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Hyaluronic acid nanocapsules for the intracellular delivery of anticancer drugs

Ana Cristina Cadete Pires

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Universidad de Santiago de Compostela

Facultad de Farmacia – Departamento de Tecnología Farmacéutica

Université d'Angers

UFR Santé – Micro et Nanomédecines Biomimétiques

Doctoral Thesis

**Hyaluronic acid nanocapsules for the intracellular
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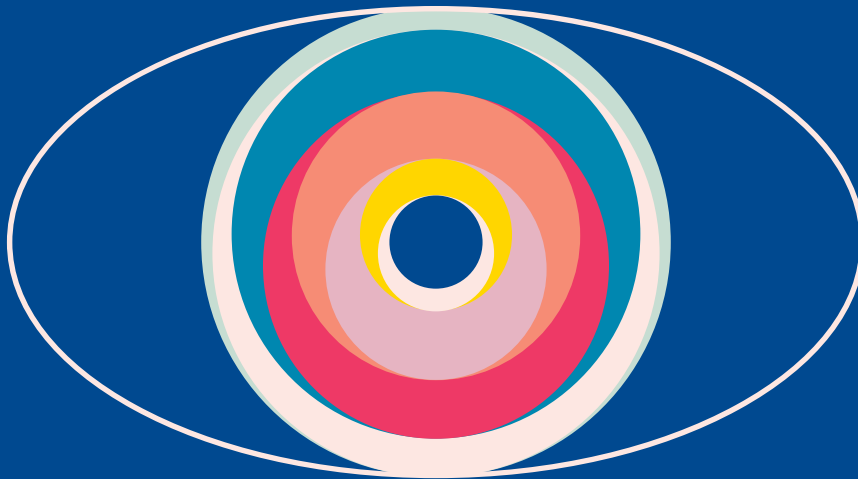
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Santiago de Compostela, 2016



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SANTIAGO DE COMPOSTELA | 2016



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Cover design: Ricardo Reis



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Facultad de Farmacia – Departamento de Tecnología Farmacéutica

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Tesis Doctoral

**Nanocápsulas de ácido hialurónico para la
liberación intracelular de fármacos antitumorales**

Ana Cadete Pires

Santiago de Compostela, 2016

Diseño portada: Ricardo Reis

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Informan:

Que la presente Memoria Experimental titulada: **“Nanocápsulas de ácido hialurónico para la liberación intracelular de fármacos antitumorales”**, elaborada por **Ana Cadete Pires**, fue realizada bajo su dirección y en el Departamento de Farmacia y Tecnología Farmacéutica de la Universidad de Santiago de Compostela y de la Universidad de Angers y, estando concluida, autorizan su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, expiden y firman el presente certificado en Angers, a 7 de marzo y en Santiago de Compostela, a 10 de marzo de 2016.

Prof. Dolores Torres

Prof. Marcos García-Fuentes

Prof. Jean Pierre Benoît

Dra. Dolores Torres, Associate Professor at the Department of Pharmacy and Pharmaceutical Technology at the University of Santiago de Compostela, Spain

Dr. Marcos García-Fuentes, Associate Professor at the Department of Pharmacy and Pharmaceutical Technology at the University of Santiago de Compostela, Spain

Dr. Jean Pierre Benoît, Full Professor at the Department of Pharmacy at the University of Angers, France

Report:

That the experimental entitled: “Hyaluronic acid nanocapsules for the intracellular delivery of anticancer drugs” presented by Ana Cadete Pires was conducted under their supervision at the Department of Pharmaceutical Technology at the University of Santiago de Compostela and the University of Angers. Being completed, they authorize its presentation and evaluation by the assigned tribunal members.

And for the record, they issue and sign the present certificate on Angers, March 7th and Santiago de Compostela, March 10th 2016.

Prof. Dolores Torres

Prof. Marcos García Fuentes

Prof. Jean Pierre Benoît

Aos meus pais.

À minha família.

*“Há um tempo em que é preciso abandonar as roupas usadas
que já tem a forma do nosso corpo,
e esquecer os nossos caminhos,
que nos levam sempre aos mesmos lugares.
É o tempo da travessia.
E se não ousarmos fazê-la,
teremos ficado, para sempre,
à margem de nós mesmos.”*

Fernando Pessoa

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Resumen/Abstract/Résumé

Resumen

En esta tesis se describe el desarrollo de un nuevo método sostenible para la elaboración de nanocápsulas de ácido hialurónico (NCs HA) como una nueva estrategia para el tratamiento del cáncer. Estas nanocápsulas permiten la incorporación de diferentes moléculas terapéuticas, tanto hidrofóbicas como hidrofílicas, y promueven su liberación en el interior de las células tumorales. En primer lugar, se desarrolló un método de auto-emulsificación para la preparación de las NCs HA sin el uso de disolventes orgánicos, temperatura o aplicación de energía. Estas condiciones son ideales para la incorporación de biomoléculas lábiles, así como para reducir el impacto medioambiental del proceso. Otra ventaja del sistema reside en el uso de un derivado de HA modificado hidrofóbicamente que permite la formulación de las nanocápsulas sin la adición de un tensoactivo catiónico, reduciendo así la posible toxicidad del sistema. Las NCs HA se mantuvieron estables en condiciones de almacenamiento y tras su dilución en plasma, manteniendo un tamaño nanométrico (130 nm) y una carga superficial negativa (-20 mV), lo que corrobora su potencial para administración intravenosa. La versatilidad de este nanosistema fue confirmada mediante la incorporación de diferentes moléculas: docetaxel, un fármaco citostático encapsulado en el núcleo oleoso, y anti-gasdermina B, un anticuerpo monoclonal asociado a la cubierta polimérica. El docetaxel fue eficazmente encapsulado, manteniendo su citotoxicidad en la línea celular de cáncer de pulmón A549, mostrando una liberación del sistema de un modo controlado. Finalmente, la anti-gasdermina B fue asociada de manera eficaz a la cubierta polimérica de las NCs HA y su liberación intracelular confirmada por microscopía confocal. Una vez en el interior de la célula, la anti-gasdermina B abandonó el compartimento endosomal y bloqueó de manera efectiva la proteína intracelular gasdermina B, promoviendo así una importante reducción de la migración e invasión de las células HCC1954 de cáncer de mama. Estos resultados ponen de manifiesto el potencial de las NCs HA, preparadas por auto-emulsificación, como sistemas multifuncionales para transportar diversos fármacos, con especial énfasis en la liberación intracelular de anticuerpos monoclonales, una estrategia ambiciosa en la lucha contra el cáncer.

Abstract

The main goal of this thesis has been the development of hyaluronic acid nanocapsules (HA NCs) as a multifunctional platform for the encapsulation and delivery of diverse anticancer drugs, such as hydrophobic drugs and hydrophilic biomolecules. The first step was the development of a spontaneous emulsification method, where HA NCs were formulated without the need of organic solvents, heat or high energy input, providing conditions for the incorporation of sensitive biomolecules while decreasing the environmental impact. Another advantage of this system is based on the use of a hydrophobically-modified HA derivative that allowed the preparation of HA NCs by hydrophobic interactions rather than electrostatic forces and thus, reducing the toxicity associated to the addition of a cationic surfactant as a counterion. Once formulated, HA NCs had a size around 130 nm and a negative zeta potential about -20 mV. Moreover, these nanocapsules were markedly stable under storage conditions and diluted in human plasma, taking forward this system as a potential carrier for intravenous administration. The versatility of this nanocarrier was confirmed by the incorporation of different molecules: docetaxel, a cytostatic drug, was incorporated into the oil core, whereas anti-gasdermin B, a monoclonal antibody, was entrapped into the polymeric shell. Docetaxel was highly encapsulated, released in a sustained manner and its cytotoxicity in A549 lung cancer cell line was maintained. Finally, anti-gasdermin B was successfully associated to the polymeric shell of HA NCs and its intracellular delivery confirmed by confocal microscopy. Once inside the cell, anti-gasdermin B was able to escape the endosomal compartment and to target the intracellular protein gasdermin B, promoting an important decrease in the migratory and invasive behavior of HCC1954 breast cancer cell line. All these results highlight the potential of self-emulsifying HA NCs as multifunctional systems to transport diverse anticancer drugs, with special emphasis in the intracellular delivery of monoclonal antibodies, an ambitious challenge that could open new avenues to fight cancer.

Résumé

Cette thèse de doctorat avait pour principal objectif le développement d'une méthode viable pour la formulation de nanocapsules d'acide hyaluronique (NCs HA) à des fins d'incorporation et de libération intracellulaire d'agents anticancéreux. La première étape de ce travail a visé le développement d'une méthode d'émulsion spontanée dans laquelle les NCs HA ont été formulées sans avoir recours à des solvants organiques, ni à un travail à haute température ou à un apport énergétique élevé, ce qui fournit des conditions optimales pour l'incorporation de biomolécules sensibles tout en diminuant l'impact environnemental. Un autre avantage de ce système est basé sur l'utilisation d'un dérivé de l'acide hyaluronique modifié hydrophobiquement, ce qui permet la formulation de NCs HA par des interactions hydrophobes, réduisant ainsi la toxicité due à l'addition d'un surfactant cationique. Une fois formulées, les NCs HA étaient caractérisées par une taille de 130 nm et un potentiel zeta négatif de -20 mV. La versatilité de ce nanotransporteur a été confirmée par l'incorporation de différentes molécules : le docétaxel, un agent cytostatique, a été incorporé au sein du cœur huileux, tandis que l'anti-gasdermin B, un anticorps monoclonal, a été piégé au sein de l'enveloppe polymérique. Le taux d'encapsulation du docétaxel était élevé, sa libération contrôlée et sa cytotoxicité maintenue sur la lignée cellulaire A549 de cancer du poumon. Enfin, l'anti-gasdermin B a été associée avec succès à l'enveloppe polymérique de NCs HA et, une fois à l'intérieur de la cellule, l'anti-gasdermin B était capable d'échapper au compartiment endosomal et d'effectivement cibler la protéine intracellulaire gasdermin B, entraînant une importante diminution du comportement migratoire et invasif des cellules de la lignée HCC1954 de cancer du sein. Tous ces résultats mettent en évidence le potentiel de NCs HA auto-émulsifiées en tant que systèmes multifonctionnels pour transporter divers agents anticancéreux, en particulier pour la libération intracellulaire d'anticorps monoclonaux, une approche ambitieuse qui pourrait passer au premier plan parmi les stratégies innovantes dans la lutte contre le cancer.

