), USA) and the ratio between those values was used to calculate the concentration in the samples.

The stability of the antigen after its inclusion in the nanoparticles was further assessed by fluorescence Western blot. Briefly, OVA-loaded nanoparticles were incubated with a reducing sample buffer containing β-mercaptoethanol for 5 min at 95 ºC. Then, samples were loaded on a 10% SDS-PAGE gel and proteins were separated by electrophoresis at room temperature (RT) for 1 hour. Proteins were then transferred to a PVDF membrane (Immobilon-FL®, Merck Millipore; Darmstadt, Germany) for 90 minutes and the membranes were subsequently blocked with SEA BLOCK blocking buffer (Thermo Fisher Scientific; Waltham (MA), USA) for two hours at RT. Finally, the membranes were incubated overnight at 4 ºC with a primary rabbit polyclonal anti-ovalbumin antibody (abcam; Cambridge, UK) (dilution 1:2000), washed extensively and then one hour at RT with a secondary donkey anti-rabbit IgG antibody, labelled with Alexa Fluor® 680 (abcam; Cambridge, UK) (dilution 1:10000). Detection of protein fluorescence was performed with an Odyssey Infrared Imaging System (LI-COR, Lincoln (NE), USA).

2.4.1. Morphological characterization

The morphology of OVA-loaded nanoparticles was evaluated by transmission and field emission scanning electron microscopy (TEM and FESEM). For TEM, nanoparticles were placed on Formvar-coated copper grids and stained with 2% (w/v) phosphotungstic acid (Sigma-Aldrich; Saint Louis, USA). Following an overnight drying step, at room temperature, the samples were observed with a JEM-2010 TEM equipment (JEOL; Peabody (MA), USA). For SEM, samples were placed in a silicon wafer of 5x5 mm and kept at room temperature overnight until complete dryness, and observed with a FESEM ULTRA Plus (Zeiss; Oberkochen, Germany).

2.4.2. Stability studies

The stability of the nanoparticles in storage (4 ºC) was evaluated through the control of the colloidal properties of the nanoparticles during time. For this purpose, at the determined time points, samples of the isolated nanoparticles were collected to measure particle size and polydispersity index, as described in section 2.3.1.

2.5. Freeze-drying of OVA-loaded CS:CMβG nanoparticles

A powder form of OVA-loaded nanoparticles was obtained through freeze-drying in the presence of cryoprotectant. A screening comparing different cryoprotectants (5% sucrose, 5% trehalose, 5% polyvinyl pyrrolidone (PVP) and 0.24% glycine + 1.2% sucrose) and initial nanoparticle concentrations (1.5 and 3 mg/mL) was performed. Nanoparticles were frozen overnight at -20 ºC and lyophilized in a VirTis Genesis 25L equipment (Model SQ EL-85, SP Scientific; Warminster (PA), USA). Initial freezing temperature was -30 ºC and the drying process lasted a total of 46 hours, divided in two steps. The samples were then recovered to initial nanoparticle concentration adding ultrapure water and vortex agitation until complete resuspension. Characterization of the nanoparticle suspensions after freeze-drying was performed as described in section 2.4.1. The integrity of the antigen after this process was also evaluated by Western blot, as described in section 2.4.

2.6. Biodistribution of CS:CMβG nanoparticles in the lymphatic system

2.6.1. Animals and administration schedule

C57/BL6 female 6-8 weeks old mice, bred in-house or acquired from Janvier labs (Le Genest-Saint-Isle, France), were used for *in vivo* experiments. Animals were kept in pathogen-free facilities at Institute for Research in Biomedicine (Bellinzona, Switzerland) with food and water *ad libitum*. All experiments complied with the Swiss Federal Veterinary Office guidelines and the veterinarian local authorities approved animal protocols.

Ten microliters of blank or OVA-loaded nanoparticles (2 µg of OVA), were diluted with 15 µL of PBS and administered to mice through a single-dose injection in each footpad. Fifteen to twenty minutes after this injection, 0.5 µg of CD169-Alexa Fluor® 647 and CD21/35-Pacific Blue, were also injected in both footpads in a final volume of PBS of 5 µL, to adequately label different cell types. Polystyrene nanoparticles of 200 nm labelled in green (Firefli™ Fluorescent Green) were co-injected with the nanoparticles, to normalize the results.

2.6.2. Ex vivo lymph node imaging

Popliteal and lumbar lymph nodes of the injected mice were harvested at 12 hours post-injection and kept in PBS at 4 ºC. As a control, one axillary lymph node was also collected at the same time point. Imaging was performed with a customised two-photon platform

(TrimScope, LaVision BioTec GmbH; Bielefeld, Germany). 2-photon excitation of the fluorescent markers and tissue second harmonic generation (SHG) were achieved using two tunable Ti:Sapphire lasers (Chamaleon Ultra I, Chamaleon Ultra II, Coherent Inc.; Santa Clara (CA), USA) with an output wavelength in the range of 690-1080 nm. Additionally, an optical parametric oscillator emitting in the range of 1010 to 1340 nm (Chamaleon Compact OPO, Coherent Inc.; Santa Clara (CA), USA) was also used as part of the system. 3D reconstruction of the lymph nodes was performed with a Nikon Plan Apo λ 10X/0.45 objective, with a mosaic of up to 4x3 adjacent field-of-view image acquisitions.

2.6.3. Image analysis

Mosaic images obtained by two-photon microscopy were reconstructed using FIJI software [37], with an automated image processing custom-developed script. The hyperstacks were then loaded on Imaris 7.7.2 (Bitplane; Zurich, Switzerland) software to obtain the 3D rendering of the lymph nodes. Throughout each experiment, fluorescence intensity obtained for each channel was fixed in a range and quantified using the Imaris software to create individualized surfaces, corresponding roughly to particles or agglomerates.

2.6.4. Flow cytometry

Lymph nodes collected previously for imaging were later disrupted with tweezers and digested for 10 min at 37 °C in an enzyme mix - DNase I [0.28 mg/ml] (Amresco; Solon, USA), Dispase [1 U/ml] (Corning; New York, USA) and Collagenase P [0.5 mg/ml] (Roche; Basel, Switzerland) - in calcium- and magnesium-free PBS (PBS-) (Sigma-Aldrich; Saint Louis, USA). Subsequently, the reaction was stopped with a solution of 2 mM EDTA (Sigma-Aldrich; Saint Louis, USA) and 2% heat-inactivated filter-sterilised Fetal Bovine Serum (Thermo Fisher Scientific; Waltham, USA) in PBS-. Different cell populations were stained using labelled antibodies [αMHCII (M5/114.15.2), αCD11c (N418), αCD11b (M1/70), αCD80 (16-10A1), αCD86 (GL-1); BioLegend, San Diego (CA), USA] and the samples were analysed by flow cytometry on LSRFortessa™ (BD Biosciences; San Jose, USA). Data were analysed with FlowJo® software (FlowJo, LLC; Ashland, USA).

2.7. *In vivo* T-cell priming response

Aiming at understanding the outcome of the developed nanocarriers in antigen delivery and effective immune system triggering, we designed an *in vivo* T-cell priming assay that

could provide this information. For this purpose, the spleen from an OT-II HZ mouse was collected and processed to obtain a single cell suspension. $CD4^+$ T cells were subsequently isolated using a specific EasySep™ isolation kit, according to manufacturer's instructions (STEMCELL Technologies; Vancouver, Canada). A total of ~$4.8x10^6$ cells were obtained and stained with CFSE (Invitrogen™, Thermo Fisher Scientific, Carlsbad (CA), USA; 0.5 µM in 2 mL PBS-) for 20 minutes at 37 ºC. After staining, excess dye was removed by an additional incubation of 5 minutes with PBS supplemented with FBS. Finally, $CD4^+$ T cells were washed and resuspended in PBS- in a total volume of 600 µl.

Approximately $8x10^5$ CFSE labelled CD45.1 OTII transgenic T cells were injected intravenously to C57/BL6 recipient mice in a total volume of 100 µl (in PBS-). Twenty-four hours later, these mice received 15 µL of OVA-loaded nanoparticles (20 µg of OVA) or alum-adsorbed OVA (10 µg of OVA) subcutaneously in each footpad and after three days, popliteal lymph nodes were harvested and single cell suspensions were obtained. Finally, cells were stained with the priming panel (0.1 µg CD45.1 PerCP Cy5.5 and 0.2 µg CD4 APC/Cy7; BioLegend, San Diego (CA), USA) for 30 minutes on ice, for further cytometry analysis. Each lymph node was treated individually to increase the sample set.

2.8. Preparation of solid-in-oil CS:CMβG nanoparticle dispersions

To obtain solid-in-oil nanoparticle dispersions, 250 µL of OVA-loaded CS:CMβG nanoparticles (3 mg/mL, OVA loading 6.7% w/w) were frozen at -80 ºC in the presence of sucrose dilaureate (D1216) at 10% (w/w) and sucrose at 4% (w/w), in a volume ratio of 1:1:0.1 (nanoparticles:D1216:sucrose). Following this, samples were freeze-dried according to the protocol described in the previous section and redispersed in 250 µL of squalene with 10% Span® 80 (w/w). To characterize these systems, an aliquot of the oil dispersion was diluted in water (40x), thoroughly mixed using vortex and centrifuged to obtain two separate phases. Through this technique, nanoparticles were extracted from the oil dispersion to the aqueous phase so their colloidal properties could be measured by dynamic light scattering as described in section 2.4.1.

2.9. Statistical analysis

Statistical analysis, as well as data graphs, were done using Prism 7 (GraphPad Software; La Jolla (CA), USA). Unpaired two-tailed t tests with Welch's correction were

applied to the different datasets and statistical significance was defined as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and ****($p<0.0001$).

3. RESULTS AND DISCUSSION

Based on the immunostimulant properties of beta glucans [22,26,32–34], the aim of this work was to develop a new type of nanoparticles combining carboxymethyl-β-glucan (CMβG) and chitosan (CS) and to explore their potential as novel antigen nanocarriers for vaccination.

The selection of beta glucans as biomaterials for antigen delivery was motivated by their observed adjuvant effect following oral administration in combination with viral or tumoral antigens to chicken or mice, respectively [38,39]. Although hollow glucan microparticles have already been described for oral antigen delivery [32,33], there is only one report describing the formation of nanoparticles composed by beta glucan from *Ganoderma lucidum* and polyI:C and their efficacy as antigen carriers using ovalbumin as model antigen [35].

In this work we chose to form hybrid nanoparticles made of CMβG and CS, based on the already shown value of CS nanoparticles for immunization [19–21,40,41]. To assess the value of these nanoparticles, we selected ovalbumin (OVA) as a model antigen, and studied their interaction with immune cells, as well as their access to the lymph nodes after subcutaneous injection to mice. Finally, taking into account the observed positive effect of the use of solid-in-oil dispersions of nanoparticles [42,43], we produced a new formulation consisting of CS:CMβG nanoparticles dispersed in squalene. This oil has been described as immunostimulant and is included in some licensed adjuvants for influenza vaccines [44,45].

3.1. Development and optimization of CS:CMβG nanoparticles

3.1.1. Preparation of blank nanoparticles

Nanoparticles were prepared by interaction of the anionic CMβG and the cationic CS, as previously described for the formation of other polysaccharidic nanoparticles [46–48]. The pH of the chitosan solution was adjusted to 5 with acetate buffer 10mM and the pH of the CMβG solution was left as was in water, at pH 7. Increasing the pH value of this solution to 8, 9 or 10 did not lead to significant changes in the physicochemical properties of the nanoparticles and, for that reason, we continued with the initial aqueous solution.

Several polymer mass ratios were evaluated (CS:CMβG 2:1, 4:1 and 1:2), using solutions of both polymers at different concentrations, and maintaining the volume ratio constant (1:1). **Figure 1** shows that the mass ratio has a major influence in particle size

and polydispersity index, with an excess of chitosan being necessary to achieve a monodisperse nanoparticle population with a small size. These results are in accordance with those reported by other authors, concerning polyelectrolyte-based nanoparticles [49,50,14].

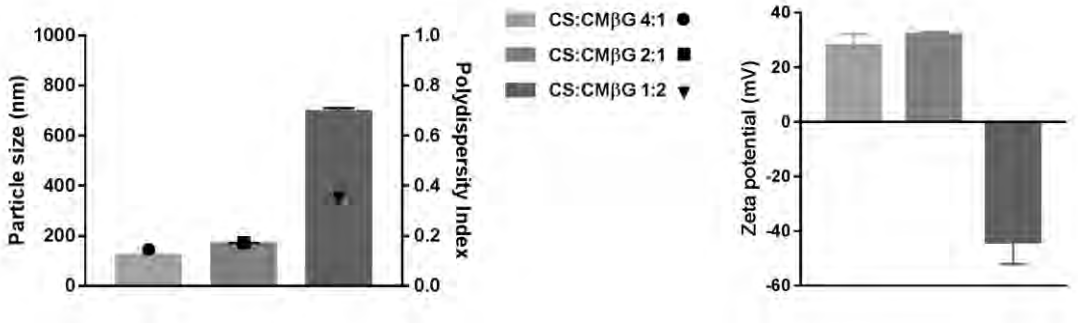


Figure 1. *Physicochemical characterization of blank CS:CMβG nanoparticles prepared at different polymer mass ratios. Results show mean ± SD of particle size (bars on left graph), polydispersity index (symbols on left graph) and zeta potential measurements (bars on right graph) of at least three independent replicates. CS, chitosan; CMβG, carboxymethyl-beta-glucan.*

Based on the physicochemical parameters of blank developed nanoparticles (small particle size and cationic charge), we selected the 2:1 CS:CMβG ratio for the formation of OVA-loaded nanoparticles. The final composition of the blank CS:CMβG nanoparticles is illustrated in **figure 2.** The results indicate that the proportion of both components in the final system was very similar to the theoretical ratio, a fact that suggests that an excess of chitosan is necessary to form nanoparticles with the adequate physicochemical properties. The yield of production was determined to be 85%, a fact that also confirms that both polysaccharides are incorporated in the nanostructures.

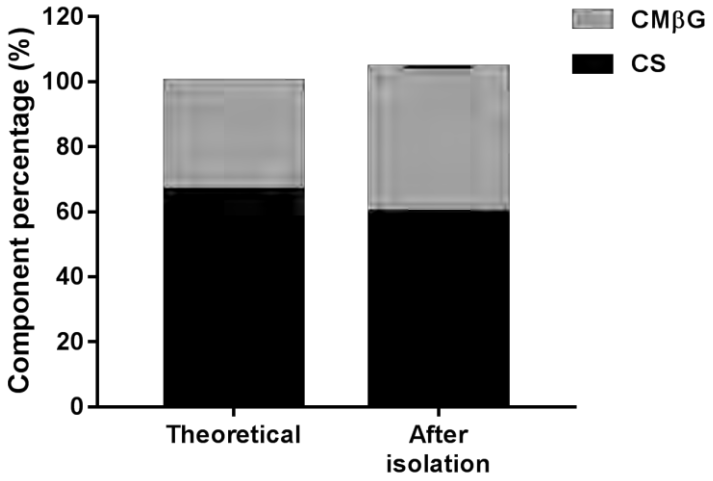


Figure 2. *Elemental analysis of the developed nanoparticles (CS:CMβG mass ratio 2:1) after the isolation process. Results are shown as mean ± SD (two pools of three replicates each) of the mass percentage corresponding to each polymer in the total nanoparticle mass obtained. CS, chitosan; CMβG, carboxymethyl-beta-glucan.*

3.2. Association of the model antigen ovalbumin (OVA)

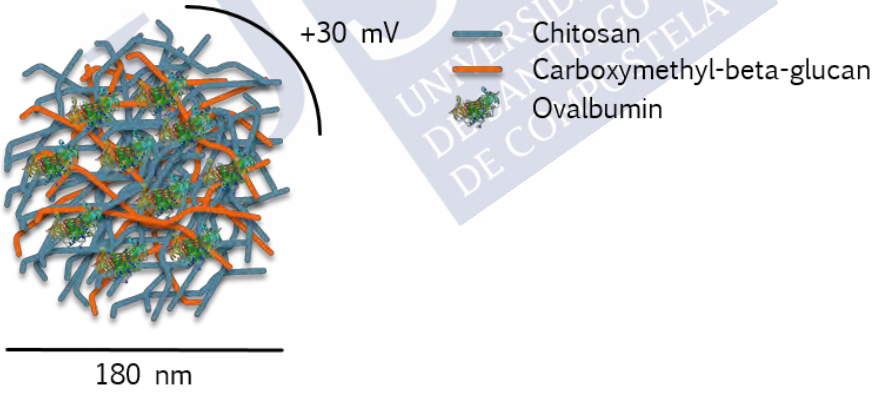
OVA has a molecular weight of 45 kDa and an isoelectric point of 4.5, being negatively charged at pH above that value. For this reason, ovalbumin was incorporated together with the anionic polysaccharide, which presented a pH around 7. As mentioned in the previous section, increasing the pH of the anionic solution to 8, 9 or 10 did not improve the OVA association efficiency nor led to significant differences in the colloidal properties of the particles. Three different theoretical loadings were tested, 1, 3 and 6.7% (w/w) with respect to the total amount of polymers present in the formulation.

As it can be observed in **table 1**, OVA-loaded CS:CMβG nanoparticles have physicochemical properties similar to those of blank nanoparticles, irrespective of the theoretical loading. Therefore, the nanoparticles with 6.7% OVA loading were selected for further evaluation.