Resumen *in extenso*

Introducción

El cáncer es una de las principales causas de morbilidad y mortalidad en todo el mundo, responsable de más de 9 millones de muertes al año. Pese a los avances en investigación y al continuo descubrimiento de nuevas dianas y moléculas terapéuticas, estamos aún lejos de que la cura del cáncer sea una realidad. Por lo tanto, sigue siendo una prioridad en investigación la búsqueda de nuevas terapias que permitan lograr resultados más prometedores en el tratamiento del cáncer.

La quimioterapia es la modalidad terapéutica más aplicada a la mayoría de los pacientes con cáncer. Sin embargo, los fármacos utilizados presentan una distribución inespecífica, que da lugar a que sólo una pequeña fracción del fármaco llegue al tumor. Esto hace que dichos tratamientos no sean lo suficientemente eficaces y que, en muchos de los casos, estén asociados con la aparición de graves efectos adversos. El conocimiento de algunos de los mecanismos asociados al crecimiento tumoral ha estimulado el descubrimiento de nuevos agentes terapéuticos, más específicos y capaces de ejercer sus efectos sobre proteínas individuales implicadas en el desarrollo tumoral. Aunque estas nuevas terapias pueden contribuir a una mayor supervivencia de los pacientes, hay una serie de barreras biológicas que dificultan su administración sistémica y por ello, necesitan de un vehículo que les permita alcanzar las células tumorales de una manera más efectiva.

La nanomedicina es la aplicación de la nanotecnología en el campo de la medicina y agrupa tres áreas principales: el diagnóstico, el transporte de fármacos (nanoterapias) y la medicina regenerativa. La nanoterapia, enfocada en cáncer, pretende utilizar plataformas nanométricas como transportadores de fármacos quimioterapéuticos, asegurando una liberación más eficaz en las células tumorales. Con esta finalidad, se han desarrollado diferentes sistemas entre los que se pueden mencionar las nanopartículas, los liposomas o las micelas. En los últimos años, la atención se ha centrado considerablemente también en las nanocápsulas poliméricas como vehículos transportadores con potencial aplicación en oncología. Las nanocápsulas son sistemas vesiculares que presentan una estructura versátil y ventajosa para la incorporación de diversas moléculas terapéuticas. Están compuestas por un núcleo oleoso, capaz de

incorporar moléculas hidrofóbicas, como la mayoría de los fármacos citostáticos convencionales, y una cubierta polimérica diseñada para asegurar una mejor protección del fármaco, controlar su liberación y lograr una acumulación selectiva en las células tumorales. Actualmente, el ácido hialurónico (HA) es uno de los polímeros más utilizados para la formulación de nanotransportadores y, en el caso de las nanocápsulas poliméricas podría incorporarse formando parte de la cubierta.

El HA es un polisacárido de origen natural constituido por unidades repetidas de ácido glucurónico y acetil glucosamina, que presenta propiedades físico-químicas adecuadas para su aplicación en nanotecnología. En primer lugar, el HA es un biomaterial biocompatible, biodegradable y sin problemas de toxicidad aparente. Además, su carácter aniónico ($pK_a = 3 - 4$) le permite interactuar con otros polímeros catiónicos, lípidos o tensoactivos, dando lugar a la formación de muchos nanosistemas. Finalmente, el HA tiene grupos funcionales reactivos, los cuales permiten su conjugación con otros fármacos o moléculas químicas. Además de sus propiedades físico-químicas, el HA posee características especiales que lo hacen atractivo para el desarrollo de nanosistemas en oncología. En primer lugar, su carácter hidrofílico genera alrededor de las partículas una repulsión estérica que puede evitar la opsonización, permitiendo un aumento en el tiempo de circulación en sangre, resultando en una mayor acumulación de fármaco en el tumor, por medio del conocido “efecto de permeabilidad y retención aumentada”. Por otra parte, el HA tiene la capacidad de interactuar con receptores celulares específicos, como el CD44, que está sobre-expresado en un gran número de tumores. Esta interacción HA-CD44 representa una estrategia muy prometedora para la orientación de moléculas terapéuticas a células cancerosas, un efecto conocido como “vectorización activa”.

Cabe destacar, además, que en la selección de un proceso de preparación de nanosistemas, no solo se tienen en cuenta las características del fármaco y la composición del nanosistema, sino que también se consideran de crítica importancia las necesidades industriales, el impacto ambiental y el coste/efectividad de la formulación. Así, surge la técnica de auto-emulsificación como una alternativa a las técnicas convencionales de preparación como, por ejemplo, el desplazamiento del disolvente.

Utilizando este método, las nanoemulsiones se forman en ausencia de disolventes orgánicos, calor o energía, proporcionando la posibilidad de incorporar moléculas lábiles como proteínas, péptidos o anticuerpos, sin que sean degradados durante el proceso de preparación. La técnica de auto-emulsificación consiste en la formación espontánea de nanoemulsiones cuando una fase oleosa, conteniendo un tensoactivo dispersable en agua, se mezcla con una fase acuosa bajo agitación magnética. El método de auto-emulsificación presenta importantes ventajas como, por ejemplo, elevado rendimiento de producción, fácil escalado industrial y bajo impacto ambiental, por lo cual es considerado como “tecnología sostenible o tecnología verde”. Como inconvenientes a mejorar, se podrían citar la importante presencia de tensoactivos, así como el hecho de que sea una técnica que exige una elevada solubilidad del fármaco en la fase oleosa.

El avance en investigación permite que sea cada vez más frecuente el descubrimiento de nuevas dianas terapéuticas como, por ejemplo, determinadas proteínas intracelulares responsables de la invasión y migración de las células tumorales. Hasta ahora, la mayoría de las terapias contra estas proteínas intracelulares se basaban en el uso de quimioterapia, terapias silenciadoras (siRNA) o inhibidores de las proteínas quinasas. Sin embargo, debido a la falta de eficacia de las mismas, persiste la necesidad de encontrar un vehículo que consiga el “targeting” de las proteínas intracelulares.

El objetivo general de este trabajo se ha orientado al desarrollo de nanocápsulas de HA, diseñadas como una plataforma multifuncional para la incorporación de fármacos antitumorales de diferente naturaleza y facilitar su acceso al interior de las células cancerosas. Los objetivos específicos se pueden describir de la siguiente manera:

1. Desarrollo de un método de auto-emulsificación para la preparación de nanocápsulas de HA, utilizando dos tipos de polímero: el HA y un HA modificado con una molécula lipídica.
2. Incorporación en el núcleo oleoso de las nanocápsulas de un fármaco antitumoral hidrofóbico, el docetaxel.
3. Asociación de una proteína terapéutica, el anticuerpo monoclonal anti-gasdermin B, en la cubierta polimérica, destinada a ser liberada en el interior de las células tumorales

y a bloquear la oncoproteína gasdermin B, responsable de la migración e invasión de las células tumorales.

1. Desarrollo de un método de auto-emulsificación para la preparación de nanocápsulas de HA

1.1 Metodología

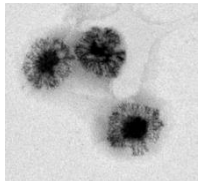
Las nanocápsulas se prepararon mediante la técnica de auto-emulsificación, utilizando el HA y un HA modificado con una cadena lipídica (mod-HA). El método se optimizó inicialmente para la formulación de nanoemulsiones y, posteriormente, se adaptó para la preparación de las nanocápsulas. En primer lugar, se seleccionaron los materiales más adecuados para la preparación de las nanoemulsiones sin disolventes orgánicos, eligiendo el núcleo oleoso y los tensoactivos más apropiados. A continuación, se estudiaron distintos parámetros clave en la formación del sistema: la cantidad de tensoactivo en la fase acuosa, la relación aceite/tensoactivo en la fase oleosa y, por último, la relación fase oleosa/fase acuosa. Una vez elegida la composición y las relaciones más adecuadas para la elaboración de las nanoemulsiones, las nanocápsulas se prepararon de la misma manera, pero incorporando el polímero en la fase acuosa. Los parámetros objeto de estudio en la preparación de las nanocápsulas fueron: la cantidad de tensoactivo catiónico en la fase oleosa y la concentración de HA en la fase acuosa. Estos parámetros fueron optimizados para conseguir formulaciones con un tamaño nanométrico inferior a 150 nm, un índice de polidispersión inferior a 0.2 y una carga superficial negativa. Una vez preparadas, las nanocápsulas se aislaron por cromatografía de exclusión de tamaño, se caracterizaron por espectroscopia de correlación fotónica (Zetasizer Nano ZS, Malvern) y su morfología se visualizó mediante microscopía electrónica de transmisión (TEM, CM12, Phillips). La toxicidad de las nanocápsulas y su capacidad de internalización en las células tumorales se evaluó *in vitro* utilizando la línea de cáncer de pulmón A549 y el método de viabilidad celular AlamarBlue®. Para los ensayos de internalización se incorporó un fluoróforo, el rojo nilo, en el núcleo oleoso de las nanocápsulas, evaluando su capacidad de internalización mediante microscopía confocal.