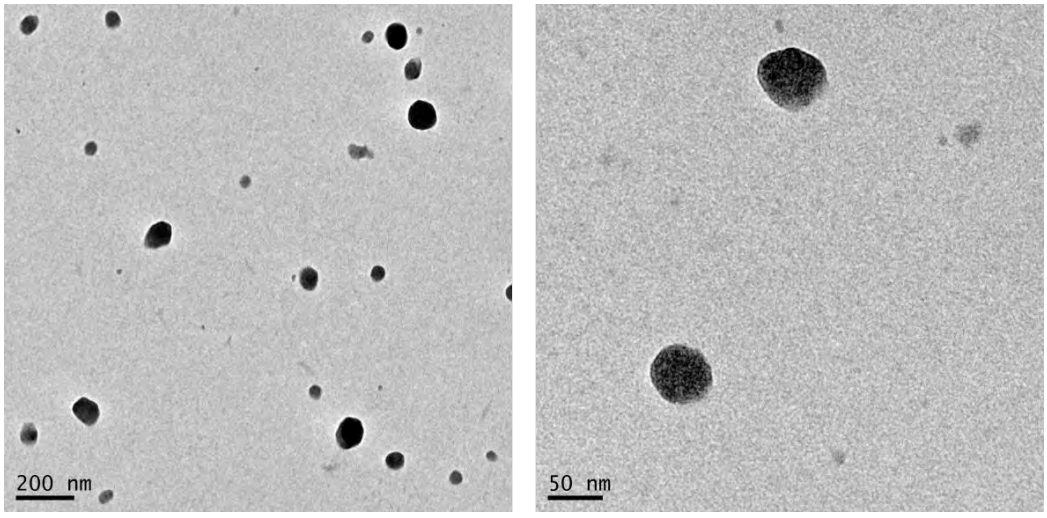
Table 1. *Summary of the physicochemical characteristics of OVA-loaded CS:CMβG 2:1 nanoparticles. Theoretical ovalbumin loading of 1, 3 and 6.7 % (w/w). Results are shown as mean ± SD (n=3). PdI, polydispersity index.*

OVA loading	Particle size (nm)	PdI	Zeta Potential (mV)
1%	164 ± 11	0.2	+33 ± 1
3%	171 ± 4	0.2	+33 ± 1
6.7%	159 ± 18	0.2	+32 ± 1

The morphology of OVA-loaded nanoparticles was evaluated by transmission and scanning electron microscopy (**figure 3**). Electron microscopy images evidence the presence of sphere-shaped structures, corresponding to the common architecture of this type of nanosystems [19,51]. It is also worth mentioning that, as expected, particle size measured by these image techniques is smaller than that observed with dynamic light scattering (DLS). This is mostly due to the characteristics of both methods, since DLS measures the hydrodynamic diameter of the particles (in suspension), while for electron microscopy the samples are left for complete dryness before observation.



B



C

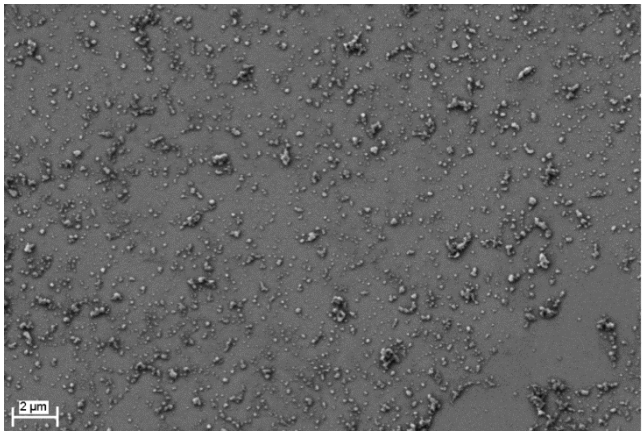


Figure 3. *Morphology of the developed nanoparticles. (**A**) Schematic representation of CS:CMβG nanoparticles loaded with ovalbumin (OVA). (**B**) Transmission and (**C**) scanning electron microscopy images of the nanoparticles (scale indicated in the figures).*

OVA-loaded CS:CMβG nanoparticles were further characterized to confirm the association of the model protein, and to determine their release profile. For this purpose, the free unbound OVA that remained in the supernatant following centrifugation of the nanoparticles was quantified by Bradford protein assay. The results showed an association efficiency value close to 50% (46±19%), with a final loading of 6.7% (w/w).

To assure the integrity of the antigen following association to the nanoparticles, we performed a Western blot assay using a specific anti-OVA primary antibody and a secondary fluorescent antibody. As depicted in **figure 4**, the protein band corresponding to ovalbumin associated to CS:CMβG nanoparticles has very similar intensity to the one corresponding to the control (ovalbumin solution at the same concentration).

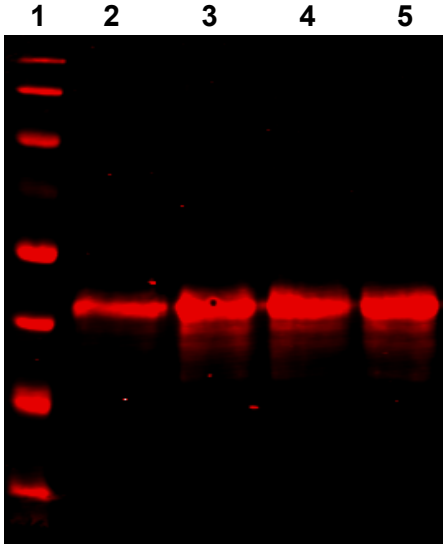


Figure 4. *Ovalbumin stability upon association to nanoparticles, analysed by fluorescence Western blot. Lane 1, molecular weight marker; lane 2, ovalbumin control (5 µg/mL); lane 3-5, three replicates of ovalbumin associated to nanoparticles (5 µg/mL).*

3.3. Stability of OVA-loaded CS:CMβG nanoparticles and freeze-drying process

One of the mandatory points in the development of nanocarriers for antigen delivery is to develop nanovaccines that are stable during storage. Considering this, we have evaluated the colloidal properties of OVA-loaded CS:CMβG nanoparticles upon storage at 4 ºC for two months. As depicted in **figure 5**, results showed that the formulations were able to maintain their mean particle size and polydispersity index in these conditions. These results are consistent with those observed for other similar polymeric particles developed in our group, which were stable for long periods of time [21,52].

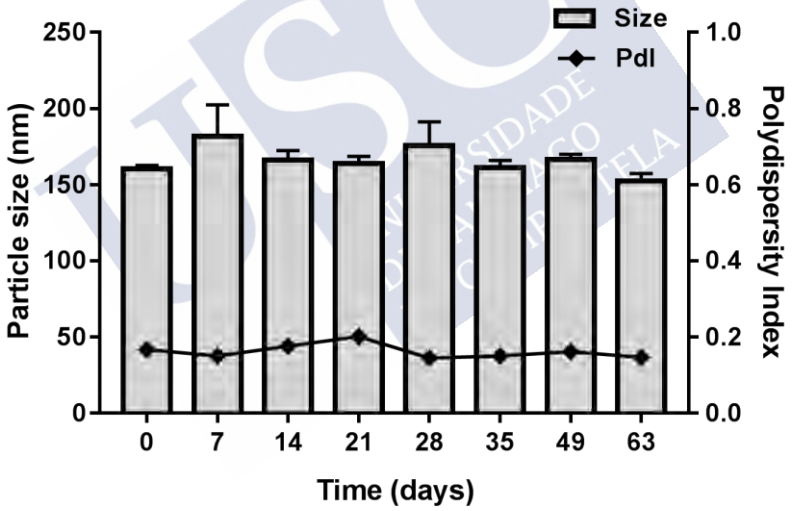


Figure 5. *Stability of OVA-loaded CS:CMβG nanoparticles (particle size and polydispersity index) in storage conditions (in suspension at 4 ºC).*

Given the importance of storage stability in the development of a vaccine prototype, we also aimed at obtaining a dry powder form of the OVA-loaded nanoparticles. The achievement of a thermostable formulation, that could overcome the current need for cold chain storage, is a major challenge in vaccine development. Based on the experience from our group in terms of converting liquid colloidal suspensions into dry powder [53–55], we performed a screening of freeze-drying conditions, varying the type

of cryoprotectant used. **Figure 6A** shows the outcome of this screening, in which we could observe that all conditions allowed the successful lyophilisation of the nanoparticles. Independently of the type and concentration of cryoprotectant used, nanoparticles could be resuspended to the initial concentration and maintained their colloidal properties.

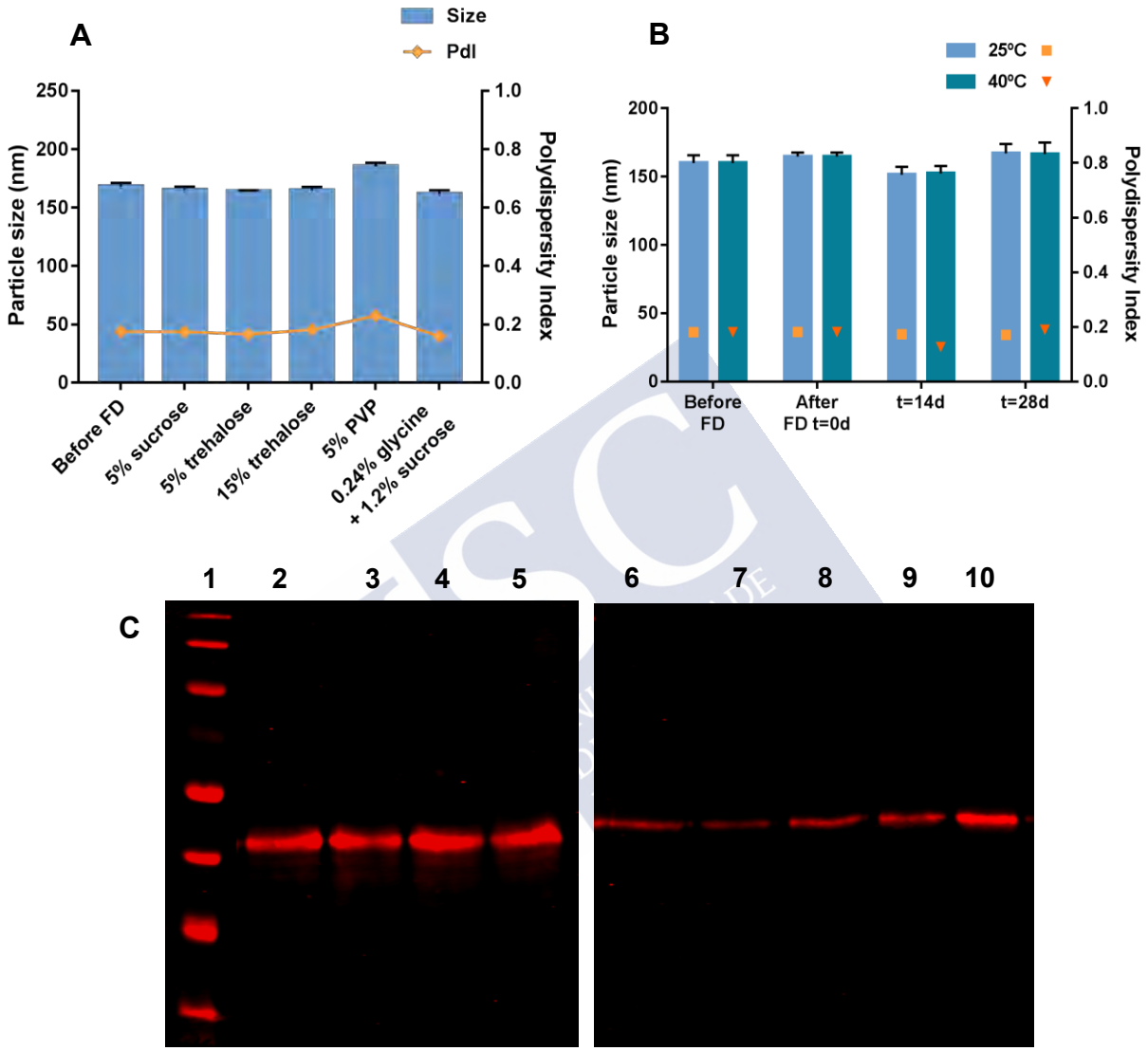


Figure 6. *Freeze-drying (FD) of OVA-loaded CS:CMβG nanoparticles.* ***(A)*** *Screening of freeze-drying conditions.* ***(B)*** *Stability of formulations freeze-dried with 15% trehalose (w/w) (particle size – bars – and polydispersity index – symbols) upon storage at 25 ºC / 60% humidity and 40 ºC / 75% humidity.* ***(C)*** *Antigen integrity following freeze-drying: lane 1, molecular weight marker; lanes 2 and 6, ovalbumin control (5 µg/mL); lanes 3-5, three replicates of freeze-dried ovalbumin-loaded nanoparticles immediately after resuspension; lanes 7-10, ovalbumin-loaded nanoparticles after freeze-drying and storage at 40 ºC (resuspension done at 7, 14, 21 and 28 days after storage).*

Next experiments were performed to further evaluate the impact of the freeze-drying process in the overall stability of these particles. For this purpose, we kept samples lyophilized with both cryoprotectants (sucrose and trehalose) in controlled temperature and humidity conditions. These parameters were set i) at 25 ºC / 60% humidity to simulate average storage conditions, and ii) at 40 ºC / 75% humidity to simulate more extreme conditions. The results showed that the colloidal stability of nanoparticles freeze-dried with 5% sucrose or trehalose, was compromised upon storage at 25ºC after two weeks and that it was necessary to add 15% trehalose to the nanoparticles suspension in order to preserve their stability. As shown in **figure 6B**, the formulations were stable at high temperature and humidity conditions for up to 28 days. The integrity of the antigen after this process was also assessed. Western blot images show similar bands for OVA in the freeze-dried nanoparticles and in solution, at the same concentration, irrespective of the tested conditions (**figure 6C**). Therefore, we can conclude that the freeze-drying process and the storage of the dry powder form does not lead to significant changes in the integrity of the associated antigen. Other authors have reported the importance of achieving a dry powder vaccine formulation that could avoid cold chain of storage and resist extreme climate conditions, while maintaining its efficacy [53,56,57]. Therefore, considering these results, we believe this prototype presents interesting characteristics for antigen delivery. Nevertheless, further studies concerning the biological efficacy of the developed freeze-dried prototype are still to be performed.

3.4. Biodistribution of CS:CMβG nanoparticles in the lymphatic system

Targeting the lymphatic system is an advantage commonly described for antigen nanocarriers, given the wide presence of antigen presenting cells in these tissues [58]. In fact, the capture and processing of particulate antigens by macrophages and dendritic cells resident in the draining lymph nodes is usually the trigger for the development of adequate cellular and humoral immune responses [59]. This targeting may be passive or active, depending if the draining to the lymph nodes is dependent only on the physicochemical characteristics of the nanosystems or if there is any ligand moiety for the receptors present in the target cells [60].

Considering this, we performed a biodistribution assay with the developed CS:CMβG nanoparticles. Fluorescent nanoparticles were prepared upon labelling chitosan with 5-

TAMRA. This molecule is a rhodamine derivative that had been previously used to label other polymers such as protamine or polyarginine [55,61]. To avoid modifying the physicochemical properties of the nanoparticles, twenty-five percent of the total amount of chitosan used in the formulation was replaced by the TAMRA-labelled polymer.

In this study, we exploited the abilities of an innovative imaging technique such as two-photon microscopy and combined it with a quantitative technique such as flow cytometry. First, we analysed the *ex vivo* images of the lymph nodes draining from the footpad, which was chosen as injection site for the subcutaneous administration of the particles. In this study, we compared blank nanoparticles and OVA-loaded ones, in order to determine the biodistribution of the nanosystem and evaluate if it could be affected by the encapsulation of the antigen. Popliteal lymph nodes were collected 12 hours post-administration of the formulation, in order to allow the accumulation and co-localization of the nanoparticles with the immune cells. In addition, this is usually the period in which the immune response is due to innate immunity mechanisms, which are the first line of response to external stimuli.

The quantification of the fluorescence intensity measured by flow cytometry showed no influence of the antigen in the accumulation of the nanoparticles in the lymph nodes studied (**figure 7A)**. As mentioned before, beta glucans have been described in the literature as natural ligands to complement receptor 3 (CR3) and Dectin-1, both present in phagocytic cells such as macrophages and dendritic cells [24,25]. Therefore, an interaction with these receptors could contribute to the accumulation of the nanoparticles in the lymph nodes. Other parameters, such as nanoparticle size and shape, could also account for this process, as has been reviewed by several authors [58,62]. **Figure 7C** shows a representative image of one of the lymph nodes, in which the accumulation of the OVA-loaded nanoparticles, labelled in red, is visible in the medullary and interfollicular regions of this lymph node. Some degree of co-localization with the control polystyrene beads is visible in yellow regions (overlapping of green and red labelling).

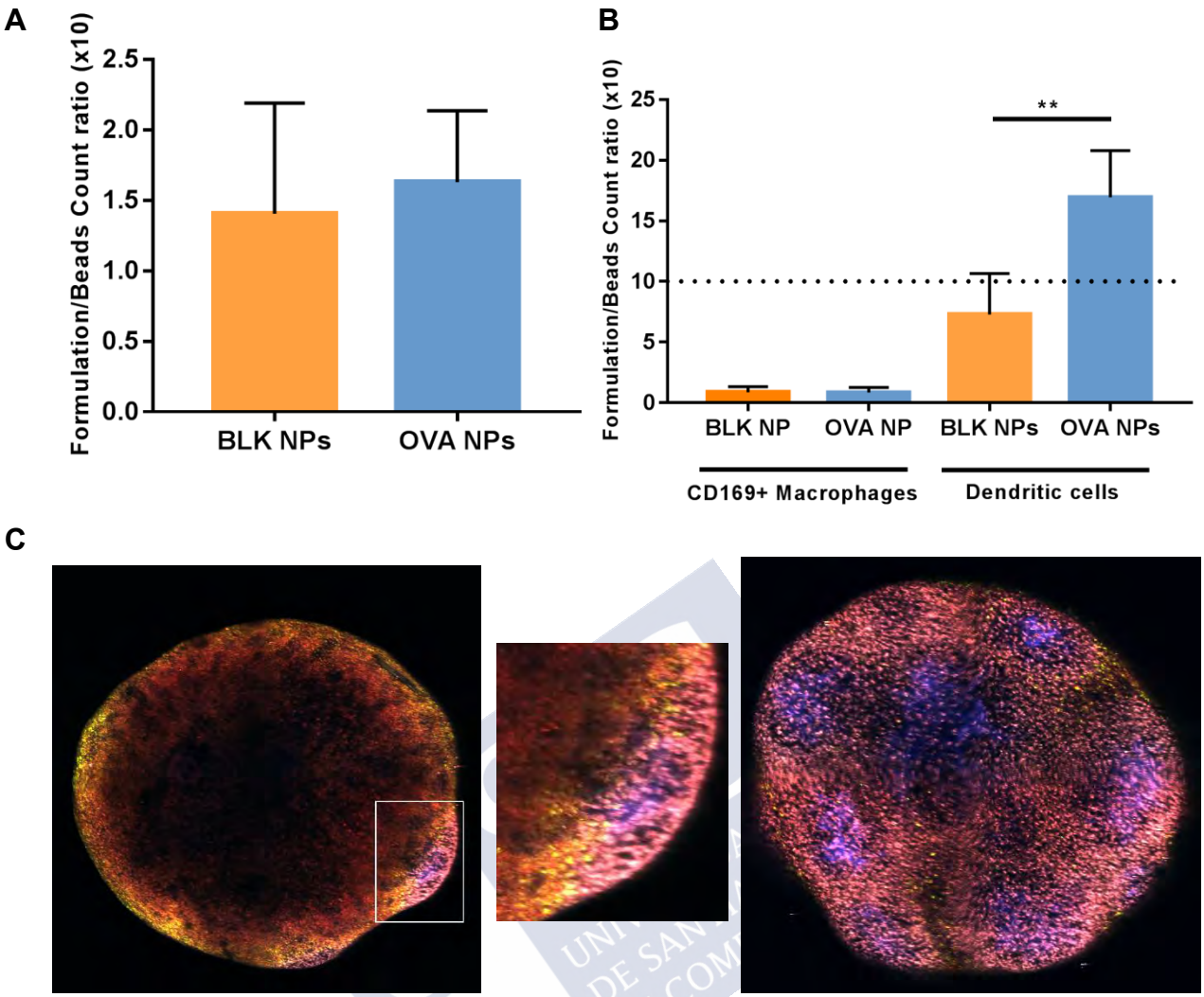


Figure 7. *Distribution of CS:CMβG nanoparticles (blank, BLK, or loaded with ovalbumin, OVA) in popliteal lymph node, after subcutaneous injection. Fluorescence intensity was quantified by flow cytometry, in the total number of cells of the lymph node (**A**) and in two cell populations, macrophages and dendritic cells (**B**). Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanocapsules and beads). Ex vivo, 2-photon microscopy images (**C**) show the accumulation of OVA-loaded nanoparticles in the medullary (left) and follicular (right) areas of popliteal lymph node. A detail of the medullary region, with a follicle in the back, is seen in the middle image. Macrophages are shown in white (CD169-AF 647), follicles in blue (CD21/35-PB), nanoparticles in red (TAMRA) and control polystyrene beads in green (Firefli™ Fluorescent Green). ** p<0.01*

With respect to the interaction of the nanoparticles with the immune cells, the results in **figure 7B** suggest a preferential interaction with dendritic cells. Indeed, the signal

attributed to the fluorescent nanoparticles was almost negligible in macrophages, and significantly higher in dendritic cells. Dendritic cells are the most efficient antigen presenting cells, with specialized abilities to recognize different types of antigens, from proteins to whole microbes. Moreover, these cells have high expression levels of dectin-1, which is the main beta glucan receptor [63,64]. Therefore, it is possible that a combination of these factors is leading to the preferential uptake of CS:CMβG nanoparticles by these cells in comparison with macrophages. In this regard, an efficient uptake of yeast glucan microparticles by DCs has been reported upon subcutaneous administration to mice, which translates in an efficient antigen presentation and immune response in an anticancer therapeutic approach [65]. Similarly, a complex between schizophyllan (a mushroom-derived beta glucan) and a CpG oligodeoxynucleotide was described by Kobiyama *et al* as capable of activating dendritic cells and inducing cytokine production by these cells [66].

Overall, our results show an adequate accumulation of the nanoparticles in the lymph nodes and efficient internalization of these carriers by antigen presenting cells, particularly dendritic cells. Therefore, we considered this prototype as interesting for further studies concerning its adjuvant potential.

3.5. *In vivo* evaluation of the T cell responses elicited by OVA-loaded CS:CMβG nanoparticles

The main goal of this work was the development of polysaccharide-based nanoparticles that would allow and efficient antigen delivery to the adequate immune cells. Considering this, we evaluated the behaviour of these cells following subcutaneous immunization of mice with the developed nanoparticles.

We performed a T-cell priming assay, collecting OVA-specific CD4+ T cells from OT II transgenic animals and labelling them with carboxyfluorescein succinimidyl ester (CFSE), an intracellular fluorescent dye. Given this stable coupling, the fluorescence can be retained in cells for long periods and is not transferrable to adjacent cells. CFSE-labelled T cells were then injected into C57/B6 mice, which were immunized with the formulations and controls 24 hours after the adoptive transfer of the cells. At day 3 post-immunization, the draining lymph nodes were collected from these mice to evaluate the degree of proliferation of the transferred cells, corresponding to an adequate presentation of the antigen by DCs. As CFSE levels within each cell decrease with its division, a decrease in median fluorescence intensity (MFI) and/or in the frequency of CFSE- cells is commonly used to indicate T cell proliferation.

As can be seen in **figure 8A**, histograms of the adoptively transferred CD4+ T cells indicate a similar proliferation rate for both treatments (OVA-loaded nanoparticles and alum-adsorbed OVA). In addition, dot plots of proliferated CD4+ T cells show similar proliferation frequencies and median CFSE fluorescence intensities for all samples (**figure 8B**), indicating similar proliferation kinetics for both study groups. Therefore, our results indicate that CS:CMβG nanoparticles were able to deliver the loaded antigen to DCs, which then present this antigen to T cells, stimulating their proliferation at least to the same level as the standard adjuvant used as positive control. This is in agreement with other published results, showing the potential of different nanocarriers to trigger this type of cell activation. For example, De Geest *et al* showed the ability of ovalbumin-loaded multilayer polymeric capsules, prepared with dextran and polyarginine, to generate CD4+ and CD8+ T cell proliferation in lymph nodes, following subcutaneous immunization [67]. Similarly, Huang *et al* showed enhanced *ex vivo* T cell proliferation following multiple subcutaneous immunizations of mice with ovalbumin-loaded beta glucan particles [68].

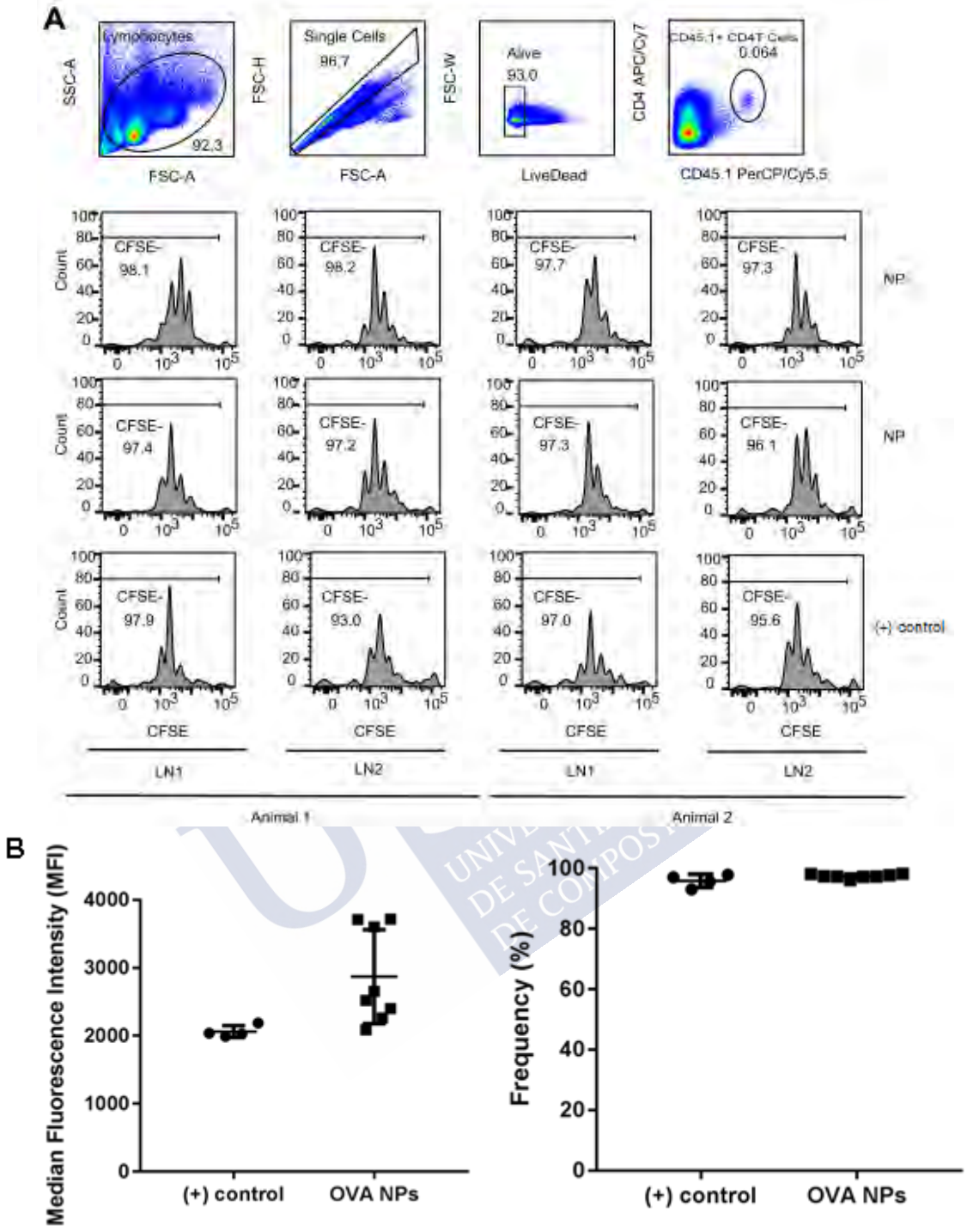


Figure 8. *Proliferation of OVA-specific CD4+ T cells collected from popliteal lymph nodes of mice at day 3 after subcutaneous administration of OVA-loaded CS:CMβG nanoparticles (20 μg of OVA per footpad) or ovalbumin (10 μg per footpad) admixed with alum (positive control).* ***(A)*** *Gating strategy and proliferation histograms of transferred CD4+ T cells from OTII HZ mice (specific for OVA)* ***(B)*** *Median fluorescence intensity (**left**) and frequency (**right**) plots for CFSE- populations in each treated group.*

Interestingly, we may propose a correlation between the results achieved in these experiments and the biodistribution observed for these particles and described in the previous section. As depicted in **figure 7C**, the accumulation of the OVA-loaded nanoparticles is particularly high in dendritic cells, possibly due to the high levels of expression of the main beta glucan receptor, dectin-1, in these cells. Correspondingly, authors have previously shown the role played by this receptor in the induction of T cell responses following subcutaneous immunization. In a study performed by Carter *et al*, results evidence high levels of CD4+ T cell proliferation induced by the administration of OVA-antibody conjugates targeted to dectin-1 expressing cells [69]. Therefore, we may hypothesize that the preferential co-localization of our nanoparticles with dendritic cells may be related to the affinity of this receptor by beta glucans and could be influencing the following T cell activation observed in our studies. Nevertheless, further studies to confirm this hypothesis and establish the adjuvant potential of these nanoparticles are still necessary.

Overall, this experiment shows that the developed CS:CMβG nanoparticles can efficiently carry the loaded ovalbumin antigen to APCs such as dendritic cells, leading to CD4+ T cell priming. Moreover, their action is similar to that of alum, possibly stimulating inflammation processes that create the immunological niche required for priming. Future studies should include more controls such as soluble antigen and higher doses of alum-associated OVA. Finally, further studies that evaluate the humoral and cellular immune response elicited by this immunization approach should also be performed.

3.6. Solid-in-oil dispersion of CS:CMβG nanoparticles

Considering the potential of the developed CS:CMβG nanoparticles for vaccine delivery, last experiments were focused in the development of a new pharmaceutical form of administration, consisting of a suspension of nanoparticles in an oil. In this regard, solid-in-oil (S/O) nanodispersions show a lot of promise, especially if the oil vehicle is selected among those with adjuvant properties, such as squalene or vitamin E. Some authors have described, so far, the delivery of drugs and model antigens using S/O nanodispersions, with adequate immune responses generated against those antigens upon transdermal administration [42,43].

To develop these nanodispersions, we prepared a mixture of cryoprotectants (sucrose laurate (D1216) and sucrose) which was added to the CS:CMβG nanoparticles before freeze-drying. Sucrose esters are nonionic surfactants particularly interesting for this application, given their emulsifying properties and ability to improve the stability of these

formulations [70]. Following lyophilisation, we tested different oils and oil-surfactant mixtures to redisperse the solid nanoparticles. Initially, we used squalene alone or mixtures of this oil with caprylic/capric triglyceride (Labrafac® Lipophile WL 1349; Gattefossé, St Priest, France) or vitamin E (α-tocopherol, Calbiochem®; Merck Millipore, Darmstadt, Germany). These mixtures were chosen due to the immunostimulant properties described for these oils. However, these conditions were not adequate for the redispersion of the nanoparticles and consequent achievement of an S/O formulation. Considering the specificities of this type of formulation, it was thought that by adjusting the hydrophilic-lipophilic balance (HLB) of the oil it would be possible to achieve an adequate dispersibility of the formulation. In this particular case, the HLB value of squalene is 10 and both mixtures (with Labrafac® and vitamin E) had HLB values around 8. Finally, we were able to redisperse the formulation in squalene with 10% Span® 80 (HLB ≈ 9.5), leading to a clear suspension similar to the one initially obtained.

Furthermore, following extraction of the nanoparticles from the dispersion, as described in section 2.7, we could characterize them in terms of particle size and polydispersity index. Results showed that the particles had similar characteristics before and after the process, evidencing the achievement of an adequate solid-in-oil nanodispersion that could be further studied for immunization purposes. This is particularly interesting for alternative routes of administration that avoid the use of injection material. Moreover, to the best of our knowledge, this is the first time this technique is applied for the dispersion of nanoparticles encapsulating a protein of interest. Combining the advantages brought by this formulation technique and those given by the nanotechnology applied to antigen delivery, previously described, this prototype could be particularly interesting for this field. Further studies would be necessary, particularly in terms of *in vitro* and *in vivo* biological activity, to assure the potential of this approach in vaccine development.

4. CONCLUSIONS

We describe in this study the successful development and characterization of novel beta glucan-based nanocarriers specifically designed for vaccination. These particles efficiently associate ovalbumin are stable under storage conditions and a thermostable dry powder form of this formulation could be achieved. *In vivo* studies showed accumulation of the nanoparticles in the draining lymph nodes upon subcutaneous administration to mice, and efficiently interaction with APCs, leading to T cell activation and proliferation. Further studies should be done with this prototype to evaluate its potential in terms of triggering an effective immune response against the loaded antigen. Finally, we have successfully prepared an innovative form of presentation of this formulation – solid-in-oil nanodispersion –, which could be of interest for non-parenteral antigen delivery studies.

5. REFERENCES

[1] Gori A, Longhi R, Peri C, Colombo G. Peptides for immunological purposes: design, strategies and applications. Amino Acids 2013;45:257268. doi:10.1007/s00726-013-1526-9.

[2] Bobbala S, Hook S. Is There an Optimal Formulation and Delivery Strategy for Subunit Vaccines? Pharm Res 2016;33:2078–97. doi:10.1007/s11095-016-1979-0.

[3] Brito LA, O'Hagan DT. Designing and building the next generation of improved vaccine adjuvants. J Control Release 2014;190:563–79. doi:10.1016/j.jconrel.2014.06.027.

[4] Reed SG, Orr MT, Fox CB. Key roles of adjuvants in modern vaccines. Nat Med 2013;19:1597–608. doi:10.1038/nm.3409.

[5] Cordeiro AS, Alonso MJ. Recent advances in vaccine delivery. Pharm Pat Anal 2016;5:49–73. doi:10.4155/ppa.15.38.

[6] Irvine DJ, Swartz MA, Szeto GL. Engineering synthetic vaccines using cues from natural immunity. Nat Mater 2013;12:978–90. doi:10.1038/nmat3775.

[7] Kreuter J, Speiser PP. New adjuvants on a polymethylmethacrylate base. Infect Immun 1976;13:204–10.

[8] Blanco M, Alonso MJ. Development and characterization of protein-loaded poly(lactide-co-glycolide) nanospheres. Eur J Pharm Biopharm 1997;43:287–94.

[9] Tobío M, Gref R, Sánchez A, Langer RS, Alonso MJ. Stealth PLA-PEG nanoparticles as protein carriers for nasal administration. Pharm Res 1998;15:270–5. doi:10.1023/A:1011922819926.