1.2 Resultados

En primer lugar, se optimizó el método de preparación de las nanoemulsiones mediante la técnica de auto-emulsificación, procediendo a la selección de los componentes y parámetros de formulación más adecuados. Así, Miglyol®812 y Tween®80 fueron los componentes que constituyeron la fase oleosa y, la fase acuosa se formó con una solución de Solutol®HS15. El Miglyol®812 se eligió como núcleo oleoso dado que es un triglicérido de cadena media, ampliamente utilizado en la formulación de este tipo de sistemas. Además, tiene la capacidad de solubilizar fármacos hidrofóbicos, como el docetaxel, permitiendo así su incorporación en el núcleo oleoso de las nanocápsulas. Respecto al tensoactivo, el Tween®80 se seleccionó porque su balance hidrofilia-lipofilia (HLB) de 15 le confiere una gran hidrofilia, favoreciendo la formación inmediata de nanoemulsiones aceite/agua. Comparándolo con otros tensoactivos similares, el Tween®80 presenta la ventaja de estar ya aprobado para administración por vía parenteral. La selección del Solutol®HS15 guarda relación con su HLB de 14-16, que facilita su incorporación en la interfaz de las nanoemulsiones y, además, presenta cadenas PEGyladas que aumentan la estabilidad del sistema en circulación. Una vez seleccionados los componentes, el método de auto-emulsificación se optimizó para la preparación de las nanoemulsiones de acuerdo con el siguiente procedimiento: la fase oleosa, compuesta por Miglyol®812/Tween®80 (relación 1:1 p/p) se añadió a la fase acuosa, constituida por una solución de Solutol®HS15 de concentración 2.5 mg/mL. La fase oleosa se añadió a la fase acuosa en una relación 1:8 (v/v), bajo agitación magnética. Las NCs HA se prepararon utilizando este procedimiento, incorporando el HA a la superficie de las nanocápsulas mediante interacciones electrostáticas entre el polímero, cargado negativamente, y la superficie de las partículas modificadas con un tensoactivo catiónico, CTAB. La cubierta de HA (0.25 mg/mL) dio lugar a una inversión del potencial zeta de +10 mV, en las nanoemulsiones catiónicas, a -18 mV tras la adsorción del polímero. Para evitar el uso del tensoactivo catiónico, cuya presencia puede estar relacionada con una posible toxicidad celular, el HA se sustituyó por un HA modificado químicamente con una cadena lipídica. Este mod-HA presenta un carácter anfifílico, lo

cual permite su incorporación en el sistema mediante interacciones hidrofóbicas. Las nanocápsulas de mod-HA presentaron características muy semejantes a las formuladas con el HA. Sin embargo, fue necesario añadir 0.5 mg/mL de mod-HA para conseguir una carga superficial en torno a -20 mV. En la **Tabla 1** se representan las características físico-químicas de los sistemas preparados por auto-emulsificación y una imagen de las nanocápsulas de mod-HA, obtenida por TEM. La imagen muestra la estructura núcleo-cubierta característica de las nanocápsulas.

Tabla 1. Caracterización físico-química de las distintas formulaciones preparadas por auto-emulsificación y fotografía de las nanocápsulas de mod-HA, obtenida por microscopía electrónica de transmisión (TEM).

Formulación	Tamaño (nm)	PDI	Potencial Zeta (mV)	Imagen
NE aniónica	145 ± 1	0.2	-15 ± 2	
NE catiónica	146 ± 3	0.2	+10 ± 1	
NCs HA	137 ± 11	0.2	-19 ± 1	
NCs mod-HA	126 ± 5	0.2	-20 ± 2	

Nota: Los resultados están expresados como media ± desviación estándar (n=3)

Abreviaturas: PDI, índice de polidispersión; NE, nanoemulsión; NCs, nanocápsulas; HA, ácido hialurónico nativo; mod-HA, ácido hialurónico modificado

La **Figura 1** muestra el perfil de toxicidad de las distintas formulaciones, en células A549, tras 72h de incubación. Se observa que, independientemente de la composición de los sistemas, las nanocápsulas preparadas con HA o mod-HA no afectan a la viabilidad de las células A549, hasta alcanzar una concentración de 350 µg/mL. Sin embargo, para concentraciones superiores (hasta 1000 µg/mL), solo las nanocápsulas preparadas con mod-HA mostraron un perfil ausente de toxicidad. Estos resultados podrían estar relacionados con la presencia del tensoactivo CTAB en las nanocápsulas de HA, y su potencial toxicidad celular. Por otro lado, la mezcla de tensoactivos compuesta por Tween®80, Solutol®HS15 y CTAB para una concentración de 350 µg/mL, dio lugar a una acentuada toxicidad celular, con un 85% de muerte celular. Esto indica que los tensoactivos libres en solución presentan una toxicidad muy elevada que se ve disminuida cuando se incorporan a la estructura de las nanocápsulas.

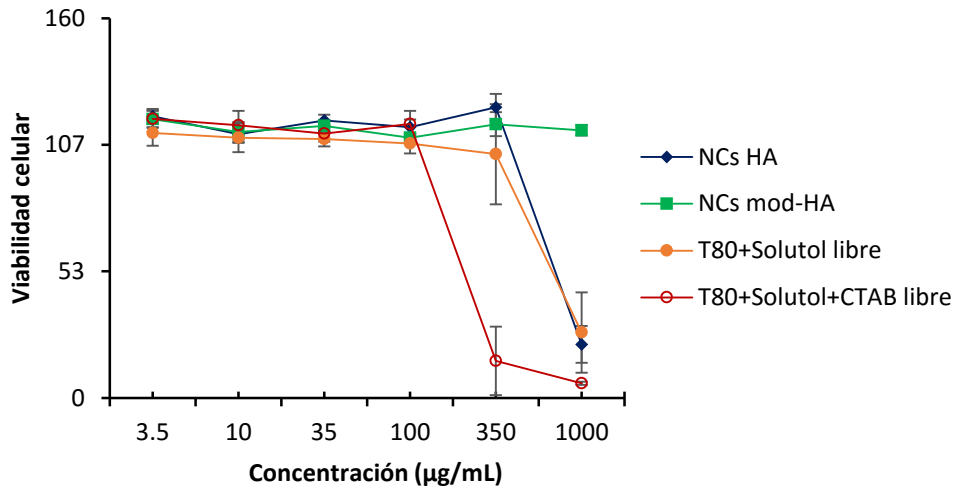


Figura 1. Viabilidad celular determinada en células de cáncer de pulmón A549, tras 72h de incubación con diferentes concentraciones de nanocápsulas de HA y mod-HA, y mezclas de tensoactivos

Abreviaturas: NCs, nanocápsulas; HA, ácido hialurónico nativo; mod-HA, ácido hialurónico modificado; T80, Tween[®]80

La capacidad de internalización de las nanocápsulas se estudió mediante microscopía confocal utilizando nanocápsulas marcadas con rojo nilo. Como control, las células se expusieron a una solución del fluoróforo libre, que no fue internalizado (**Figura 2 A**). Sin embargo, las nanocápsulas consiguieron penetrar en el interior celular y liberar dentro de las células una gran cantidad del marcador, como así lo confirmó la elevada fluorescencia observada en el citoplasma celular (**Figura 2 B**). Esta internalización podría estar probablemente mediada por un proceso de endocitosis asociado a los receptores CD44 expresados en la superficie de las células A549.

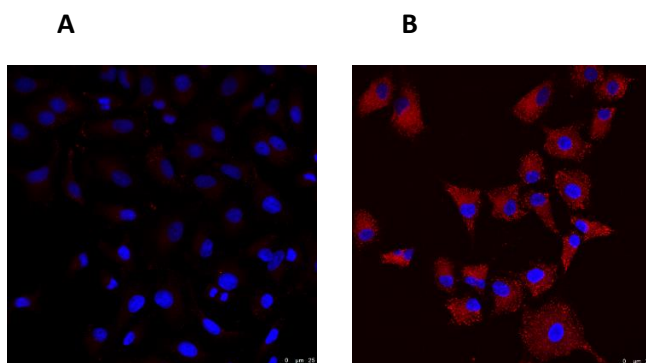


Figura 2. Estudio de internalización del fluoróforo rojo nilo solo (a la izquierda) o incluido en las NCs HA (a la derecha).

2. Incorporación del docetaxel en las nanocápsulas de HA

2.1 Metodología

En primer lugar, se hicieron estudios de solubilidad del docetaxel en Miglyol®812 para proceder a su incorporación directa en el aceite. Para ello, se puso en contacto un exceso de docetaxel con un determinado volumen de Miglyol®812 bajo agitación magnética. Tras 24h, la suspensión se centrifugó y el docetaxel solubilizado fue cuantificado mediante una técnica de HPLC. Las nanocápsulas de HA conteniendo docetaxel se prepararon de acuerdo con el procedimiento anterior, utilizando como núcleo oleoso el Miglyol®812 con el docetaxel solubilizado. Estos nanosistemas se caracterizaron en cuanto a tamaño, índice de polidispersión y potencial zeta. Tras separar el fármaco libre del encapsulado mediante cromatografía de exclusión por tamaño, la eficacia de encapsulación se determinó de un modo directo, valorando el docetaxel encapsulado. Así, se utilizó la siguiente ecuación: $[\text{fármaco encapsulado}] / [\text{fármaco total}] \times 100$. La liberación del docetaxel a partir de las nanocápsulas se cuantificó tras dilución en PBS a 37°C, siguiendo un método en el que se evaluó el reparto del fármaco desde una suspensión de nanocápsulas hacia una fase oleosa externa, capaz de solubilizar el fármaco libre. La actividad del fármaco encapsulado se confirmó mediante ensayos de toxicidad en células A549.

2.2 Resultados

La solubilidad del docetaxel en Miglyol®812 fue de 2.03 ± 0.2 mg/mL. De acuerdo con estos resultados, se preparó una solución madre de docetaxel en Miglyol®812 de 1.8 mg/mL, garantizando la solubilidad total del fármaco y evitando su precipitación. Las nanocápsulas conteniendo docetaxel mantuvieron sus características físico-químicas iniciales, mostrando una elevada eficacia de encapsulación, en torno al 90%, que se corresponde con una concentración de docetaxel en las nanocápsulas de 100 µg/mL (**Tabla 2**).