[10] Tobío M, Nolley J, Guo Y, McIver J, Alonso MJ. A Novel System Based on a Poloxamer/ PLGA Blend as a Tetanus Toxoid Delivery Vehicle. Pharm Res 1999;16:682–8. doi:10.1023/a:1018820507379.

[11] Vila A, Sánchez A, Perez C, Alonso MJ. PLA-PEG nanospheres: new carriers for transmucosal delivery of proteins and plasmid DNA. Polym Adv Technol 2002;13:851–8. doi:10.1002/pat.280.

[12] Csaba N, González L, Sánchez A, Alonso MJ. Design and characterisation of new nanoparticulate polymer blends for drug delivery. J Biomater Sci Polym Ed 2004;15:1137–51.

[13] Cuña M, Alonso-Sande M, Remuñán-López C, Pivel J, Alonso-Lebrero JL, Alonso MJ. Development of Phosphorylated Glucomannan-Coated Chitosan Nanoparticles as Nanocarriers for Protein Delivery. J Nanosci Nanotechnol 2006;6:2887–95.

[14] Alonso-Sande M, Cuña M, Remuñán-López C, Teijeiro-Osorio D, Alonso-Lebrero JL, Alonso MJ. Formation of New Glucomannan−Chitosan Nanoparticles and Study of Their Ability To Associate and Deliver Proteins. Macromolecules 2006;39:4152–8. doi:10.1021/ma060230j.

[15] Petrovsky N, Cooper PD. Carbohydrate-based immune adjuvants. Expert Rev Vaccines 2011;10:523–37. doi:10.1586/erv.11.30.

[16] Mahla RS, Reddy MC, Prasad DVR, Kumar H. Sweeten PAMPs: Role of sugar complexed PAMPs in innate immunity and vaccine biology. Front Immunol 2013;4:1–16. doi:10.3389/fimmu.2013.00248.

[17] Cordeiro AS, Alonso MJ, de la Fuente M. Nanoengineering of vaccines using natural polysaccharides. Biotechnol Adv 2015;33:1279–93. doi:10.1016/j.biotechadv.2015.05.010.

[18] García-Fuentes M, Alonso MJ. Chitosan-based drug nanocarriers: Where do we stand? J Control Release 2012;161:496–504. doi:http://dx.doi.org/10.1016/j.jconrel.2012.03.017.

[19] Prego C, Paolicelli P, Díaz B, Vicente S, Sánchez A, González-Fernández Á, et al. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. Vaccine 2010;28:2607–14. doi:10.1016/j.vaccine.2010.01.011.

[20] Vicente S, Peleteiro M, Díaz-Freitas B, Sánchez A, González-Fernández Á, Alonso MJ, et al. Co-delivery of viral proteins and a tlr7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy. J Control Release 2013;172:773–81. doi:http://dx.doi.org/10.1016/j.jconrel.2013.09.012.

[21] Correia-Pinto JF, Csaba N, Schiller J, Alonso MJ. Chitosan-Poly (I:C)-PADRE Based Nanoparticles as Delivery Vehicles for Synthetic Peptide Vaccines. Vaccines 2015;3:730–50. doi:10.3390/vaccines3030730.

[22] Novak M, Vetvicka V. Beta-glucans, history, and the present: immunomodulatory aspects and mechanisms of action. J Immunotoxicol 2008;5:47–57. doi:10.1080/15476910802019045.

[23] Demento SL, Siefert AL, Bandyopadhyay A, Sharp FA, Fahmy TM. Pathogen-

associated molecular patterns on biomaterials: a paradigm for engineering new vaccines. Trends Biotechnol 2011;29:294–306.

[24] Goodridge HS, Wolf AJ, Underhill DM. β-glucan recognition by the innate immune system. Immunol Rev 2009;230:38–50. doi:10.1111/j.1600-065X.2009.00793.x.

[25] Huang H, Ostroff GR, Lee CK, Agarwal S, Ram S, Rice PA, et al. Relative contributions of Dectin-1 and complement to immune responses to particulate beta-glucans. J Immunol 2012;189:312–7. doi:10.1115/1.3071969.Automating.

[26] Samuelsen A, Schrezenmeir J, Knutsen S. Effects of orally administered yeast-derived beta-glucans: A review. Mol Nutr Food Res 2014;58:183–93. doi:10.1002/mnfr.201300338.

[27] Whitehead A, Beck EJ, Tosh S, Wolever TM. Cholesterol-lowering effects of oat beta-glucan: a meta-analysis of randomized controlled trials. Am J Clin Nutr 2014;100:1413–21. doi:10.3945/ajcn.114.086108.

[28] Du B, Bian Z, Xu B. Skin health promotion effects of natural Beta-Glucan derived from cereals and microorganisms: A review. Phyther Res 2014;28:159–66. doi:10.1002/ptr.4963.

[29] Anusuya S, Sathiyabama M. Preparation of β-D-glucan nanoparticles and its antifungal activity. Int J Biol Macromol 2014;70:440–3. doi:10.1016/j.ijbiomac.2014.07.011.

[30] Rice PJ, Adams EL, Ozment-Skelton T, Gonzale AJ, Goldman MP, Lockhart BE, et al. Oral Delivery and Gastrointestinal Absorption of Soluble Glucans Stimulate Increased Resistance to Infectious Challenge. J Pharmacol Exp Ther 2005;314:1079–86. doi:10.1124/jpet.105.085415.

[31] Pinto RJ, Almeida A, Fernandes SC, Freire CS, Silvestre AJ, Neto CP, et al. Antifungal activity of transparent nanocomposite thin films of pullulan and silver against Aspergillus niger. Colloids Surfaces B Biointerfaces 2013;103:143–8. doi:10.1016/j.colsurfb.2012.09.045.

[32] De Smet R, Demoor T, Verschuere S, Dullaers M, Ostroff GR, Leclercq G, et al. β-Glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination. J Control Release 2013;172:671–8. doi:10.1016/j.jconrel.2013.09.007.

[33] Baert K, De Geest BG, De Rycke R, Antunes AB da F, De Greve H, Cox E, et al.

β-glucan microparticles targeted to epithelial APN as oral antigen delivery system. J Control Release 2015;220:149–59. doi:10.1016/j.jconrel.2015.10.025.

[34] Huang H, Ostroff GR, Lee CK, Specht C a, Levitz SM. Characterization and optimization of the glucan particle-based vaccine platform. Clin Vaccine Immunol 2013;20:1585–91. doi:10.1128/CVI.00463-13.

[35] Tincer G, Yerlikaya S, Yagci FC, Kahraman T, Atanur OM, Erbatur O, et al. Immunostimulatory activity of polysaccharide–poly(I:C) nanoparticles. Biomaterials 2011;32:4275–82. doi:10.1016/j.biomaterials.2011.01.028.

[36] Ernst O, Zor T. Linearization of the Bradford Protein Assay. J Vis Exp JoVE 2010:e1918. doi:doi:10.3791/1918.

[37] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012;9:676–82. doi:10.1038/nmeth.2019.

[38] Harnack U, Eckert K, Fichtner I, Pecher G. Oral administration of a soluble 1-3, 1-6 beta-glucan during prophylactic survivin peptide vaccination diminishes growth of a B cell lymphoma in mice. Int Immunopharmacol 2009;9:1298–303. doi:10.1016/j.intimp.2009.07.013.

[39] Le TH, Le TB, Doan THT, Quyen D Van, Le KXT, Pham VC, et al. The adjuvant effect of Sophy β-glucan to the antibody response in poultry immunized by the avian influenza A H5N1 and H5N2 vaccines. J Microbiol Biotechnol 2011;21:405–11. doi:10.4014/jmb.1011.11024.

[40] Lebre F, Borchard G, Faneca H, Pedroso de Lima M, Borges OM. Intranasal Administration of Novel Chitosan Nanoparticle/DNA Complexes Induces Antibody Response to Hepatitis B Surface Antigen in Mice. Mol Pharm 2016;13:472–82. doi:10.1021/acs.molpharmaceut.5b00707.

[41] Vicente S, Peleteiro M, González-Aramundiz JV, Díaz-Freitas B, Martínez-Pulgarín S, Neissa JI, et al. Highly versatile immunostimulating nanocapsules for specific immune potentiation. Nanomedicine 2014;9:2273–89. doi:10.2217/nnm.14.10.

[42] Kitaoka M, Naritomi A, Hirakawa Y, Kamiya N, Goto M. Transdermal Immunization using Solid-in-oil Nanodispersion with CpG Oligodeoxynucleotide Adjuvants. Pharm Res 2015;32:1486–92. doi:10.1007/s11095-014-1554-5.

[43] Hirakawa Y, Wakabayashi R, Naritomi A, Sakuragi M, Kamiya N, Goto M.

Transcutaneous immunization against cancer using solid-in-oil nanodispersions. Med Chem Commun 2015;6:1387–92. doi:10.1039/C5MD00168D.

[44] Roman F, Vaman T, Gerlach B, Markendorf A, Gillard P, Devaster J-MM. 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;28:1740–5. doi:10.1016/j.vaccine.2009.12.014.

[45] Esposito S, Tagliaferri L, Daleno C, Valzano A, Picciolli I, Tel F, et al. Pandemic influenza A/H1N1 vaccine administered sequentially or simultaneously with seasonal influenza vaccine to HIV-infected children and adolescents. Vaccine 2011;29:1677–82. doi:10.1016/j.vaccine.2010.12.047.

[46] Drogoz A, Munier S, Verrier B, David L, Domard A, Delair T. Towards biocompatible vaccine delivery systems: interactions of colloidal PECs based on polysaccharides with HIV-1 p24 antigen. Biomacromolecules 2008;9:583–91. doi:10.1021/bm701154h.

[47] Sharma S, Mukkur TK, Benson HA, Chen Y. Enhanced immune response against pertussis toxoid by IgA-loaded chitosan-dextran sulfate nanoparticles. J Pharm Sci 2012;101:233–44. doi:10.1002/jps.22763.

[48] Pawar D, Jaganathan K. Mucoadhesive glycol chitosan nanoparticles for intranasal delivery of hepatitis B vaccine: enhancement of mucosal and systemic immune response. Drug Deliv 2014:1–11. doi:10.3109/10717544.2014.908427.

[49] Drogoz A, David L, Rochas C, Domard A, Delair T. Polyelectrolyte complexes from polysaccharides: Formation and stoichiometry monitoring. Langmuir 2007;23:10950–8. doi:10.1021/la7008545.

[50] de la Fuente M, Seijo B, Alonso MJ. Design of novel polysaccharidic nanostructures for gene delivery. Nanotechnology 2008;19:75105. doi:10.1088/0957-4484/19/7/075105.

[51] de la Fuente M, Seijo B, Alonso MJ. Novel hyaluronan-based nanocarriers for transmucosal delivery of macromolecules. Macromol Biosci 2008;8:441–50. doi:10.1002/mabi.200700190.

[52] González-Aramundiz JV, Olmedo MP, González-Fernández Á, Alonso Fernández MJ, Csaba NS. Protamine-based nanoparticles as new antigen delivery systems. Eur J Pharm Biopharm 2015;97:51–9.

doi:10.1016/j.ejpb.2015.09.019.

[53] Vicente S, Díaz-Freitas B, Peleteiro M, Sanchez A, Pascual DW, González-Fernández Á, et al. A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach. PLoS One 2013;8:e62500. doi:10.1371/journal.pone.0062500.

[54] Lozano MV, Esteban H, Brea J, Loza MI, Torres D, Alonso MJ. Intracellular delivery of docetaxel using freeze-dried polysaccharide nanocapsules. J Microencapsul 2013;30:181–8. doi:10.3109/02652048.2012.714411.

[55] González-Aramundiz JV, Presas E, Dalmau-Mena I, Martínez-Pulgarín S, Alonso C, Escribano JM, et al. Rational design of protamine nanocapsules as antigen delivery carriers. J Control Release 2017;245:62–9. doi:10.1016/j.jconrel.2016.11.012.

[56] Tonnis W, Amorij J-P, Vreeman M, Frijlink H, Kersten GF, Hinrichs WL. Improved storage stability and immunogenicity of hepatitis B vaccine after spray-freeze drying in presence of sugars. Eur J Pharm Sci 2014;55:36–45. doi:10.1016/j.ejps.2014.01.005.

[57] Mody KT, Mahony D, Cavallaro AS, Stahr F, Qiao SZ, Mahony TJ, et al. Freeze-drying of ovalbumin loaded mesoporous silica nanoparticle vaccine formulation increases antigen stability under ambient conditions. Int J Pharm 2014;465:325–32. doi:10.1016/j.ijpharm.2014.01.037.

[58] Thomas SN, Schudel A. Overcoming transport barriers for interstitial-, lymphatic-, and lymph node-targeted drug delivery. Curr Opin Chem Eng 2015;7:65–74. doi:10.1016/j.coche.2014.11.003.

[59] Gause KT, Wheatley AK, Cui J, Yan Y, Kent SJ, Caruso F. Immunological Principles Guiding the Rational Design of Particles for Vaccine Delivery. ACS Nano 2017;11:54–68. doi:10.1021/acsnano.6b07343.

[60] Gutjahr A, Phelip C, Coolen A-L, Monge C, Boisgard A-S, Paul S, et al. Biodegradable Polymeric Nanoparticles-Based Vaccine Adjuvants for Lymph Nodes Targeting. Vaccines 2016;4:34. doi:10.3390/VACCINES4040034.

[61] Reimóndez-Troitiño S, Alcalde I, Csaba N, Íñigo-Portugués A, de la Fuente M, Bech F, et al. Polymeric nanocapsules: a potential new therapy for corneal wound healing. Drug Deliv Transl Res 2016:708–21. doi:10.1007/s13346-016-0312-0.

[62] Benne N, van Duijn J, Kuiper J, Jiskoot W, Slütter B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. J Control Release 2016;234:124–34. doi:10.1016/j.jconrel.2016.05.033.

[63] Brown GD, Herre J, Williams DL, Willment JA, Marshall ASJ, Gordon S. Dectin-1 Mediates the Biological Effects of β-Glucans. J Exp Med 2003;197:1119–24. doi:10.1084/jem.20021890.

[64] Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, et al. Dectin-1 is required for β-glucan recognition and control of fungal infection. Nat Immunol 2007;8:31–8. doi:10.1038/ni1408.

[65] Pan Y, Li X, Kang T, Meng H, Chen Z, Yang L, et al. Efficient delivery of antigen to DCs using yeast-derived microparticles. Sci Rep 2015;5:10687. doi:10.1038/srep10687.

[66] Kobiyama K, Temizoz B, Kanuma T, Ozasa K, Momota M, Yamamoto T, et al. Species-dependent role of type I IFNs and IL-12 in the CTL response induced by humanized CpG complexed with B-glucan. Eur J Immunol 2016;46:1142–51. doi:10.1002/eji.201546059.

[67] De Geest BG, Willart MA, Hammad H, Lambrecht BN, Pollard C, Bogaert P, et al. Polymeric multilayer capsule-mediated vaccination induces protective immunity against cancer and viral infection. ACS Nano 2012;6:1–4. doi:10.1021/nn205099c.

[68] Huang H, Ostroff GR, Lee CK, Specht CA, Levitz SM. Robust Stimulation of Humoral and Cellular Immune Responses following Vaccination with Antigen-Loaded B-Glucan Particles. MBio 2010;1:e00164-10-e00164-16. doi:10.1128/mBio.00164-10.

[69] Carter R, Thompson C, Reid D, Wong S, Tough D. Preferential Induction of CD4+ T Cell Responses through In Vivo Targeting of Antigen to Dendritic Cell-Associated C-Type Lectin-1. J Immunol 2006;177:2276–84. doi:10.4049/jimmunol.177.4.2276.

[70] Szuts A, Szabó-Révész P. Sucrose esters as natural surfactants in drug delivery systems - A mini-review. Int J Pharm 2012;433:1–9. doi:10.1016/j.ijpharm.2012.04.076.

General Discussion

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

General Discussion

The search for novel vaccine adjuvants that may contribute to the efficiency of immunization approaches has been one of the great challenges in vaccine development in the last decades. Despite the undeniable potential of vaccination for the control of infectious and non-infectious diseases all over the world, limitations including the need for a cold chain of storage and low immunogenicity of modern antigens raised the need for innovative solutions [1,2]. Nanotechnology has played a major role in this field, given the versatility and potential of nanocarriers for antigen delivery [3,4].

In the scope of this work, we have focused on polymer-based nanosystems specifically engineered for immunization, with special interest in polysaccharides. These natural polymers hold great promise as modulators of the immune system, given their wide distribution in the structure of several pathogenic species [5]. Our group was pioneer in the development of polysaccharide-based nanoparticles [6] and has published several reports on the potential of chitosan-based prototypes for immunization [7–9]. Other polysaccharides such as dextran, alginate, inulin and mannans have also attracted attention from researchers in this field [10–12].

Considering this background, this work aimed at the development and optimization of polymeric nanosystems to evaluate the impact of their specific composition and physicochemical properties regarding their biodistribution and adjuvant potential. In this line, we performed *in vitro* and *in vivo* studies, regarding the interaction of a panel of polymer-based nanocapsules with immune cells, with the aim of generating a deeper understanding of the parameters influencing these processes. Particularly, the effects of particle size, presence of a polymeric shell, and surface charge of the nanocarriers, were determined to be key parameters affecting the biodistribution of these vehicles and their interaction with antigen presenting cells (APCs). On the other hand, we developed and characterized a new thermostable prototype of nanoparticles, composed of chitosan and carboxymethyl-β-glucan, and evaluated its behaviour in a mouse model, in terms of biodistribution and capacity to elicit an immune response. The summary of the prototypes and experiments described in this thesis is graphically represented in **figure 1**.

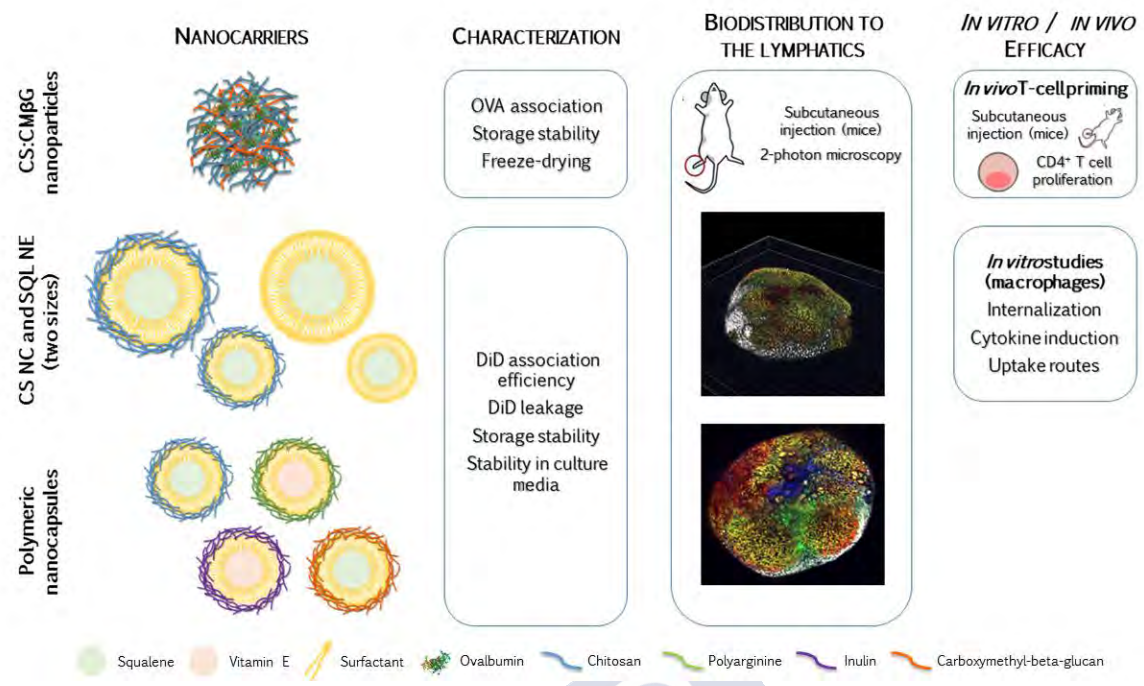


Figure 1. *Summary of the nanocarriers developed in the scope of this thesis and the experimental work performed with each formulation. CS, chitosan; CMβG, carboxymethyl-β-glucan; NC, nanocapsules; SQL, squalene; NE, nanoemulsions; OVA, ovalbumin*

1. Engineering of nanocarriers for lymphatic targeting and internalization by macrophages

Several authors have described the influence of the physicochemical characteristics of nanosystems on their efficient drainage from the injection site, with particular interest in particle size and surface charge [13–16]. Nevertheless, the lack of systematic comparisons may lead to generalized observations that do not always apply to other antigen nanocarriers. Therefore, we selected polymer-based nanocapsules with different sizes and surface coatings to perform comparisons that may provide some insights into the potential of these nanosystems for antigen delivery.

Starting from 200 nm chitosan nanocapsules, with a squalene core stabilized by lecithin and previously evaluated as antigen carriers [8,17,18], we were able to develop another prototype with the same composition but less than half of the particle size. This was possible through a reduction in the amount of components down to 25% of the initial values, as well as with the injection of the organic phase in the aqueous one. These results were in accordance with what was previously reported by our group for size reduction of polymeric nanocapsules [19]. In addition, we have also developed control

nanoemulsions, lacking the chitosan shell, to evaluate the influence of the polymeric coating in the behaviour of the nanocarriers. Finally, we prepared four nanocapsule prototypes with small particle size (below 100 nm) and different polymeric coatings (chitosan, polyarginine, inulin and carboxymethyl-β-glucan), to compare the effects of surface charge and specific polymer composition in the biodistribution of these carriers. These polymers were selected mainly given their demonstrated adjuvant potential in a variety of compositions [12,20–22]. Moreover, the use of carboxymethyl-β-glucan in nanocapsules is another innovative approach to the potential adjuvant role played by beta glucan-based antigen carriers. **Table 1** summarizes the physicochemical characteristics of these vehicles, which were labelled with the fluorescent marker DiD for *in vivo* imaging studies.

Table 1. *Physicochemical characterization of DiD-loaded nanocapsules and DiD-loaded control nanoemulsions developed and optimized for in vitro and in vivo imaging studies. SQL, squalene; NE, nanoemulsions; CS, chitosan; NC, nanocapsules; PArg, polyarginine; CMβG, carboxymethyl-β-glucan; PdI, polydispersity index.*

Nanocarrier	Particle size (nm)	PdI	Zeta Potential (mV)	DiD encapsulation efficiency (%)
SQL NE S	87 ± 12	0.2	-45 ± 9	95.7 ± 0.9
CS NC S	93 ± 9	0.2	+22 ± 2	94.7 ± 0.3
SQL NE M	166 ± 13	0.1	-66 ± 3	93.2 ± 0.2
CS NC M	238 ± 19	0.2	+32 ± 5	97.2 ± 0.3
PArg NC	90 ± 11	0.2	+55 ± 4	93.8 ± 0.7
Inulin NC	80 ± 5	0.2	-23 ± 5	100
CMβG NC	96 ± 8	0.2	-38 ± 3	84.6 ± 1.9

To evaluate the influence of this modulation of physicochemical parameters of nanocarriers on their in vitro and in vivo behaviour, we began evaluating their *in vitro* uptake by macrophages. Moreover, we studied the role played by two surface receptors, scavenger receptor A (SR-A) and Toll-like receptor 4 (TLR-4) in the uptake process, as well as the triggering of cytokine secretion following uptake, and the routes used by the cells for internalization. For this purpose, we selected chitosan nanocapsules and control nanoemulsions, with two particle sizes, to evaluate the influence of the chitosan coating

and particle size in these processes. Four uptake routes were studied using chemical inhibitors: cytochalasin D for phagocytosis, 5-(N,N-dimethyl)amiloride hydrochloride (DMA) for macropinocytosis, chlorpromazine hydrochloride for clathrin-mediated endocytosis and filipin III for caveolae-mediated endocytosis. Furthermore, we used macrophages from TLR-4 knockout mice and wildtype macrophages in which SR-A expression was silenced using siRNA.

In general, none of the four nanosystems generated a significant cytokine response, either in wildtype or in TLR-4 KO macrophages. However, in the case of MCP-1, smaller nanocapsules were able to elicit higher levels of this cytokine than the medium-sized ones, particularly in the case of TLR-4 KO cells. Despite the previous description of the role played by this receptor in mediating immunomodulating effects of chitosan [23,24], other cytokines such as TNF-α are usually highly secreted in those studies, contrarily to what we observed. This may be related to slight variations in the characteristics of the polymer, including purity and actual concentration of chitosan at the surface of each nanocarrier.

Concerning the role of the scavenger receptor, which is known to participate in the internalization of several pathogens and apoptotic cells [25], we observed that the uptake of all formulations was slightly reduced upon inhibition of this pathway. This effect was particularly noticeable in the case of smaller prototypes, and with nanocapsules in comparison with corresponding nanoemulsions. This was somehow unexpected, considering the reported affinity of scavenger receptors for polyanionic surfaces. However, the formation of a protein corona around nanocarriers in contact with complete cell media has also been well described for several carriers [26,27]. Therefore, we consider that this process of protein coating of the nanocapsules could be favouring their recognition by scavenger receptors and, consequently, increasing the influence of this pathway in the uptake of these formulations. In fact, scavenger-mediated uptake has been described in the literature for a wide range of nanosystems, including polymeric, metallic and nucleic acid-based nanoparticles [28–30].

Finally, we studied the routes involved in the internalization of the nanocarriers by macrophages, using specific pathway inhibitors. It is worth mentioning that different intracellular trafficking of nanovaccines in APCs may lead to different immune response pathways [31,32]. Overall, we observed some level of uptake inhibition in all routes, with particular relevance in the case of phagocytosis (inhibited by cytochalasin D). This compound interferes with phagocytic and macropinocytic processes, though, in view of the low inhibition we have observed with DMA (specific for micropinocytosis) we may

conclude that phagocytosis is probably the most relevant pathway used by the cells for particle internalization. Nevertheless, a combination of routes has been described for a variety of nanocarriers [33–36] and is probably responsible for the results we have obtained in these studies.

Particle size only influenced significantly the internalization via phagocytosis, which showed higher inhibition levels for smaller nanosystems than for their medium-sized counterparts. This is particularly interesting considering the common description of phagocytosis as an endocytic process specialized for larger particles (> 500 nm). However, in accordance with other published work [30,37], receptor-mediated phagocytosis may be relevant also for smaller particles. Interestingly, the role played by SR was only significant in the case of clathrin-mediated endocytosis, especially for medium-sized nanosystems. In view of previous works, describing the participation of scavenger receptors in the recognition of particles for clathrin coating [38–40], we may assume that in this case a combination of factors involving particle size and SR-A recognition is responsible for the uptake of our nanocarriers.

In general, the results obtained in these studies and summarized in **table 2**, show the complexity of the internalization process, with several routes playing a role in the uptake of the studied nanosystems. However, we could observe a more interesting profile for smaller chitosan nanocapsules, considering that the have the ability to elicit higher MCP-1 levels than their medium-sized counterparts. Moreover, smaller nanocapsules seemed to be more efficiently internalized than medium-sized ones. Nevertheless, we should consider the possibility of some differences in the chitosan shell density among the nanocapsule prototypes, which could also influence their internalization. It is also worth mentioning that the different uptake routes may assume some degree of compensation when one is inhibited [41–43], leading to this multifactorial profile observed in the inhibition studies.

Table 2. *Summary of the results obtained in the in vitro evaluation of the uptake routes and scavenger receptor (SR) influence in the internalization of chitosan nanocapsules (CS NC) and control nanoemulsions (SQL NE) by RAW 264.7 macrophages. Arrows indicate inhibition of uptake upon incubation with pathway inhibitor; (SR) indicates stronger uptake inhibition in cells with silenced SR expression, in comparison with wild-type cells.*

Formulation	Phagocytosis	Macropinocytosis	Clathrin-mediated	Caveolin-mediated
SQL NE S	↓	-	↓	↓
CS NC S	↓↓	-	↓	↓
SQL NE M	↓	-	↓ (SR)	↓
CS NC M	↓	-	↓ (SR)	↓

In a similar fashion than for CS:CMβG nanoparticles, studies were designed to evaluate the influence of particle size and polymer coating in the biodistribution of chitosan nanocapsules to the lymphatic system. For that, DiD-labelled nanosystems were subcutaneously administered to mice. Twelve hours later, we extracted the draining lymph nodes, popliteal and lumbar, for multiphoton fluorescence imaging and flow cytometry quantification. Regarding the influence of particle size, results showed significantly higher accumulation of the smaller nanocapsules (below 100 nm) in the popliteal lymph node (**figure 2**). Since this is the first node draining from the injection site, these results establish the facilitated access of smaller particles to the lymphatic system, though it is yet to be determined the exact way in which this drainage occurs. Fluorescence quantification from the microscopy images showed similar tendencies, therefore highlighting the potential of this technique for semi-quantitative biodistribution studies. Similar results had been already described for other type of nanocarriers, with sizes below 100 nm, being pointed as a favouring characteristic for free drainage of the particles from the administration site [19,44,45].

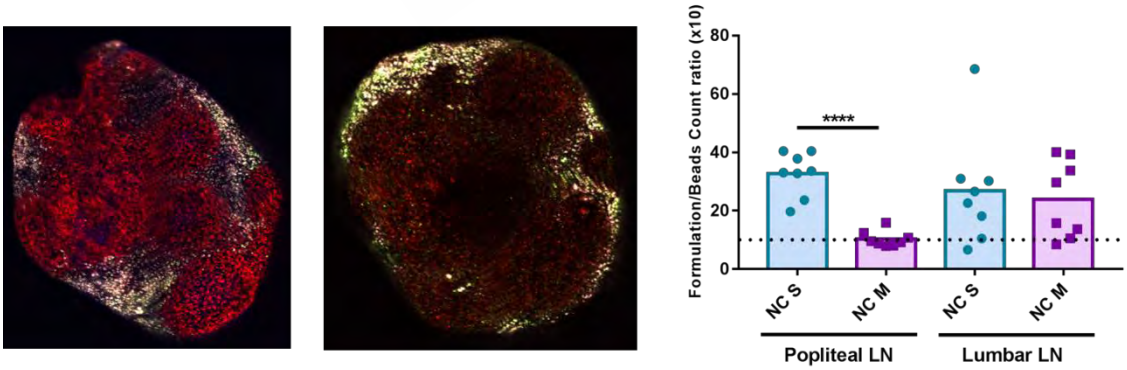


Figure 2. *Distribution of DiD-labelled CSNC of two particle sizes (NC S, below 100 nm and NC M, above 200 nm) in popliteal and lumbar lymph nodes, after subcutaneous injection. Ex vivo, 2-photon microscopy images show the accumulation of the*

*nanosystems (**left**, NC S and **center**, NC M) in the popliteal lymph node. Macrophages are shown in red (CD169-PE), nanocapsules in white (DiD) and control polystyrene beads in green (Firefli™ Fluorescent Green). Fluorescence intensity was quantified by flow cytometry (**right**). Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanocapsules and beads). **** p<0.0001.*

In view of these results, we additionally studied the kinetics of this distribution, through an *in vivo* imaging assay. For this purpose, we co-administered chitosan nanocapsules with both sizes subcutaneously to mice and acquired real-time videos of the arrival of those nanocarriers to the popliteal lymph node. In accordance with the previous *ex vivo* results, the videos showed a quick arrival of the smaller prototypes to the lymph node, about 8-10 minutes after the injection, as depicted in **figure 3**. Contrarily, the drainage was much slower for the larger nanocapsules, which also presented much lower accumulation levels in the lymph node at the end of the video timeframe (4 hours post-injection). The non-immediate arrival of the nanocapsules to the lymph node allows us to conclude that there should be no influence of the injection pressure in this process. For this reason, we can be more certain of the fundamental role played by the intrinsic characteristics of the nanocarriers in their drainage from the injection site and accumulation in the popliteal lymph node.

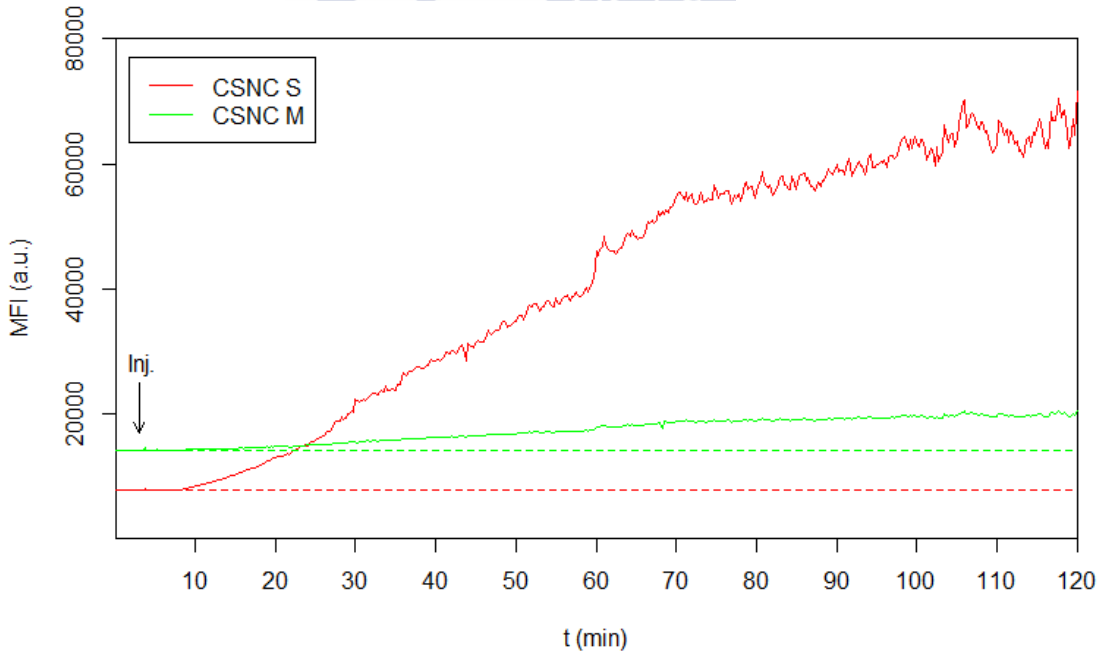


Figure 3. *Dynamics of the arrival of chitosan nanocapsules (CSNC S, below 100 nm and M, above 200 nm) to the popliteal lymph node after subcutaneous injection. Graphical representation of the median fluorescence intensity acquired for DiD- (CSNC S) and Nile Red- (CSNC M) labelled nanocapsules as a function of time (injection time is indicated with an arrow).*

Apart from the distribution of the nanocapsules to the lymphatic system following administration, it was also important to understand the level of co-localization of these formulations with the main cell populations of the lymph nodes. In these organs, the subcapsular sinus is usually rich in macrophages that sample the flowing lymph to capture antigens and other foreign materials for processing. Furthermore, in the medullary region, the populations of macrophages and dendritic cells are also in majority, providing antigen presentation to the effector T and B lymphocytes present in the lymph node [46]. For this reason, we selected these three populations – subcapsular sinus and medullary macrophages, as well as dendritic cells – to study their co-localization with the injected nanocarriers. Results showed higher accumulation of smaller nanocapsules with all three types of cells, and particularly in the case of medullary macrophages and dendritic cells. Considering that the drainage of the formulations from the injection site is quite rapid, it is expected that, at the time point we have selected, the nanocapsules had already passed the subcapsular sinus region, and were mostly accumulated in the medullary region, co-localizing with the macrophages and DCs present in that area. Manolova *et al* have described similar results, with small polystyrene nanoparticles (20 nm) accumulating at the subcapsular sinus region at an early time post-injection (2 hours), and later draining to other lymph node areas [45]. Overall, our results show an interesting biodistribution profile for particles with size below 100 nm, both in terms of the kinetics of their drainage to the lymph nodes and of their accumulation in these organs and co-localization with the main APC populations.