Tabla 2. Caracterización físico-química de las nanocápsulas de HA conteniendo docetaxel.

Formulación	Tamaño (nm)	PDI	Potencial Zeta (mV)	EE (%)
NCs HA	140 ± 1	0.2	-18 ± 2	88 ± 9
NCs mod-HA	145 ± 3	0.2	-20 ± 1	86 ± 3

Nota: Los resultados están expresados como media \pm desviación estándar (n=3)

Abreviaturas: PDI, índice de polidispersión; mod-HA, ácido hialurónico modificado; EE, eficacia de encapsulación

En ambas formulaciones preparadas con HA o mod-HA, se produjo una liberación rápida inicial de 45% y 55% de docetaxel, respectivamente. Sin embargo, en ambos prototipos la liberación del docetaxel se prolongó hasta las 24h, alcanzando un valor del 70% de ambos sistemas. Este perfil de liberación se puede justificar por la propia estructura de las nanocápsulas, que favorece un reparto del fármaco entre el núcleo oleoso y el medio acuoso.

Las nanocápsulas de HA con docetaxel demostraron una mejor inhibición de la viabilidad celular (IC50) en comparación con el fármaco libre. La concentración IC50 para el fármaco encapsulado en las nanocápsulas de HA se correspondió con un valor de 10µM tras 48h de incubación. Sin embargo, con el fármaco libre no se llegó a alcanzar el valor de IC50 en el rango de concentraciones estudiadas (**Figura 3**). Las nanocápsulas de HA pueden ser consideradas, por lo tanto, como nanosistemas prometedores para la

liberación del docetaxel en el interior de las células tumorales, promoviendo una mayor toxicidad que el fármaco libre.

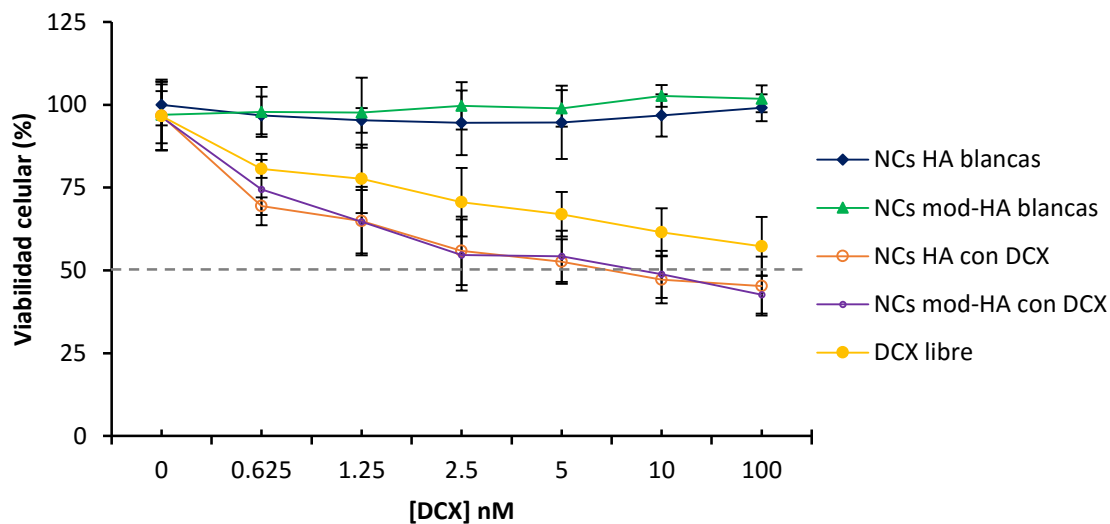


Figura 3. Viabilidad celular determinada en células de cáncer de pulmón A549, tras 48h de incubación con diferentes concentraciones de nanocápsulas de HA, mod-HA, y fármaco libre.

Abreviaturas: NCs, nanocápsulas; mod-HA, ácido hialurónico modificado; DCX, docetaxel

3. Asociación del anticuerpo monoclonal anti-gasdermina B a las nanocapsulas de ácido hialurónico modificado

3.1 Metodología

La concentración del anticuerpo monoclonal (mAb), anti-gasdermina B (anti-GSDMB), se midió utilizando el equipo Nanodrop® 2000 y su pureza e integridad se analizó por SDS-PAGE. La afinidad del mAb por el antígeno (la oncoproteína gasdermina B, GSDMB) se midió mediante la técnica ELISA. La asociación de la anti-GSDMB a las nanocápsulas se llevó a cabo mediante un proceso de adsorción, incubando las nanocápsulas de mod-HA con concentraciones crecientes de proteína, bajo agitación magnética, promoviendo interacciones tanto iónicas como hidrofóbicas. La asociación se evaluó tanto con el anticuerpo protonado como con el no-protonado. El punto isoeléctrico de la anti-GSDMB se encuentra entre 6.5 y 8.1, y por lo tanto a $\text{pH} < 6.5$ se encuentra cargada positivamente. Así, la anti-GSDMB protonada se preparó por acidificación con una solución de acetato de sodio/ ácido acético a $\text{pH} 3.8$, hasta alcanzar un pH final de 4.5.

Las nanocápsulas con el mAb asociado se caracterizaron con respecto al tamaño, índice de polidispersión y potencial zeta, como se ha descrito previamente. La eficacia de asociación se determinó mediante ELISA una vez separado el mAb asociado del libre por centrifugación, utilizando filtros Nanosep®300K.

La internalización de la anti-GSDMB se evaluó mediante inmunofluorescencia en células de cáncer de mama, HCC1954. Para ello, tanto la anti-GSDMB libre como la asociada a las nanocápsulas se incubaron con las células HCC1954 durante 2h. Tras el período de incubación, las células se fijaron con paraformaldehído al 4% durante 15 min y se permeabilizaron con tritón Triton X-100 al 0.1% en PBS durante 10 min. La anti-GSDMB marcada con un anticuerpo secundario acoplado a una molécula fluorescente (Alexafluor) se visualizó mediante microscopia confocal. Por último, la eficacia de la anti-GSDMB para bloquear la oncoproteína intracelular se estudió mediante un ensayo de migración en un modelo de herida celular, en células HCC1954.

3.2 Resultados

Una de las dificultades más grandes en el tratamiento del cáncer es el “targeting” de las dianas intracelulares. De hecho, muchas de las oncoproteínas responsables de la invasión y migración de las células tumorales están en su citoplasma. En este trabajo, hemos utilizado un mAb, la anti-GSDMB, diseñada para bloquear una oncoproteína, la GSDMB, localizada en el compartimento celular de las células HCC1954 de cáncer de mama. Los mAbs libres no son capaces de atravesar la membrana celular y por ello, el objetivo de este trabajo se ha dirigido a la asociación de la anti-GSDMB a las nanocápsulas de HA (mod-HA) como estrategia para promover su acceso intracelular y bloquear así la oncoproteína diana y, consecuentemente, inhibir la migración de las células tumorales.

Una vez caracterizada la pureza e integridad de la anti-GSDMB, se incubaron concentraciones crecientes del mAb con las nanocápsulas de mod-HA por medio de un proceso de adsorción físico, evitando el uso de reactivos agresivos y garantizando así la integridad y conformación de la misma. En estos ensayos, se evaluó el efecto de la

incorporación del mAb sin protonar o protonado. En el caso del mAb protonado (pH 4.5), su interacción con el polímero de las nanocápsulas (cargado negativamente) debería ser fundamentalmente iónica. Por otro lado, con la anti-GSDMB sin protonar, se sugiere que las fuerzas hidrofóbicas son las que deberían gobernar el proceso. Los resultados indicaron que, independientemente del tipo de interacción, tanto el mAb protonado como el sin protonar, se asoció eficazmente a la cubierta de las nanocápsulas de mod-HA (80%). De igual manera, el tamaño y el índice de polidispersión se mantuvieron sin alteraciones significativas. Con respecto al potencial zeta, éste se vio ligeramente modificado (de -20 mV para las blancas a -10 mV para las que contenían el mAb), corroborando la asociación eficaz de la proteína. Una vez que se comprobó que la protonación del mAb no presentaba ninguna ventaja para la encapsulación del mismo, las nanocápsulas de mod-HA con la anti-GSDMB sin protonar fueron las elegidas para llevar a cabo los ensayos de internalización celular y eficacia. De este modo, el mAb se utilizó en sus condiciones óptimas, ya que el medio ácido a largo plazo podría interferir con la estabilidad e integridad del sistema.

Los ensayos de internalización demostraron, según lo esperado, la incapacidad del mAb sin encapsular para atravesar la membrana celular. Por el contrario, su asociación a las nanocápsulas de mod-HA hizo posible su internalización y su liberación en el citoplasma de las células HCC1954. La internalización de las nanocápsulas puede justificarse mediante la afinidad del HA por los receptores CD44, expresados en la membrana de las células HCC1954, que favorece la entrada por endocitosis. Uno de los retos de la terapia biológica es, no solo conseguir que la proteína, el mAb en este caso, entre en las células, sino también garantizar que consigue escapar de la degradación por los lisosomas. El estudio de eficacia consistió en la evaluación de la capacidad de la anti-GSDMB para bloquear la oncoproteína intracelular, mediante ensayos de migración. La GSDMB se caracteriza por promover la invasión de las células tumorales que resulta en una migración acentuada de las mismas. La liberación del mAb en el compartimento intracelular de las células HCC1954 dio lugar a un bloqueo efectivo de la GSDMB, resultando inhibida de manera significativa la migración e invasión de estas células tumorales.

Conclusiones

Este trabajo demuestra el potencial de las nanocápsulas de HA como sistemas multifuncionales capaces de promover la liberación intracelular de fármacos antitumorales de diferente naturaleza. Las nanocápsulas de HA se desarrollaron mediante un nuevo método de auto-emulsificación que emerge como una tecnología sostenible que evita el uso de solventes orgánicos. Por un lado, el fármaco antitumoral docetaxel se encapsuló eficazmente en el núcleo oleoso de las nanocápsulas, dando lugar a una disminución de la viabilidad de las células de cáncer de pulmón A549, en comparación con el fármaco libre. Por otro lado, las nanocápsulas de HA constituyeron una plataforma eficaz para la liberación intracelular de proteínas terapéuticas, como los mAb. Así, se ha demostrado que la internalización celular del mAb, anti-GSDMB, en células de cáncer de mama HCC1954, sólo fue posible al ser incorporado a las nanocápsulas de HA. Además, su eficacia al interactuar con la oncoproteína intracelular GSDMB, se ha puesto de manifiesto al inhibir de forma significativa la migración e invasión de las células tumorales.

En conclusión, este sistema representa una estrategia prometedora en el tratamiento del cáncer, constituyendo una plataforma capaz de combinar la terapia tradicional de citostáticos con nuevas inmunoterapias, al facilitar el acceso intracelular de biomoléculas terapéuticas que por sí solas no serían capaces de atravesar la membrana celular.

Introduction

Cancer is one of the worst diseases we are facing nowadays and exert an enormous global toll. In 2015, about 9 million people worldwide died from some source of cancer. The progress in cancer genomics had push research to a point where new targets, molecules and pathways are constantly coming up. This “boom” in the backstage of research gave us, pharmacists, the responsibility of finding a way to take to patients these new treatments and nanotechnology was, undoubtedly, essential to achieve our goals. Many drug delivery systems have been designed in the last few years. However, development and innovation are not anymore the only concern of the pharmaceutical industry when we talk about new nanotechnologies but has been an increased attention to “green technology” and the development of environmentally friendly techniques. Furthermore, nanotechnology has led to the development of versatile drug delivery systems, intended not only for the encapsulation of cytostatic drugs but also for the delivery of complex biologic molecules, such as monoclonal antibodies.

The aim of this introduction is to give an overview of how important is green technology for industries and what is its impact in formulation development. Additionally, it would be interesting to evaluate the importance of nanotechnology in the development of new delivery systems and to assess the undergoing clinical candidates for docetaxel. Finally, we discuss whether it is feasible the use of monoclonal antibodies (mAb) as a promising strategy for the targeting of intracellular cancer proteins.

1. Green technology – the impact of sustainable methodologies in the pharmaceutical industry

“Nanotechnology and green chemistry have an intimate relationship and great potential to do good.” John C. Warner, University of Massachusetts Center for Green Chemistry

In November 2015, the G20 summit joined the most powerful countries to discuss, among others, a global solution to climate change. Although a drop in the ocean, pharmaceutical companies are responsible for an environmental footprint and the chemical industry is directly responsible for adverse impacts in the environment and public health. A change in work mentalities started two decades ago with the release of

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the “Twelve principles of green chemistry” and since then, this field has received great attention from the scientific community due to its capability to design alternative, safer, energy efficient, and less toxic routes towards synthesis [1]. Nowadays, it is visible the commitment of global healthcare companies by developing environmentally favorable techniques. The biggest examples come from Pfizer, Merck and GlaxoSmithKline (GSK). For example, by applying the principles of green chemistry, Pfizer dramatically improved the manufacturing process of sertraline which offered pollution prevention benefits, including both workers and environment safety. That success inspired Pfizer to start a “Green Journey” and look to other manufacturing processes in order to integrate environmental sustainability into its business and supplier network [2–3]. Additionally, GSK started a “green chemistry initiative” applied to the discover new medicines while reducing the environmental impact of their manufacture. Scientists come up with new ways of making medicines by using “greener” solvents (less toxic, easy to dispose and recycle), reducing waste and balance water consume [4]. Additionally, GSK had developed “Green technology guides” to move the company towards more sustainable business practices [5].

The increasing awareness and desire for green technology have emerged not only into the field of chemistry but is also becoming of full importance in the design of new nanotechnologies. If four years ago green nanotechnology was not widespread and popular in the scientific and business communities, nowadays the formulation of nanocarriers with sustainable materials and methodologies is an industrial priority [6]. Three main reasons have motivated this change: (i) emerged nanotechnologies can be made clean from the start, breaking a whole set of environmental problems; (ii) adopting green nano-approaches to technology development would shift society to look at nanotechnology with a new proactive paradigm; and (iii) investors are looking at sustainable technologies as the largest economic opportunity of the 21st century [7]. There have been many advances in greener synthesis of nanoparticles, especially in the reduction of solvents use, energy and water consumption and the hazards of reagents disposed. A successful study was the design and synthesis of gold and silver nanoparticles using green chemistry and the same accomplishment can be applied to

polymeric nanocarriers, for example by using polysaccharides as green capping agents [1]. The pharmaceutical industry is one of the larger users of organic solvents and companies are constantly attempting to eliminate its excessive usage [8]. Alongside with the environmental impact, solvents are expensive to use, to store and to dispose [9]. By avoiding or reducing the use of solvents, pharmaceutical industries would improve its business strategy and sustainable policy.

It is clear the influence of green technologies in chemistry, formulation and nanotechnology. As such, the design of new nanoparticles that meet specific requirements and pose a minimal manufacturing impact are gaining special attention from the pharmaceutical industry, with environmental sustainability and business costs playing the major role to make better, healthy and innovative science [10].

2. Spontaneous emulsification method

“It is not as though nanotechnology will be an option; it is going to be essential for coming up with sustainable technologies.” Paul Anastas, ACS Green Chemistry Institute

2.1 Overview

The preparation of nanoemulsions or nanoparticles can be done by means of several methodologies while, nowadays, a special focus has been given to the use of the so-called low energy methods. Self or spontaneous emulsification method has drawn a great deal of attention in the pharmaceutical field as it generates nanoemulsions at room temperature without the use of any organic solvent or heat [11]. Using this method, the nanoemulsions are created as a result of mixing an organic phase (containing the oil and a hydrophilic surfactant) with an aqueous phase [12]. Without organic solvents or high energy input, the formation of nanoemulsions would be governed by the intrinsic characteristics of the components that will change the free energy of the system favoring dispersion and droplets formation [13]. The two phases, thermodynamically stable alone, are brought to a non-equilibrium state when they are mixed. Thus, the rapid transfer of hydrophilic materials from the oil to the water phase

Introduction

results in a dramatic increase of the interfacial area, leading to the spontaneous formation of fine oil droplets in the oil-water boundary (**Figure 1**) [14]. Moreover, spontaneous emulsification has been related to phase transitions during the emulsification process involving lamellar liquid crystalline phases [15–17]. Thus, the ease of formulation was suggested to be related to the ease of water penetration into the various liquid crystals formed on the surface of the droplet, leading to interface disruption and the consequent droplet formation [18].

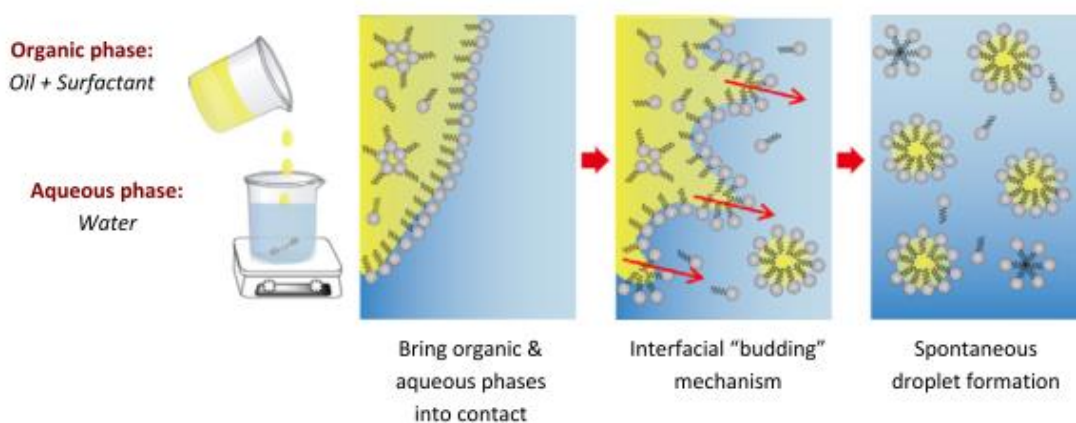


Figure 1. Schematic representation of a proposed mechanism for spontaneous emulsification: fine oil droplets are spontaneously formed when an organic phase containing a surfactant is mixed with an aqueous phase. The surfactant moves from the organic phase to the water phase (red arrows), leading to interfacial turbulence and spontaneous oil droplet formation. Adapted from [12].

The spontaneous emulsification is a technique mainly described for the preparation of nanoemulsions [12][19–21]. However, nanoemulsions can be used as a template for nanoparticle formulation. By establishing a link between nanoemulsion and nanoparticle preparation, the experimental process can be modified by including additional components such as surfactants, monomers, polymers or other macromolecules [22]. For example, Hossein et al have described the preparation of nanocapsules using spontaneous emulsification. In this study, multilayered nanoemulsions were fabricated in two steps and coated with the anionic biopolymer, pectin [23].

2.2 Components choice

The self-emulsification process depends on the nature of the oil/surfactant pair, surfactant concentration and oil/surfactant ratio. Only very specific pharmaceutical excipient combinations lead to efficient self-emulsifying systems [24].

Oil phase

The choice of the oil phase is often a compromise between its ability to solubilize the drug and its capacity to formulate a nanoemulsion with desired characteristics. Oils with excessively long hydrocarbon chains or long-chain triglycerides are difficult to nanoemulsify, whereas oils with moderate or short chain length (medium-chain triglycerides) and fatty acid esters (e.g., ethyl oleate) are easy to nanoemulsify [11]. Medium-chain triglycerides are preferred due to higher fluidity, better solubilization properties and chemical stability, as well as safe regulatory status and low cost [25]. Furthermore, a mixed lipid phase composed of long chain triglycerides and medium chain mono- and diglycerides can have a beneficial impact on the self-emulsifying properties of a system in comparison with a single lipid phase. Mixed lipid formulations can allow the development of small and monodisperse self-emulsifying systems with lower surfactant content and no added co-solvents incorporation [26].