To evaluate the influence of the chitosan shell in this biodistribution process, we also performed *ex vivo* studies with control nanoemulsions, with the same composition and similar particle size as the previously described nanocapsules. In this case, we found unexpected results, since the larger nanoemulsions accumulated more in the draining lymph nodes following subcutaneous administration than all other prototypes. This trend was observed in both lymph nodes analysed (popliteal and lumbar), as can be seen in **figure 4**, and could be related with the easier flow of the nanoemulsions from the injection site due to their anionic nature. Moreover, nanoemulsions have been developed by

several authors as lymph node-targeting systems for imaging and theranostic purposes [47,48]. Alternatively, as described by Makidon *et al* for similar lipid-based nanoemulsions [49], these formulations could also be efficiently captured by APCs at the injection site and therefore be rapidly transported to the lymph nodes.

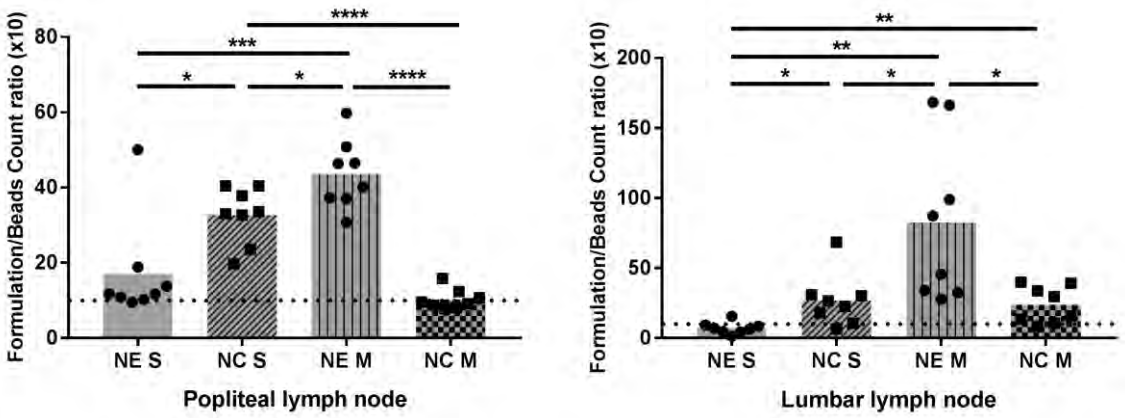


Figure 4. *Distribution of DiD-labelled chitosan nanocapsules (NC) and control nanoemulsions (NE) with two particle sizes (S, below 100 nm and M, around 200 nm) in popliteal and lumbar lymph nodes, after subcutaneous injection. Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanosystems and beads). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

Considering the relevant role played by the polymers and the surface charge they confer to the nanocapsules in their interaction with cells and tissues, previously reported by other authors [15,50,51], we completed this study with a comparison of nanocapsules with different polymeric shells. For this purpose, we used the previously mentioned chitosan nanocapsules, as well as polyarginine (cationic), inulin (neutral) and carboxymethyl-β-glucan (anionic). In the case of the popliteal lymph node, results showed a higher accumulation of the cationic nanocapsules, with particular significance for the polyarginine ones. As previously discussed, this could be due to the charge interactions between cell membranes and cationic materials [15,52], while anionic prototypes could flow further along the lymphatic system due to electrostatic repulsion which would hinder the interactions with immune cells [53,54].

Interestingly, as seen in **figure 5**, the accumulation levels in the lumbar lymph nodes showed a different trend. In this case, the nanocapsules with higher accumulation were the ones coated with polyarginine and carboxymethyl-β-glucan, showing a less important

effect of the surface charge. In fact, these results raise the possibility of a combined effect between different parameters including surface charge and intrinsic characteristics of the polymer used in the coating of the nanocapsules.

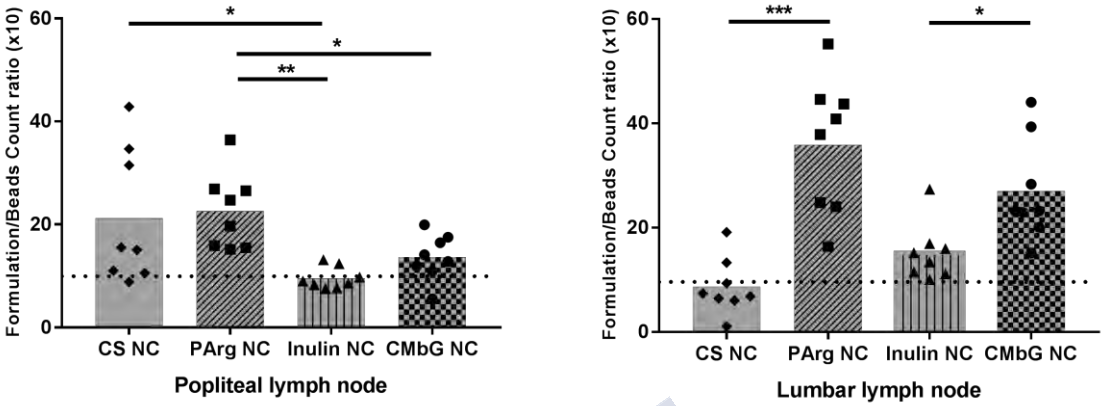


Figure 5. *Distribution of DiD-labelled chitosan (CS), polyarginine (PArg), inulin and carboxymethyl-β-glucan (CMbG) coated nanocapsules (60-90 nm) in popliteal (**left**) and lumbar (**right**) lymph nodes, after subcutaneous injection. Fluorescence intensity was quantified in total by flow cytometry. Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanocapsules and beads). * p<0.05, ** p<0.01, *** p<0.001.*

Studies comparing the internalization of different cationic polymers by immune cells have shown better performance of polyarginine [55,56], which might help explain our data. On the other hand, beta glucan particles injected to mice also showed fast drainage to lymph nodes and co-localization with macrophages in these organs [57–59]. Considering this, and in the absence of specific mechanistic reports comparing the biodistribution of different polymer-based nanocapsules upon administration to animal models, we may speculate that APCs possibly express specific surface receptors for these polymers, which would be responsible for their increased accumulation in lymph nodes.

Overall, our studies allowed the observation of a very important role played by both particle size and surface charge in the biodistribution of polymer-coated nanocapsules to the lymphatic system and in their interaction with the main immune cell populations. Further studies concerning the translation of these results in terms of the immune response elicited against these potential adjuvants are required to establish the real value of this modulation strategy.

2. Development of novel polymeric antigen nanocarriers

As previously mentioned, polysaccharides are particularly interesting in vaccine development given their action as pathogen-associated molecular patterns that can be recognized by APCs and therefore be processed for antigen presentation [5,60]. Specifically, we focused on carboxymethyl-β-glucan (CMβG), from the beta glucan family, which is known for their immunostimulant properties [61,62], though to the best of our knowledge, only two reports refer to its application in nanocarriers [63,64]. Beta glucan microparticles, obtained from the cell wall of fungi such as *Saccharomyces cerevisae*, have also been proposed for antigen delivery, particularly through the oral route, with promising results [22,65]. Moreover, this family of polymers has also shown interesting immunostimulant properties in a variety of animal models and through different administration routes [66].

On the other hand, as described in the previous section of this chapter, chitosan is a cationic polysaccharide widely explored in the field of nanoparticle development, and particularly in antigen delivery. Chitosan has been described as a mucoadhesive and immunostimulant polymer, and it was used in a variety of nanocarriers for the delivery of different antigens, including tetanus toxoid, hepatitis B and influenza [11,67]. In view of this background, we engineered an innovative nanoparticle formulation combining CMβG with chitosan (CS). A screening of the optimal conditions for the preparation of CS:CMβG nanoparticles was performed by varying the mass ratio of the polymers, while keeping constant the volumes and the initial concentration of each polymer solution (4 mg/mL of chitosan and 2 mg/mL of CMβG). The main physicochemical characteristics of the developed formulations are summarized in **table 3**.

Table 3. *Physicochemical characteristics of the CS:CMβG nanoparticles developed with variable mass ratios of both polymers. CS, chitosan; CMβG, carboxymethyl-β-glucan; PdI, polydispersity index.*

CS:CMβG w/w ratio	Particle size (nm)	PdI	Zeta Potential (mV)
4:1	120 ± 7	0.1	+28 ± 4
2:1	168 ± 3	0.2	+32 ± 1
1:2	695 ± 15	0.4	-44 ± 8

As shown by the physicochemical characterization, a small excess of chitosan (CS:CMβG mass ratio 2:1) was required to obtain monodisperse populations of particles

with sizes below 200 nm. An equal mass ratio (1:1) led to precipitation of the particles, probably due to charge neutralization. This in accordance with previous results obtained with a variety of polyelectrolyte-based nanosystems in which an excess of one of the components is usually required to avoid this phenomenon [68,69]. Moreover, results obtained in elemental analysis studies, as well as the high yield of production determined for this formulation, corroborated its theoretical composition, with efficient incorporation of both polymers in the final nanoparticles.

As a proof-of-concept of the ability of these particles to carry and deliver protein antigens, we have efficiently associated ovalbumin (OVA) as a model antigen, within the polymeric matrix of these systems. Given the low isoelectric point of this protein and, consequently, its anionic charge in aqueous solution, we have included it in the CMβG phase prior to the formation of the nanoparticles, to favour its incorporation. Irrespective of the theoretical loading (1, 3 and 6.7% w/w), OVA-loaded nanoparticles exhibited similar physicochemical properties as the blank nanocarriers. For this reason, we selected the highest loading for further studies. Moreover, antigen release from the nanoparticles in water at 37 ºC was negligible for up to 24 hours, providing good basis for further studies with this system.

To complete the characterization of this prototype, we assessed the morphology of the OVA-loaded nanoparticles using transmission (TEM) and scanning (SEM) electron microscopy techniques. Both techniques allowed a corroboration of the expected spherical structure of the nanoparticles, with some dispersion in particle size. In particular, it is worth mentioning the smaller size observed with these techniques, commonly attributed to the dryness of the sample in comparison with the hydrated status in DLS measurements. **Figure 6** shows an example of a TEM image and the proposed structure of the developed nanoparticles, as well as their main physicochemical characteristics.

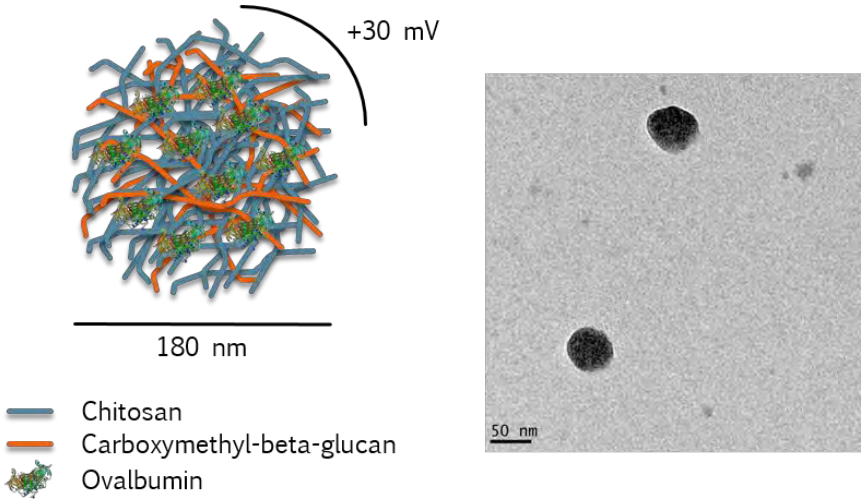


Figure 6. *Schematic representation (**left**) and transmission electron microscopy image (**right**) of CS:CMβG nanoparticles loaded with ovalbumin (scale indicated in the figure).*

Finally, the stability of these nanoparticles in storage conditions (4 ºC) was in accordance with results obtained for similar carriers [9,68], as they were able to maintain their physicochemical properties (particle size, PdI and zeta potential) for up to two months in the studied conditions. In addition, it is known that the need to keep a cold chain of storage for these products is one of the main challenges still to be achieved in vaccine development. In this regard, nanotechnology has emerged as an alternative to provide thermostability to vaccine formulations, through the lyophilisation of antigen-loaded nanosystems achieving a dry powder form that could be resuspended immediately before administration [70,71].

Bearing in mind this goal, we developed a freeze-dried form of the previously described OVA-loaded CS:CMβG nanoparticles. For this purpose, we tested a variety of cryoprotectants commonly used in this process, as well as different concentrations of these components. Ultimately, saccharides such as sucrose or trehalose are usually the most efficient excipients in lyophilisation processes, allowing the formation of adequate powder forms and the maintenance of the physicochemical characteristics of the nanosystems upon resuspension [18,72,73]. In our study, 15% (w/v) of trehalose was necessary to maintain the stability of the nanoparticles dry powder for at least one month, both at 25 ºC and at 40 ºC, in this case with humidity levels as high as 75% (**figure 7**). This stability is a good indication of the potential of this formulation to be kept in less adequate conditions and to avoid the costly cold chain of vaccine storage and transport. Moreover, our results have shown the conservation of the integrity of the antigen

throughout this process, as seen by Western blot images, which is an additional interesting result for this prototype. Nevertheless, *in vivo* studies should be performed to assess the efficacy of this powder formulation as an antigen carrier and adjuvant.

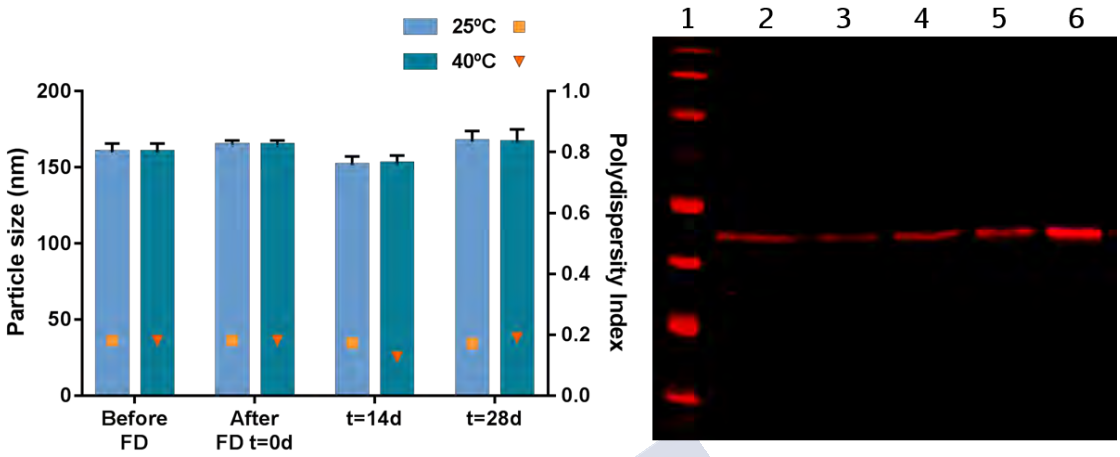


Figure 7. *Stability of OVA-loaded nanoparticles freeze-dried with 15% trehalose (w/v) (**left**: particle size – bars – and polydispersity index – symbols) upon storage at 25 ºC / 60% humidity and 40 ºC / 75% humidity. Antigen integrity following freeze-drying (**right**): lane 2, molecular weight marker; lane 2, ovalbumin control (5 µg/mL); lanes 3-6, ovalbumin-loaded nanoparticles after freeze-drying and storage at 40 ºC (resuspension done at 7, 14, 21 and 28 days after storage).*

Another important challenge in the field of vaccine delivery is the achievement of efficient non-parenteral vaccine formulations. This strategy has been considered fundamental to increase patient compliance and facilitate the administration of vaccines in developing countries, reducing the costs associated to sterile material and healthcare professionals required for parenteral immunization. Considering this, and in view of the results obtained with the OVA-loaded CS:CMβG nanoparticles, we prepared a solid-in-oil nanodispersion of these particles. These formulations, initially described as vehicles for catalytic enzymes, have also been described as drug and antigen carriers, mainly for the transdermal route [74–77]. For example, Kitaoka *et al* reported efficient transdermal immunization of mice with a solid-in-oil nanodispersion containing OVA and CpG as an adjuvant, which was able to overcome the skin barrier to generate a systemic immune response [76].

In our case, we chose squalene and vitamin E as starting oils to resuspend the freeze-dried nanoparticles, given the immunomodulatory properties presented by these

materials, which are currently included in the adjuvants of a few commercial vaccines [78,79]. Therefore, we attempted using a combination of these oils with or without different surfactants in order to redisperse the nanoparticles, which had been freeze-dried in presence of sucrose and sucrose esters. In this regard, we were able to obtain a clear dispersion of the nanoparticles in a mixture of squalene and 10% (w/w) Span® 80 (sorbitan oleate). Moreover, we could extract the nanoparticles from the oily dispersion and verify their physicochemical properties, which were maintained throughout the process. This leads us to believe this formulation could be optimized for administration through non-invasive routes and should be further studied in terms of the immune response elicited against the transported antigen.