Surfactants

Non-ionic surfactants, with hydrophilic-lipophilic balance (HLB) values between 12-16 are usually applied for the formulation of self-emulsifying systems [27]. The commonly used emulsifiers are various ethoxylated polyglycolized glycerides and polyoxyethylene esters, such as Tween®80, Labrasol® and Cremophor® [11]. Surfactants with a high HLB have a high hydrophilicity, which promotes the formation of o/w droplets and rapid spreading of the formulation in the aqueous media. For the formation of stable self-emulsifying systems, the usual surfactant strength ranges between 30-60% w/w of the formulation [28]. Thus, the main drawback of the self-emulsification process when compared to high energy methods is the use of high surfactant concentrations, which

can be associated to possible toxic effects [29]. Nevertheless, this toxic impact is generally less problematic than in the case of ionic surfactants. As such, the selected surfactant must be approved for the intended route of administration and used at the lowest concentration needed [25].

Co-surfactants and co-solvents

In general, the surfactant alone cannot low the oil–water interfacial tension sufficiently to yield a microemulsion, which can make necessary the addition of an amphiphilic short chain molecule or co-surfactant to bring about the surface tension close to zero. Co-surfactants penetrate into the surfactant monolayer providing additional fluidity to the interfacial film and disrupting the liquid crystalline phases [30]. In general, medium chain length alcohols (8 to 12 Carbon atoms) are adequate, otherwise, derivatives of ethylene-glycol, glycerol and propylene glycol can be also included [25]. These solvents may help to dissolve large amounts of the hydrophilic surfactant or the drug in the lipid phase [15].

2.3 Application in cancer

The majority of anticancer drugs used in clinic are hydrophobic and the effective delivery of them to its target cells has been hampered by its low aqueous solubility [31]. Hydrophobic drugs are not soluble enough to be directly administered by intravenous (i.v.) administration and, orally, their high lipophilicity results in poor oral bioavailability [18]. One of the most popular approaches for solubility enhancement is the development of lipid-based drug delivery systems. Self-emulsifying formulations have been explored as an efficient approach to improve the dissolution rate and bioavailability of poorly water soluble drugs [25]. Because they led to the formation of o/w nanoemulsions upon mild agitation in an aqueous environment, spontaneous emulsifying formulations have been explored for both oral and i.v. administration, being most described for the oral route.

Enhanced oral bioavailability

Self-emulsifying drug delivery systems (SEDDS) spread readily in the gastrointestinal tract, where the highest motility of the stomach and the intestine provide the necessary agitation for self-emulsification [32]. The lipid droplets formed upon dispersion in the gastrointestinal fluids may directly improve the chemical/enzymatic stability, enhance drug dissolution and permeation, increase interfacial area for absorption, reduce drug efflux and promote lymphatic transport [33]. The main limitation of SEDDS is related to the intrinsic lipophilicity of the drug since the active ingredient should be dissolved in a limited amount of oil [34].

Several studies present the preparation of SEDDS to enhance the oral bioavailability of chemotherapeutic drugs, mainly paclitaxel [33–35], docetaxel (docetaxel) [36–18] and curcumin [39–41]. For example, paclitaxel was self-emulsified using Triton WR-1339, sodium deoxycholate and D-alpha-tocopheryl polyethylene glycol 1000 succinate. As a result, the drug in the SEDDS was chemically stable for a year, the loading was increased by approximately five-fold compared to the marketed formulation and the excipients presented a significantly reduced cytotoxicity [35]. In another study, 9-Nitrocamptothecin (9-NC), an orally administered Topoisomerase-I inhibitor, was prepared by self-emulsification for the treatment of pancreatic carcinoma. *In vivo* studies showed an increased oral bioavailability and significant tumor shrinkage when compared to 9-NC suspension in nude mice bearing human ovarian cancer xenografts [44]. Recently, SEDDS were formulated for the oral delivery of indirubin and 3,3-Diindolylmethane-14 with improved results in the solubility and oral bioavailability of both hydrophobic components, as well as an increased antitumor activity [45][46]. Moreover, Devarajan and co-workers have reported the formulation of SEEDS for the oral administration of doxorubicin. In this work, the incorporation of doxorubicin in the oil phase was enhanced by the formation of an *in situ* ion pair between doxorubicin and docusate. The resulted formulation exhibited a high drug loading, adequate stability, low cytotoxicity and improved oral bioavailability [47].

Parenteral administration

Contrarily to the oral administration of SEDDS, where the system self-emulsify in the gastrointestinal tract, the parenteral administration of a self-emulsifying system requires its previously preparation upon administration. As such, spontaneous emulsification can generate nanoemulsions intended for parenteral delivery. These nanoemulsions are thermodynamically stable, transparent upon dilution and isotropic. An advantage of these systems is its high stability. They can be stored and diluted with injection media such as 0.9% saline just before their administration and maintain its physicochemical properties. One of the main drawbacks is related to the stringent requirements of parenteral products. Comparing with the oral route, only few excipients are acceptable for parenteral delivery, which can restrict the components choice and limit the possibilities for formulating these systems [30].

From a formulation point of view, spontaneous emulsions are advantageous as the low-energy process make possible the incorporation of thermolabile drugs, such as nucleic acids, enzymes and proteins [48]. For hydrophobic compounds, its incorporation into the oil phase can provide high encapsulation efficiency, great stability and avoid drug precipitation [49]. Additionally, the preparation process without solvents or heat can greatly decrease the production cost [50].

Spontaneous emulsification offers several advantages for the delivery of drugs, and thus, hold significant promise in the area of oncology. Nornoo et al have developed biocompatible Cremophor®-free microemulsions containing paclitaxel for i.v. administration. The selection of lecithin and Myvacet™ as the surfactant/oil mixture resulted in a stable formulation, with 110 nm droplets and into which 12mg/g of paclitaxel was incorporated [51]. In another study, paclitaxel was incorporated into self-emulsifying nanoemulsions containing PLGA. This system was able to control the release of paclitaxel without changing the inherent properties of the drug [52].

3. Nanotechnologies to improve docetaxel delivery

“Even though significant progress has been made in precision therapy and immunotherapy for the treatment of cancer, traditional chemotherapy continues to form the foundation of treatment for almost all patients” AACR, Cancer Progress Report 2015

Docetaxel has been recognized as one of the most efficient anticancer drugs over the past decades; however, its clinical application has been limited owing to its poor water solubility and systemic toxicity. Since 1995, the only available commercial formulation for docetaxel is Taxotere[®], which is composed of docetaxel and high quantities of surfactant and ethanol. As a consequence of the formulation composition, its efficacy is counterbalanced with serious side effects, including acute hypersensitivity reactions, cumulative fluid retention, neurotoxicity, among others [53]. To overcome secondary effects and improve docetaxel efficacy, much attention has been given to the design of improved formulations and nanotechnology has emerged as a fundamental tool to create alternative delivery systems [54]. If we look at the literature, we can find almost 1000 publications (research on Scopus with the words “docetaxel” and “nanoparticles” or “liposomes”) covering the development of multiple nanoformulations for docetaxel, most of them emphasizing the advantages of these nanoscale constructs in drug delivery. These nanocarriers can improve the solubility and protect the drug from degradation, enhance blood circulation time and be decorated with specific ligands, which favored the accumulation of docetaxel into the tumors through passive and active targeting strategies [55].

Although most of the current research is still done at very early stages, it is exciting to realize that several docetaxel formulations are currently in clinical trials, as summarized in **Table 1**.

Table 1. Nanoformulations for docetaxel under clinical development

Name	Type of nanocarrier	Developer	Status	Ref
BIND-014	PLGA-PEG NPs	Bind Therapeutics	Phase II	[54–57]
CriPec	Polymeric Micelles	Cristal Therapeutics	Phase I	[60]
Docetaxel-PNP	Polymeric NPs	Samyang	Phase I/II	[59–60]
CRLX-301	NP-drug-conjugates	Cerulean	Phase I/IIa	[63]
DEP-Docetaxel	Dendrimers	Starpharma	Phase I	[64]
AT-1123	Liposomes	Azaya Therapeutics	Phase II (soon)	[65]
Docecal	Micelles	Oasmia	Phase I (soon)	[66]

One of the most promising formulations is BIND-014, from Bind Therapeutics. BIND-014 is a polymeric PLGA-PEG nanoparticle decorated with a small molecule (ACUPA) target ligand that binds prostate specific membrane antigen (PSMA). These nanoparticles present a hydrophobic biodegradable core that allows the encapsulation and controlled release of docetaxel, a hydrophilic corona that promotes long circulation time and a targeting ligand that mediates interactions between the nanoparticles and the PSMA receptor, expressed in the extracellular domain of cancer cells. Pre-clinical studies showed that BIND-014 remained in plasma at concentrations at least one order of magnitude higher than equal doses of commercialized docetaxel, leading consequently to a higher tumor accumulation and improved anti-tumor efficacy [67]. Preliminary Phase II studies in 40 patients with advanced metastatic non-small cell lung cancer (NSCLC) treated with 60mg/m² on day 1 of a 21-day cycle demonstrated that BIND-014 was well tolerated with clinically meaningful anti-tumor activity at a lower dose than conventional docetaxel [68]. BIND-014 is currently in Phase II clinical development for squamous histology NSCLC and urothelial carcinoma, cholangiocarcinoma, cervical cancer, and squamous cell carcinoma of the head and neck.

Cristal Therapeutics have developed CriPec[®], a docetaxel loaded core-cross linked micelles (CCL-PMs) composed of mPEG-b-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-b-p(HPMAm-Lacn)) copolymers. The clinical phase I study had started in

2015 after passing successfully non-clinical and safety studies. The covalent conjugation of docetaxel to CCL-PM resulted in small-sized (66 nm) and stable micellar nanoparticles with prolonged circulation time, controlled release and high tumor accumulation. A single dose of CriPec resulted in complete xenograft tumor regression, providing 100% tumor-free survival to these animals [69]. Cerulean has developed CRLX301, a self-assembled docetaxel formulation that significantly enhanced antitumor efficacy and improved pharmacokinetics compared to the conventional drug. Currently in Phase I/IIa, CRLX301 showed in preclinical studies ability to deliver up to 10 times more docetaxel than the marketed formulation and lead to significant survival rate, without remarkable toxicity [70]. DEP-docetaxel comprises the drug attached to a dendrimer scaffold, with a linker designed to release the drug in a controlled manner. In pre-clinical studies DEP-docetaxel showed substantially better efficacy and lower toxicity than Taxotere® [71]. ATI-1123 is a liposomal formulation of docetaxel and its Phase II clinical trials are being planned. The Phase I study revealed acceptable tolerability and favorable pharmacokinetic profile in patients with solid tumors, as well as promising antitumor activity [72]. Finally, Docecal from Oasmia will start a Phase I clinical stage this year [66].

4. Monoclonal antibodies for intracellular delivery

“Just because people assume oncoproteins are too difficult to target doesn’t mean that scientists should give up. Dogma is a moving target.” Channing Der, University of North Carolina

4.1 Overview

Since the early 80’s, when the first anti-human leucocyte antigen (HLA) monoclonal antibody (mAb) was used to target HT29R human colon adenocarcinoma [73], researchers and industry have focused in how to target cancer cells. As a result, the FDA have actually approved 17 antibody therapies for cancer treatment, five of them in the last two years [74] and we can find more than 400 clinical studies ongoing [75]. Antibodies can be used alone, in their full length or antibody fragments, or be conjugated with a cytotoxic drug to overcome undesired side effects and increase drug efficacy [76]. They can combine more than one binding site and interfere with multiple

cancer pathways, the so called bispecific antibodies [77]. In addition, antibodies can be developed to induce immunostimulatory activity by the activation or blocking of mechanisms involved in the anticancer immune response and enhance the therapeutic efficacy of antibodies when combining immunotherapy with targeted therapies [78].

Although with different purposes and mechanisms of action, all the antibody therapies described above are defined as active targeting because of the binding affinity and specificity of an antibody for a membrane receptor [79]. However, cancer receptors are not only expressed in the surface of cells but they are within the cell compartment, the so called intracellular oncoproteins.

4.2 Intracellular cancer-causing proteins (oncoproteins)

Oncogenes are a family genes responsible for the expression of proteins that contribute to the development of cancer. Those oncogenes encode for cell surface receptors that bind communications between the extracellular environment and the intracellular compartment [80]. Vascular endothelial growth factor receptors (VEGFR), epidermal growth factor receptors (EGFR, ErbB-1), and human epidermal growth factor receptors 2 (ErbB-2, i.e. HER2) are some of the main receptors signaling pathways in cancer [81]. Moreover, proto-oncogenes also encode for intracellular proteins. These molecules are found exclusively inside cancer cells and its overexpression is responsible for the development of cancer [80]. RAS (GTPases) [82], non-receptor tyrosine kinases (like Bcr-Abl) [83], BRAF [84] or heat shock proteins (like HSP90 that interacts and stabilize mutant p53) [85] are some examples of these proteins.

Gasdermin B

Gasdermin-like proteins (GSDML) are a family of cancer associated proteins localized in the cytoplasm of tumor cells whose expression is associated to the development and progression of cancer [86]. Gasdermin B (GSDMB), a protein member of the Gasdermin family, has been described in human cancer tissues, including gastric, hepatic, colon, uterine, cervical and cancer-derived cell lines [87]. Recently, Moreno-Bueno's group has

discovered the functional implication of GSDMB in breast cancer. Overexpression of GSDMB promotes cell motility and invasion, while its silencing suppresses a migratory and invasive phenotype. GSDMB could be considered a new marker of invasiveness and metastasis in breast cancer; nevertheless, additional studies are ongoing to fully understand its role as an intracellular cancer protein [88].

4.3 Targeting intracellular oncoproteins

The intracellular localization of proteins is a challenge and new therapies might be found in order to overcome the main cellular barriers [89]. So far, the most studied intracellular agents are small hydrophobic molecules or small interference RNA (siRNA). Additionally, protein kinases inhibitors are an alternative approach to inhibit oncogenic proteins. The main challenges involving siRNA therapies are related to its physicochemical characterization, high hydrophilicity and low negative charge, as well as its poor plasma stability and rapid RNase degradation [90]. Moreover, protein tyrosine kinases are attractive cancer targets as they are closely involved with tumor cell proliferation and survival [91]. Tyrosine kinases are classified in receptor tyrosine kinases, with an extracellular domain, and non-receptor tyrosine kinases, exclusively founded in the cytoplasm or nucleus. Epidermal growth factor receptor (EGFR) and Human epidermal growth factor receptor 2 (HER2) are two well-known receptor tyrosine kinases, which inhibition is mainly done in two ways, with an antibody that binds selectively to the extracellular receptor or a kinase inhibitor that blocks the active site inside the cell [92]. The advantage of the antibody therapy is the high specificity and affinity for the receptor [93]. On the other hand, tyrosine kinase inhibitors present a lack of specificity, as they can interact with the same active site of different kinases, and its inhibition might be or not reversible [94]. As such, new strategies need to be set in order to overcome drug resistance and to find alternatives to protein kinases inhibitors.

An alternative strategy to target intracellular proteins is the use of intrabodies. An intrabody is an antibody that has been designed to be expressed intracellularly and to affect protein functions [91–92]. Single-chain variable fragments (scFv) produced by phage display are the most usual and studied intrabodies [97]. The small size of scFv and

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its intracellular location make it suitable for gene therapy. Contrarily to siRNA that mediates down regulation of gene expression at the post-transcriptional level, intrabodies knockout the protein function at the post-translational level thus overcoming the off-target effects of siRNA and its reversible effect, as well as benefiting from a specific inactivation of the protein [98]. Other advantages comprise the high stability and active half-life of intrabodies and its possibility to interact with more than one active site of the protein, promoting a higher selectivity and efficiency [99]. The major downsides involving the clinical development of intrabodies are the efficient and specific delivery of the intrabody or the genetic material encoding the intrabody to *in vivo* tumor cells and the instability and unfolding conformation of intrabodies in the redox-state of the cytosol [100]. So far, these difficulties have limited the clinical application of intrabody therapies.

4.4 Intracellular delivery of monoclonal antibodies

Main barriers and delivery strategies

The intracellular transport of mAbs is one of the key problems for its therapeutic action inside the cell. Being high molecular weight and hydrophilic, mAbs do not diffuse across the cell barrier and the only way they have for reaching the intracellular compartment is through a receptor-mediated transport [101]. Without surface receptors and with the inability to penetrate the cell membrane on their own, the *in vitro* access of antibodies to intracellular targets was primarily achieved by methods such as electroporation or microinjection, or by permeabilization of the cell membrane with detergents, consequently leading to cell damage and, obviously, lacking from a viable clinical application [102]. Suitable delivery strategies must go through the development of nanocarriers designed to overcome the cell barrier and transport the antibody to the intracellular compartment [103]. Recently, Gdowski et al have published the encapsulation of anti-AnxA2 antibody within PLGA nanoparticles using the water/oil/water double emulsion evaporation technique. The antibody was released from the nanoparticles in a sustained manner and maintained its functionality, thus indicating that PLGA-based nanoparticles must be a promising intracellular delivery

vehicle [104]. Moreover, BioCellChallenge, a biotechnology company developing biological molecule delivery systems, have created ImmunoCellin[®], an antibody intracellular delivery technology based on a liposomal formulation [105]. This system was intended for the encapsulation of antibodies through non-covalent interactions, thus retaining the structure and function of the mAb. The company have proved its efficiency *in vitro* and now is working on a specific formulation adapted for *in vivo* approaches [106].

Scientific evidence

To our knowledge, Sorrento Therapeutics was the first company presenting data about the development of antibodies intended to target intracellular proteins. In collaboration with City of Hope, Sorrento has developed a new technology based on a chemical modification of mAbs that allows their penetration into the cell while maintaining their ability to binding specific target proteins. This data was recently released to the press; however, there are no available publications about the technology [107]. Recently, BioCell Challenge released data about the intracellular delivery of a specific monoclonal antibody directed against the Ras oncoprotein. They have developed lipid-based formulations able to incorporate antibodies and promote its internalization across the cell membrane. *In vivo* data were very promising, showing a recovery in 33% of cases and a prolonged survival by up to 30% [108].

Looking at the literature and published studies, there are only few authors working in this field. Nevertheless, none of them clearly justify the internalization of a mAb specifically intended to target an intracellular protein that do not express a surface receptor. Dao et al came up with the development of a new mAb, known as ESK1, able to target the intracellular Williams Tumor 1 (WT1) oncoprotein overexpressed in a wide range of leukemias. ESK1 was engineered to mimic the functions of a T cell receptor specific for the WT1 RMF peptide/HLA-A0201 complex. ESK1 mediated antibody-dependent cytotoxicity in WT1 and HLA-A0201 in a restricted manner *in vitro* and showed potent antitumor efficacy against established disseminated human leukemia xenografts [105–106]. Guo and co-workers have targeted intracellular phosphatase of

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regenerating liver protein tyrosine phosphatases with a mAb and inhibited experimental metastasis [111]. Here, the target activity was presumed to occur by different mechanisms and hypothesized that the intracellular tumor antigen was shed into blood circulation to form complexes with circulating mAbs and activate a localized tumoral immune response [112]. Another study revealed that NY-ESO-1 mAb in combination with anticancer drugs induced a strong antitumor effect by the development of NY-ESO-1 specific CD8⁺ T cells. The group hypothesized that the mAb formed immune complexes with the released tumor antigens. These data showed that intracellular NY-ESO-1 can be targeted with mAbs and chemotherapy, thus improving antitumor capacity and inducing T-cell response [113].