3. Biodistribution and efficacy of CS:CMβG nanoparticles as antigen carriers

Currently, most vaccines are administered through parenteral routes such as subcutaneous or intramuscular. This approach is generally preferred since it grants easier access of the vaccine formulations to the lymphatic system, given the widespread lymphatic vessels network [44,80]. However, vaccines usually form a depot at the injection site, creating an inflammatory niche that leads to recruitment of immune cells. These cells then capture antigens and transport them to lymph nodes, which are the main centres of immune response [81]. Alternatively, certain nanoparticles, especially those with very small size (below 100 nm) and negative or neutral charge, can drain directly to the lymph nodes, without being transported for APCs. Once in the lymph nodes, nanoparticles are captured by resident APCs and the response obtained through each pathway can substantially differ. For this reason, it is essential to understand the biodistribution patterns of antigen nanocarriers following parenteral administration, particularly their access to the lymphatic system.

To provide a proof-of-concept of the adjuvant potential for the new CS:CMβG nanoparticles developed, we evaluated the biodistribution and interaction of these carriers with immune cells. For this purpose, we efficiently labelled chitosan with a fluorescent marker, TAMRA, through a covalent bond, therefore increasing the stability of the labelling. Following, we used an innovative imaging technique – multiphoton microscopy – combined with conventional flow cytometry fluorescence quantification. Multiphoton microscopy, through the use of multiple photons with lower individual energy levels, allows deeper tissue penetration with reduced photobleaching and lower risk of tissue damage [82]. For this reason, this technique has been used in particle tracking and imaging of biological events with high resolution [83–85]. Using these techniques,

we studied the biodistribution of blank and OVA-loaded nanoparticles to the lymphatic system upon subcutaneous administration to mice. Our results showed similar levels of accumulation of both formulations in the draining lymph node (popliteal), therefore leading to the observation of a low or even absent influence of the antigen in this process. Given the ability of APCs such as macrophages and DCs to recognize beta glucans through complement and Dectin-1 receptors [62,86], this accumulation could be due to this process rather than to antigen recognition. Furthermore, the cationic charge of the particles could also be positively influencing this process, given the well-known interactions between the negative surface of cell membranes and cationic materials [15,52].

As depicted in microscopy images, nanoparticles accumulated mostly in the medullary region of the lymph nodes, where an important population of DCs resides. Accordingly, as shown in **figure 8**, flow cytometry results showed a significantly higher co-localization of nanoparticles, particularly OVA-loaded ones, with these cells, in comparison with macrophages. Other authors had shown a high expression of Dectin-1, the main beta glucan receptor, in T-cell and medullary regions of lymph nodes, co-localizing with DC markers [87]. Although it is worth exploring if there is a targeted interaction between our nanocarriers and DCs, the improved interaction with this DCs may probably be related to the natural antigen recognition characteristics of these cells [46], which would probably be more efficient in internalizing the antigen-loaded particles than macrophages.

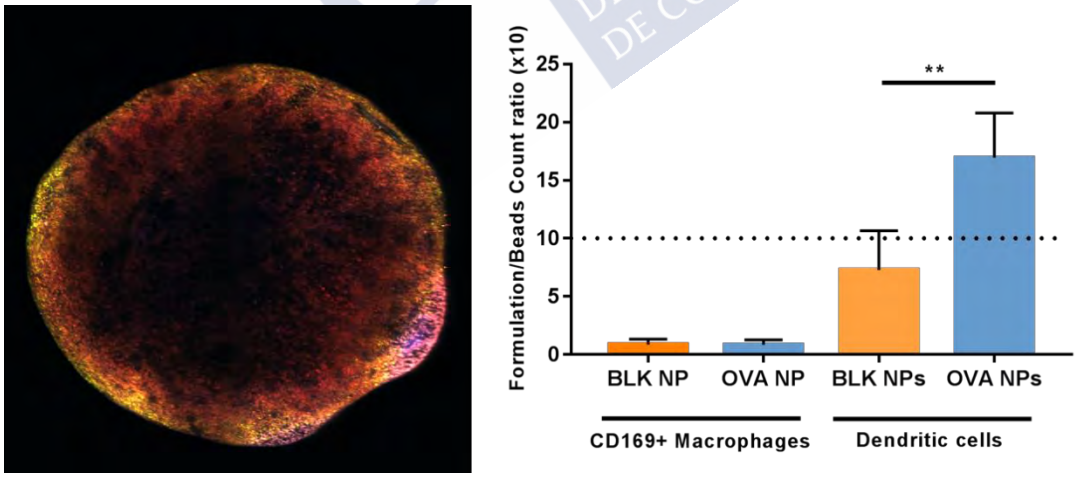


Figure 8. *Distribution of CS:CMβG nanoparticles (blank, BLK, or loaded with ovalbumin, OVA) in popliteal lymph node, after subcutaneous injection. Ex vivo, 2-photon microscopy image (**left**) show the accumulation of OVA-loaded nanoparticles in the medullary area of popliteal lymph node. Macrophages are shown in white (CD169-AF 647), follicles in blue (CD21/35-PB), nanoparticles in red (TAMRA) and control*

*polystyrene beads in green (Firefli™ Fluorescent Green). Fluorescence intensity was quantified by flow cytometry, in two cell populations, macrophages and dendritic cells (**right**). Results were normalized using the signal obtained for fluorescent polystyrene beads co-administered with the nanosystems (dot line represents equal accumulation of nanocapsules and beads). ** p<0.01.*

Considering this interesting biodistribution profile and the efficient co-localization of the nanoparticles with APCs, we studied the adjuvant potential of these carriers through an *in vivo* T-cell priming assay. In this study, we evaluate the ability of DCs from mice vaccinated with our formulations to present the antigen to specific CD4+ T cells adoptively transferred from OTII animals, inducing their proliferation. Furthermore, we compared these results with those elicited by alum-associated ovalbumin, to provide an adequate positive control using this well-known adjuvant. Interestingly, results showed at least equal ability of CS:CMβG nanoparticles to deliver the antigen to DCs and therefore induce T cell proliferation as alum-adsorbed antigen (**figure 9**), probably through the creation of the inflammatory niche required for induction of this type of immune response. Other authors had already shown the potential of beta glucan-based particles to induce T cell immunity [88,89], though this is, to the best of our knowledge, the first report using polyelectrolyte complexes containing a beta glucan derivative.

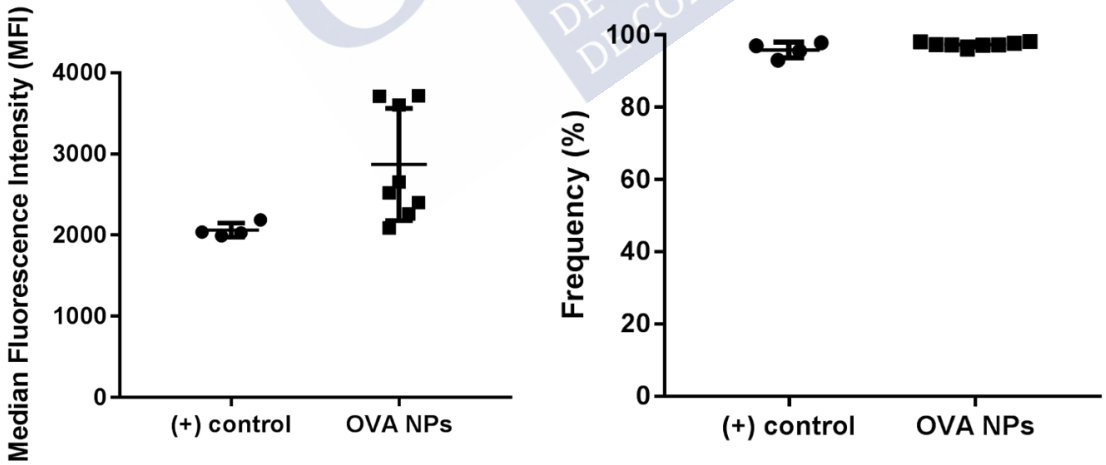


Figure 9. *In vivo priming of OVA-specific CD4+ T cells upon subcutaneous administration of OVA-loaded CS:CMβG nanoparticles (OVA NPs) (20 µg of ovalbumin per footpad). Positive control was performed with 10 µg of ovalbumin admixed with alum. Median fluorescence intensity (**left**) and frequency (**right**) plots for CFSE- populations in each treated group.*

Combining the results obtained in the biodistribution and T-cell priming assays, we may hypothesize that the increased co-localization of OVA-loaded nanoparticles with DCs may lead to an efficient induction of specific T cell responses. The role played by this receptor in the induction of T cell immunity has already been described [90] and should be confirmed for our nanoparticles through specific mechanistic studies.

Overall, we were able to show the adjuvant potential of the developed nanoparticles, through the triggering of a specific T cell response comparable to that achieved with alum. Studies concerning the ability of these formulations to elicit antibody responses and/or to be administered through non-parenteral routes are planned, to establish the real potential of this approach as a nanovaccination strategy.

As a general conclusion, the work developed in this thesis allowed the modulation of the biodistribution of polymer-coated nanocapsules depending on their particle size, surface charge and coating polymer, with interesting results both for polyarginine- and CMβG-coated prototypes. Future studies include the evaluation of the adjuvant potential of these carriers, namely to understand whether the differences observed in their biodistribution may lead to different immune response profiles. On the other hand, we were able to develop an innovative polysaccharide-based nanocarrier for antigen delivery containing carboxymethyl-β-glucan. The nanoparticles prepared with chitosan and CMβG presented interesting characteristics in terms of thermostability and biodistribution to the lymphatic system, as well as an adequate ability to transport and deliver a model antigen to APCs, leading to T cell proliferation.

REFERENCES

[1] Rappuoli R, Mandl CW, Black S, De Gregorio E. Vaccines for the twenty-first century society. Nat Rev Immunol 2011;11:865–72. doi:10.1038/nri3085.

[2] Nabel GJ. Designing tomorrow's vaccines. N Engl J Med 2013;368:551–60. doi:10.1056/NEJMra1204186.

[3] Irvine DJ, Swartz MA, Szeto GL. Engineering synthetic vaccines using cues from natural immunity. Nat Mater 2013;12:978–90. doi:10.1038/nmat3775.

[4] Cordeiro AS, Alonso MJ. Recent advances in vaccine delivery. Pharm Pat Anal 2016;5:49–73. doi:10.4155/ppa.15.38.

[5] Mahla RS, Reddy MC, Prasad DVR, Kumar H. Sweeten PAMPs: Role of sugar complexed PAMPs in innate immunity and vaccine biology. Front Immunol 2013;4:1–16. doi:10.3389/fimmu.2013.00248.

[6] Calvo P, Remuñán-López C, Vila-Jato JL, Alonso MJ. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. J Appl Polym Sci 1997;63:125–32. doi:10.1002/(SICI)1097-4628(19970103)63:1<125::AID-APP13>3.0.CO;2-4.

[7] Prego C, Paolicelli P, Díaz B, Vicente S, Sánchez A, González-Fernández Á, et al. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. Vaccine 2010;28:2607–14. doi:10.1016/j.vaccine.2010.01.011.

[8] Vicente S, Peleteiro M, Díaz-Freitas B, Sánchez A, González-Fernández Á, Alonso MJ, et al. Co-delivery of viral proteins and a tlr7 agonist from polysaccharide nanocapsules: A needle-free vaccination strategy. J Control Release 2013;172:773–81. doi:http://dx.doi.org/10.1016/j.jconrel.2013.09.012.

[9] Correia-Pinto JF, Csaba N, Schiller J, Alonso MJ. Chitosan-Poly (I:C)-PADRE Based Nanoparticles as Delivery Vehicles for Synthetic Peptide Vaccines. Vaccines 2015;3:730–50. doi:10.3390/vaccines3030730.

[10] Petrovsky N, Cooper PD. Carbohydrate-based immune adjuvants. Expert Rev Vaccines 2011;10:523–37. doi:10.1586/erv.11.30.

[11] Cordeiro AS, Alonso MJ, de la Fuente M. Nanoengineering of vaccines using natural polysaccharides. Biotechnol Adv 2015;33:1279–93. doi:10.1016/j.biotechadv.2015.05.010.

[12] Cristillo AD, Ferrari MG, Hudacik L, Lewis B, Galmin L, Bowen B, et al. Induction of mucosal and systemic antibody and T-cell responses following prime-boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. J Gen Virol 2011;92:128–40. doi:10.1099/vir.0.023242-0.

[13] Benne N, van Duijn J, Kuiper J, Jiskoot W, Slütter B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. J Control Release 2016;234:124–34. doi:10.1016/j.jconrel.2016.05.033.

[14] Lebre F, Hearnden CH, Lavelle EC. Modulation of Immune Responses by Particulate Materials. Adv Mater 2016:5525–41. doi:10.1002/adma.201505395.

[15] Fromen CA, Rahhal TB, Robbins GR, Kai MP, Shen TW, Luft JC, et al. Nanoparticle surface charge impacts distribution, uptake and lymph node trafficking by pulmonary antigen-presenting cells. Nanomedicine Nanotechnology, Biol Med 2016;12:677–87. doi:10.1016/j.nano.2015.11.002.

[16] Singh I, Swami R, Khan W, Sistla R. Lymphatic system: a prospective area for advanced targeting of particulate drug carriers. Expert Opin Drug Deliv 2014;11:211–29. doi:10.1517/17425247.2014.866088.

[17] Vicente S, Peleteiro M, González-Aramundiz JV, Díaz-Freitas B, Martínez-Pulgarín S, Neissa JI, et al. Highly versatile immunostimulating nanocapsules for specific immune potentiation. Nanomedicine 2014;9:2273–89. doi:10.2217/nnm.14.10.

[18] Vicente S, Díaz-Freitas B, Peleteiro M, Sanchez A, Pascual DW, González-Fernández Á, et al. A Polymer/Oil Based Nanovaccine as a Single-Dose Immunization Approach. PLoS One 2013;8:e62500. doi:10.1371/journal.pone.0062500.

[19] Abellán-Pose R, Teijeiro-Valiño C, Santander-Ortega MJ, Borrajo E, Vidal A, García-Fuentes M, et al. Polyaminoacid Nanocapsules for Drug Delivery to the Lymphatic System: Effect of the Particle Size. Int J Pharm 2016;509:107–17. doi:10.1016/j.ijpharm.2016.05.034.

[20] Ghendon Y, Markushin S, Vasiliev Y, Akopova I, Koptiaeva I, Krivtsov G, et al. Evaluation of Properties of Chitosan as an Adjuvant for Inactivated Influenza Vaccines Administered Parenterally. J Med Virol 2009;81:494–506. doi:10.1002/jmv.21415.

[21] Firbas C, Boehm T, Buerger V, Schuller E, Sabarth N, Jilma B, et al. Immunogenicity and safety of different injection routes and schedules of IC41, a Hepatitis C virus (HCV) peptide vaccine. Vaccine 2010;28:2397–407. doi:10.1016/j.vaccine.2009.12.072.

[22] De Smet R, Demoor T, Verschuere S, Dullaers M, Ostroff GR, Leclercq G, et al. β-Glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination. J Control Release 2013;172:671–8. doi:10.1016/j.jconrel.2013.09.007.

[23] Dang Y, Li S, Wang W, Wang S, Zou M, Guo Y, et al. The effects of chitosan oligosaccharide on the activation of murine spleen CD11c+ dendritic cells via Toll-like receptor 4. Carbohydr Polym 2011;83:1075–81. doi:10.1016/j.carbpol.2010.08.071.

[24] Zhang P, Liu W, Peng Y, Han B, Yang Y. Toll like receptor 4 (TLR4) mediates the stimulating activities of chitosan oligosaccharide on macrophages. Int Immunopharmacol 2014;23:254–61. doi:10.1016/j.intimp.2014.09.007.

[25] Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. Nat Rev Immunol 2013;13:621–34. doi:10.1038/nri3515.

[26] Yan Y, Gause KT, Kamphuis MM, Ang C-S, O'Brien-Simpson NM, Lenzo JC, et al. Differential roles of the protein corona in the cellular uptake of nanoporous polymer particles by monocyte and macrophage cell lines. ACS Nano 2013;7:10960–70. doi:10.1021/nn404481f.

[27] Albanese A, Walkey CD, Olsen JB, Guo H, Emili A, Chan WC. Secreted biomolecules alter the biological identity and cellular interactions of nanoparticles. ACS Nano 2014;8:5515–26. doi:10.1021/nn4061012.

[28] Kanno S, Furuyama A, Hirano S. A murine scavenger receptor MARCO recognizes polystyrene nanoparticles. Toxicol Sci 2007;97:398–406. doi:10.1093/toxsci/kfm050.

[29] Ezzat K, Aoki Y, Koo T, McClorey G, Benner L, Coenen-Stass A, et al. Self-Assembly into Nanoparticles Is Essential for Receptor Mediated Uptake of Therapeutic Antisense Oligonucleotides. Nano Lett 2015;15:4364–73. doi:10.1021/acs.nanolett.5b00490.

[30] França A, Aggarwal P, Barsov E V, Kozlov S V, Dobrovolskaia MA, González-

Fernández Á. Macrophage scavenger receptor A mediates the uptake of gold colloids by macrophages in vitro. Nanomedicine 2011;6:1175–88. doi:10.2217/nnm.11.41.

[31] Tonigold M, Mailänder V. Endocytosis and intracellular processing of nanoparticles in dendritic cells: routes to effective immunonanomedicines. Nanomedicine 2016;11:2625–30.

[32] Silva JM, Vandermeulen G, Oliveira VG, Pinto SN, Rodrigues C, Salgado A, et al. Development of functionalized nanoparticles for vaccine delivery to dendritic cells: a mechanistic approach. Nanomedicine 2014;9:2639–56. doi:10.2217/nnm.14.135.

[33] Firdessa R, Oelschlaeger TA, Moll H. Identification of multiple cellular uptake pathways of polystyrene nanoparticles and factors affecting the uptake: Relevance for drug delivery systems. Eur J Cell Biol 2014;93:323–37. doi:10.1016/j.ejcb.2014.08.001.

[34] Kastl L, Sasse D, Wulf V, Hartmann R, Mircheski J, Ranke C, et al. Multiple Internalization Pathways of Polyelectrolyte Multilayer Capsules into Mammalian Cells. ACS Nano 2013;7:6605–18. doi:10.1021/nn306032k.

[35] Ferreira SA, Correia A, Madureira P, Vilanova M, Gama FM. Unraveling the uptake mechanisms of mannan nanogel in bone-marrow-derived macrophages. Macromol Biosci 2012;12:1172–80. doi:10.1002/mabi.201200075.

[36] Tiffany M, Szoka FC. Co-localization of Fluorescent Labeled Lipid Nanoparticles with Specifically Tagged Subcellular Compartments by Single Particle Tracking at Low Nanoparticle to Cell Ratios. J Drug Target 2016;2330:1–21. doi:10.1080/1061186X.2016.1233976.

[37] Lunov O, Syrovets T, Loos C, Beil J, Delacher M, Tron K, et al. Differential uptake of functionalized polystyrene nanoparticles by human macrophages and a monocytic cell line. ACS Nano 2011;5:1657–69. doi:10.1021/nn2000756.

[38] Lunov O, Zablotskii V, Syrovets T, Röcker C, Tron K, Nienhaus GU, et al. Modeling receptor-mediated endocytosis of polymer-functionalized iron oxide nanoparticles by human macrophages. Biomaterials 2011;32:547–55. doi:10.1016/j.biomaterials.2010.08.111.

[39] Herrmann M, Schäfer C, Heiss A, Gräber S, Kinkeldey A, Büscher A, et al.

Clearance of fetuin-A-containing calciprotein particles is mediated by scavenger receptor-A. Circ Res 2012;111:575–84. doi:10.1161/CIRCRESAHA.111.261479.

[40] Orr GA, Chrisler WB, Cassens KJ, Tan R, Tarasevich BJ, Markillie LM, et al. Cellular recognition and trafficking of amorphous silica nanoparticles by macrophage scavenger receptor A. Nanotoxicology 2011;5:296–311. doi:10.3109/17435390.2010.513836.

[41] Ivanov AI. Pharmacological Inhibition of Endocytic Pathways: Is It Specific Enough to Be Useful? In: Ivanov AI, editor. Methods Mol. Biol. - Exocytosis Endocytosis, vol. 440. 1st ed., Totowa (NJ), USA: Humana Press; 2008, p. 15–34. doi:10.1007/978-1-59745-178-9.

[42] Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, et al. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. Mol Ther 2010;18:561–9. doi:10.1038/mt.2009.281.

[43] Chang TZ, Stadmiller SS, Staskevicius E, Champion JA. Effects of ovalbumin protein nanoparticle vaccine size and coating on dendritic cell processing. Biomater Sci 2017;5:223–33. doi:10.1039/C6BM00500D.

[44] Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat Biotechnol 2007;25:1159–64. doi:10.1038/nbt1332.

[45] Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol 2008;38:1404–13. doi:10.1002/eji.200737984.

[46] Abbas AK, Lichtman AH, Pillai S. Cellular and Molecular Immunology. 8th ed. Philadelphia (PA): Elsevier; 2015.

[47] Bae PK, Jung J, Chung BH. Highly enhanced optical properties of indocyanine green/perfluorocarbon nanoemulsions for efficient lymph node mapping using near-infrared and magnetic resonance imaging. Nano Converg 2014;1:6. doi:10.1186/s40580-014-0006-6.

[48] Kim H, Lee SK, Kim YM, Lee E-H, Lim S-J, Kim SH, et al. Fluorescent Iodized Emulsion for Pre- and Intraoperative Sentinel Lymph Node Imaging: Validation in a Preclinical Model. Radiology 2015;275:196–204. doi:10.1148/radiol.14141159.

[49] Makidon PE, Belyakov IM, Blanco LP, Janczak KW, Landers JJ, Bielinska AU, et al. Nanoemulsion mucosal adjuvant uniquely activates cytokine production by nasal ciliated epithelium and induces dendritic cell trafficking. Eur J Immunol 2012;42:2073–86. doi:10.1002/eji.201142346.

[50] Gutjahr A, Phelip C, Coolen A-L, Monge C, Boisgard A-S, Paul S, et al. Biodegradable Polymeric Nanoparticles-Based Vaccine Adjuvants for Lymph Nodes Targeting. Vaccines 2016;4:34. doi:10.3390/VACCINES4040034.

[51] van Riet E, Ainai A, Suzuki T, Kersten GF, Hasegawa H. Combatting infectious diseases; Nanotechnology as a platform for rational vaccine design. Adv Drug Deliv Rev 2014;74:28–34. doi:10.1016/j.addr.2014.05.011.

[52] Verma A, Stellacci F. Effect of Surface Properties on Nanoparticle-Cell Interactions. Small 2010;6:12–21. doi:10.1002/smll.200901158.

[53] Rao DA, Forrest ML, Alani AW, Kwon GS, Robinson JR. Biodegradable PLGA based nanoparticles for sustained regional lymphatic drug delivery. J Pharm Sci 2010;99:2018–31. doi:10.1002/jps.21970.

[54] Reddy ST, Berk DA, Jain RK, Swartz MA. A sensitive in vivo model for quantifying interstitial convective transport of injected macromolecules and nanoparticles. J Appl Physiol 2006;101:1162–9. doi:10.1152/japplphysiol.00389.2006.

[55] Mitchell D, Kim D, Steinman L, Fathman C, Rothbard J. Polyarginine enters cells more efficiently than other polycationic homopolymers. J Pept Res 2000;56:318–25. doi:10.1034/j.1399-3011.2000.00723.x.

[56] Maubant S, Banissi C, Beck S, Chauvat A, Carpentier AF. Adjuvant properties of Cytosine-phosphate-guanosine oligodeoxynucleotide in combination with various polycations in an ovalbumin-vaccine model. Nucleic Acid Ther 2011;21:231–40. doi:10.1089/nat.2011.0291.

[57] Mochizuki S, Morishita H, Kobiyama K, Aoshi T, Ishii KJ, Sakurai K. Immunization with antigenic peptides complexed with B-glucan induces potent cytotoxic T-lymphocyte activity in combination with CpG-ODNs. J Control Release 2015;220:495–502. doi:10.1016/j.jconrel.2015.11.008.

[58] Kobiyama K, Aoshi T, Narita H, Kuroda E, Hayashi M, Tetsutani K, et al. Nonagonistic Dectin-1 ligand transforms CpG into a multitask nanoparticulate

TLR9 agonist. Proc Natl Acad Sci 2014;111:3086–91. doi:10.1073/pnas.1319268111.

[59] Vu-Quang H, Muthiah M, Lee HJ, Kim Y-K, Rhee JH, Lee J-H, et al. Immune cell-specific delivery of beta-glucan-coated iron oxide nanoparticles for diagnosing liver metastasis by MR imaging. Carbohydr Polym 2012;87:1159–68. doi:10.1016/j.carbpol.2011.08.091.

[60] Demento SL, Siefert AL, Bandyopadhyay A, Sharp FA, Fahmy TM. Pathogen-associated molecular patterns on biomaterials: a paradigm for engineering new vaccines. Trends Biotechnol 2011;29:294–306.

[61] Novak M, Vetvicka V. Beta-glucans, history, and the present: immunomodulatory aspects and mechanisms of action. J Immunotoxicol 2008;5:47–57. doi:10.1080/15476910802019045.

[62] Goodridge HS, Wolf AJ, Underhill DM. β-glucan recognition by the innate immune system. Immunol Rev 2009;230:38–50. doi:10.1111/j.1600-065X.2009.00793.x.

[63] Tincer G, Yerlikaya S, Yagci FC, Kahraman T, Atanur OM, Erbatur O, et al. Immunostimulatory activity of polysaccharide–poly(I:C) nanoparticles. Biomaterials 2011;32:4275–82. doi:10.1016/j.biomaterials.2011.01.028.

[64] Anusuya S, Sathiyabama M. Preparation of β-D-glucan nanoparticles and its antifungal activity. Int J Biol Macromol 2014;70:440–3. doi:10.1016/j.ijbiomac.2014.07.011.

[65] Baert K, De Geest BG, De Rycke R, Antunes AB da F, De Greve H, Cox E, et al. β-glucan microparticles targeted to epithelial APN as oral antigen delivery system. J Control Release 2015;220:149–59. doi:10.1016/j.jconrel.2015.10.025.

[66] Soltanian S, Stuyven E, Cox E, Sorgeloos P, Bossier P. Beta-glucans as immunostimulant in vertebrates and invertebrates. Crit Rev Microbiol 2009;35:109–38. doi:doi:10.1080/10408410902753746.

[67] García-Fuentes M, Alonso MJ. Chitosan-based drug nanocarriers: Where do we stand? J Control Release 2012;161:496–504. doi:http://dx.doi.org/10.1016/j.jconrel.2012.03.017.

[68] González-Aramundiz JV, Olmedo MP, González-Fernández Á, Alonso

Fernández MJ, Csaba NS. Protamine-based nanoparticles as new antigen delivery systems. Eur J Pharm Biopharm 2015;97:51–9. doi:10.1016/j.ejpb.2015.09.019.

[69] Drogoz A, David L, Rochas C, Domard A, Delair T. Polyelectrolyte complexes from polysaccharides: Formation and stoichiometry monitoring. Langmuir 2007;23:10950–8. doi:10.1021/la7008545.

[70] Abdelwahed W, Degobert G, Stainmesse S, Fessi H. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv Drug Deliv Rev 2006;58:1688–713. doi:10.1016/j.addr.2006.09.017.

[71] Wang SH, Thompson AL, Hickey AJ, Staats HF. Dry powder vaccines for mucosal administration: critical factors in manufacture and delivery. Curr Top Microbiol Immunol 2012;354:121–56. doi:10.1007/82_2011_167.

[72] Lozano MV, Esteban H, Brea J, Loza MI, Torres D, Alonso MJ. Intracellular delivery of docetaxel using freeze-dried polysaccharide nanocapsules. J Microencapsul 2013;30:181–8. doi:10.3109/02652048.2012.714411.

[73] González-Aramundiz JV, Presas E, Dalmau-Mena I, Martínez-Pulgarín S, Alonso C, Escribano JM, et al. Rational design of protamine nanocapsules as antigen delivery carriers. J Control Release 2017;245:62–9. doi:10.1016/j.jconrel.2016.11.012.

[74] Tahara Y, Kamiya N, Goto M. Solid-in-oil dispersion: A novel core technology for drug delivery systems. Int J Pharm 2012;438:249–57. doi:10.1016/j.ijpharm.2012.09.007.

[75] Martins M, Loureiro A, Azoia NG, Silva C, Cavaco-Paulo A. Protein Formulations for Emulsions and Solid-in-Oil Dispersions. Trends Biotechnol 2016;34:496–505. doi:10.1016/j.tibtech.2016.03.001.

[76] Kitaoka M, Naritomi A, Hirakawa Y, Kamiya N, Goto M. Transdermal Immunization using Solid-in-oil Nanodispersion with CpG Oligodeoxynucleotide Adjuvants. Pharm Res 2015;32:1486–92. doi:10.1007/s11095-014-1554-5.

[77] Hirakawa Y, Wakabayashi R, Naritomi A, Sakuragi M, Kamiya N, Goto M. Transcutaneous immunization against cancer using solid-in-oil nanodispersions. Med Chem Commun 2015;6:1387–92. doi:10.1039/C5MD00168D.

[78] Roman F, Vaman T, Gerlach B, Markendorf A, Gillard P, Devaster J-MM. 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;28:1740–5. doi:10.1016/j.vaccine.2009.12.014.

[79] Esposito S, Tagliaferri L, Daleno C, Valzano A, Picciolli I, Tel F, et al. Pandemic influenza A/H1N1 vaccine administered sequentially or simultaneously with seasonal influenza vaccine to HIV-infected children and adolescents. Vaccine 2011;29:1677–82. doi:10.1016/j.vaccine.2010.12.047.

[80] Johansen P, Mohanan D, Martínez-Gómez JM, Kündig TM, Gander B. Lympho-geographical concepts in vaccine delivery. J Control Release 2010;148:56–62. doi:10.1016/j.jconrel.2010.05.019.

[81] De Temmerman M-L, Rejman J, Demeester J, Irvine DJ, Gander B, De Smedt SC. Particulate vaccines: on the quest for optimal delivery and immune response. Drug Discov Today 2011;16:569–82. doi:10.1016/j.drudis.2011.04.006.

[82] Amornphimoltham P, Masedunskas A, Weigert R. Intravital microscopy as a tool to study drug delivery in preclinical studies. Adv Drug Deliv Rev 2011;63:119–28. doi:10.1016/j.addr.2010.09.009.

[83] Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. Nat Biotechnol 2003;21:1369–77. doi:10.1038/nbt899.

[84] Stein J V, González SF. Dynamic intravital imaging of cell-cell interactions in the lymph node. J Allergy Clin Immunol 2017;139:12–20. doi:10.1016/j.jaci.2016.11.008.

[85] Kirui DK, Ferrari M. Intravital Microscopy Imaging Approaches for Image-Guided Drug Delivery Systems. Curr Drug Targets 2015;16:528–41.

[86] Huang H, Ostroff GR, Lee CK, Agarwal S, Ram S, Rice PA, et al. Relative contributions of Dectin-1 and complement to immune responses to particulate beta-glucans. J Immunol 2012;189:312–7. doi:10.1115/1.3071969.Automating.

[87] Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, et al. Dectin-1 is required for β-glucan recognition and control of fungal infection. Nat Immunol 2007;8:31–8. doi:10.1038/ni1408.

[88] De Geest BG, Willart MA, Hammad H, Lambrecht BN, Pollard C, Bogaert P, et al. Polymeric multilayer capsule-mediated vaccination induces protective immunity against cancer and viral infection. ACS Nano 2012;6:1–4. doi:10.1021/nn205099c.

[89] Huang H, Ostroff GR, Lee CK, Specht CA, Levitz SM. Robust Stimulation of Humoral and Cellular Immune Responses following Vaccination with Antigen-Loaded B-Glucan Particles. MBio 2010;1:e00164-10-e00164-16. doi:10.1128/mBio.00164-10.

[90] Carter R, Thompson C, Reid D, Wong S, Tough D. Preferential Induction of CD4+ T Cell Responses through In Vivo Targeting of Antigen to Dendritic Cell-Associated C-Type Lectin-1. J Immunol 2006;177:2276–84. doi:10.4049/jimmunol.177.4.2276.

Conclusions

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Conclusions

The experimental work described in this thesis aimed at evaluating the influence of the composition and physicochemical characteristics of polymer-based nanocarriers in their biodistribution and adjuvant potential. Overall, the results obtained allowed us to withdraw the following conclusions:

1. Polymer-based nanocapsules were developed and optimized to achieve a panel of physicochemical characteristics that would allow a rational comparison of the influence of those parameters in the biodistribution of the prototypes. In particular, chitosan nanocapsules of two particle sizes, as well as control nanoemulsions with the same characteristics and alternative coatings were developed. Novel nanocapsules with neutral (inulin) and anionic (carboxymethyl-β-glucan) coatings were originally described in this work.
2. Biodistribution of nanocapsules to the lymphatic system following subcutaneous administration to mice showed strong influence of particle size and surface coating. In this case, small chitosan nanocapsules, with particle size below 100 nm, were more efficiently drained from the injection site to the lymph node and co-localized in higher levels with both macrophages and DCs, in comparison with ≈200 nm ones. However, the opposite occurred with control nanoemulsions, suggesting a combination of factors influencing this process, including particle size and surface charge. Finally, the polymer used in the coating of nanocapsules also influenced targeting to the lymphatics, with polyarginine and carboxymethyl-β-glucan nanocapsules being the most promising ones in terms of their biodistribution profile.
3. Polysaccharide-based nanoparticles, composed of carboxymethyl-β-glucan and chitosan, exhibited a particle size of around 180 nm, a cationic surface charge, and a high protein loading capacity (close to 7% for OVA). This formulation was stable in storage conditions (4º C) for at least nine weeks.
4. A thermostable formulation of OVA-loaded CS:CMβG nanoparticles was achieved through freeze-drying, and maintained its colloidal properties and antigen integrity for up to one month at 40 ºC / 75% humidity. Moreover, we developed a solid-in-oil nanodispersion of the nanoparticles, which could be of interest for non-parenteral administration of antigens.
5. OVA-loaded CS:CMβG nanoparticles showed adequate draining to the lymphatic system following subcutaneous immunization to mice, being found mainly in the medullary region of the draining lymph node and preferentially co-localized with

dendritic cells. This biodistribution profile translated into an efficient delivery of the model antigen to these APCs, which were able to present it to CD4+ T cells, inducing their proliferation. These results provide proof-of-.concept of the adjuvant potential of the developed nanoparticles, described for the first time here.