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Chapter 1

Targeting cancer with hyaluronic acid-based nanocarriers: recent advances and translational perspectives

Targeting cancer with hyaluronic acid-based nanocarriers: recent advances and translational perspectives

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Abstract

Hyaluronic acid (HA) is a natural polysaccharide that has been widely explored for the development of anticancer therapies due to its ability to target cancer cells. Moreover, advances made in the last decade have revealed the versatility of this biomaterial in the design of multifunctional structures able to carry a variety of bioactive molecules, including polynucleotides, immunomodulatory drugs and imaging agents. In this review, we aim to provide an overview of the state of the art of hyaluronic acid-based nanocarriers for the design of oncological nanotherapies, highlighting their application to targeted delivery of cytostatic drugs, polynucleotides, combination therapies, immunomodulation and theranostics. Finally, we will discuss the main technological advances that will allow these carriers to be considered as candidates for clinical development.

Key words: hyaluronic acid, cancer nanotechnology, immunotherapy, translational research

1. Introduction

Progress made in cancer research has led to the development of a wide array of anti-cancer agents, ranging from simple molecules, such as the well-known cytostatic drugs, to complex ones, such as peptides, proteins and polynucleotides. Unfortunately, the pharmacological effect of those molecules has been highly compromised by several factors, including their poor solubility, inadequate biodistribution and, ultimately, their limited efficacy together with a significant toxicity. To address these limitations, diverse drug delivery systems have been designed for a safe and controlled delivery of the therapeutic drugs [1]. Among them, polymeric nanocarriers have been extensively studied, especially those made of biocompatible and biodegradable polymers. In particular, HA-based nanocarriers have gained a significant attention, especially in the last five years, judging by the increase number of publications in this field (**Figure 1**). The versatility of HA has allowed the design of multifunctional nanocarriers specifically tailored for the incorporation of a wide array of molecules. In this context, although cytostatic drugs continue to be of great interest, other molecules, such as immunomodulators and polynucleotides, hold considerable promise. In this review, we will first outline the physicochemical and functional characteristics of HA that make it a suitable biomaterial for the design of the anticancer targeted nanocarriers. Then, we will critically analyze the potential capacity of the most recent nanocarriers developed for the delivery of cytostatic drugs and polynucleotides, single or in combination, as well as immunostimulants and imaging agents. Finally, we will evaluate the candidates undergoing clinical assessments and the potential therapeutic impact that HA-based nanocarriers may have in successful anticancer therapies.

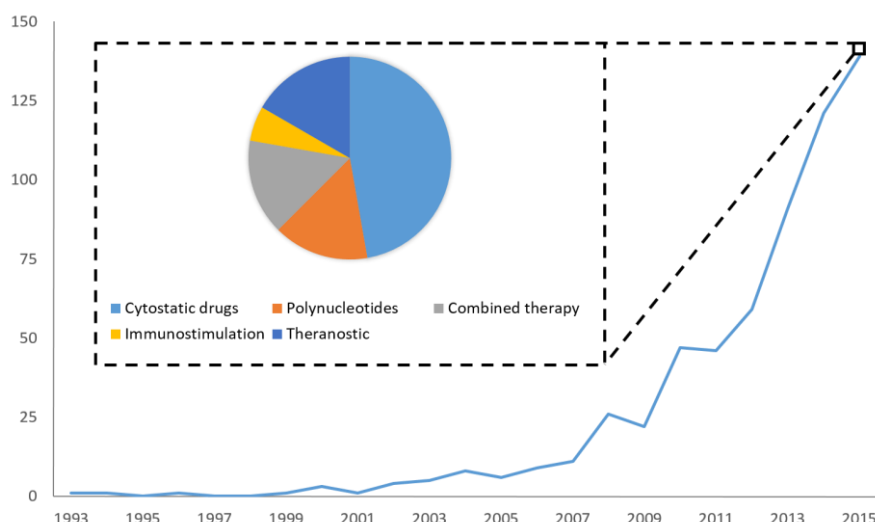


Figure 1. Evolution in the number of studies on HA-based nanocarriers for anticancer drug delivery since the 90's. In 2015, almost 150 articles were published on the application of HA for the delivery of cytostatic drugs or polynucleotides, for combined therapy, as immunostimulating vehicles and for theranostic. Data from Scopus (1993-2015) with the words “hyaluronic acid” and “delivery” and “cancer”.

2. Functional and Physicochemical Properties of HA

HA (also referred to as hyaluronan) is a naturally occurring polysaccharide composed of repeated units of N-acetyl- d- glucosamine and β -glucuronic acid [2]. Endogenous HA $> 10^6$ Da is the main component of the extracellular matrix in mammals and it is responsible, among other functions, for cell division, adhesion and matrix renovation [3]. These cellular events are mainly regulated by two major HA cell-surface receptors: CD44 and RHAMM (or CD168) [4]. The interaction of HA with CD44, LYVE-1, RHAMM and other HA-binding proteins is essential for a number of physiological processes, however its abnormal production or binding activity can cause irregular cell proliferation, migration and differentiation [5–6]. From the drug delivery perspective, the CD44 receptor has received the most attention, due to its abnormal overexpression in a large number of solid tumors [7].

From a physicochemical point of view, HA exhibits a number of key advantages. First, its hydrophilicity makes it an attractive material for the formation a protein-repellent shield around drug nanocarriers [8]. On the other hand, its anionic character ($pka = 3-4$) [9] enables its interaction with cationic polymers, lipids and surfactants, which results in the formation of a variety of nanostructures. Finally, HA has reactive functional groups,

which offers the possibility of obtaining a variety of HA-based derivatives with modulated properties and targeting capacities [10].

In general, HA is known to be a non-toxic, biocompatible and biodegradable biomaterial [11–12]. Some recent studies have claimed that low molecular weight (LMW) HA is able to stimulate the immune system [13–14] and promote the polarization of tumor-associated macrophages (TAM) through a pro-inflammatory prototype, M1 anti-tumoral [15]. However, this specific behavior in TAMs, which may need to be further validated, should not lead us to consider HA as an immunostimulatory material.

The good biocompatibility and immunotolerance of HA is further illustrated by the fact that it has been used in several marketed products since 2003, first as a dermal filler and, later as a biomaterial for surgery, and ocular and intra-articular applications [16].

Finally, a positive feature of HA from the translational point of view is the fact that it is abundant in nature, and can be extracted either from animal tissues or produced by microbial fermentation. Nowadays, the latter method is the main source of HA for pharmaceutical purposes because it yields reproducible batches of highly purified polymer [17], with a broad range of molecular weight grades ranging from 4 Da to 5,000 KDa [18].

3. Design of HA-based Nanocarriers for Cancer Therapy

HA has been engineered to deliver anticancer drugs using different strategies. HA can be conjugated directly to therapeutic molecules, assembled with different materials or used to decorate the surface of pre-formed carriers. The association of a drug to HA, either by direct conjugation or through a carrier, offers interesting opportunities in the development of new oncological therapies. So far, this technological approach has resulted in: (i) drug solubility and stability enhancement in biological fluids, (ii) improvement of the pharmacokinetic profile due to an increase in the blood circulation time (passive targeting) and, (iii) improvement of the biodistribution pattern, based on the HA ability to target tumor cells (active targeting).

The concept of **passive targeting** is associated with the ability of the nanocarrier to circulate in the blood stream for extended periods of time, which increases the

nanocarrier's chance to diffuse passively through the leaky tumor vasculature, and accumulate in the tumor due to the so-called "enhanced permeability and retention" (EPR) effect. Such passive mechanism of access to the tumor has been classically achieved by providing the nanocarriers with a hydrophilic polyethyleneglycol (PEG) coating [19]. By analogy, some authors have argued that HA could also provide this stealth role [20]. However, this needs to be further explored because this behavior may be highly dependent on the HA molecular weight (MW). For example, LMW HA (up to 150 KDa) was found to be comparable to PEG, in terms of by-passing the complement activation system [13], whereas HA of a higher MW (1,200 KDa) is known to be rapidly eliminated through the liver and kidneys. This mechanism of elimination has been associated with the high affinity of HA to the HA endocytosis receptor, HARE-1, located mainly in the liver and spleen [21].

While the passive targeting mechanism needs to be further elucidated, the **active targeting** can be explained by the binding affinity of HA to specific receptors overexpressed in cancer cells [22]. Indeed, it is well known that HA can recognize and bind to CD44, a cell surface glycoprotein overexpressed in a wide variety of solid tumors and associated with tumor progression and metastasis. Additionally, it has been reported that HA can interact with other receptors expressed in cancer cells, such as RHAMM, HARE-1 and LYVE-1. However, the contribution of this interaction to the potential targeting capacity of HA-based nanocarriers is less known [23]. The main strategy to target CD44 has been described by using HA as a drug carrier. As such, HA can bind CD44 receptors, be internalized and effectively transport anticancer drugs into the intracellular compartment [24]. It is important to emphasize that the binding affinity of HA-based nanocarriers for the CD44 receptor is largely influenced by the molecular weight (MW) of the polymer. For example, an *in vitro* CD44-mediated cell uptake study with HA-coated liposomes concluded that the binding affinity was higher for high MW (HMW) HA (175-350 KDa) than for LMW HA (up to 150 KDa) [25]. These results are in agreement with a previous study which showed that HMW HA-coating lipid nanoparticles improved ligand accessibility to CD44 [13]. The authors justified these results by the fact that larger molecules may have a greater probability to interact with CD44 receptors than smaller molecules [26]. Nevertheless, *in vivo*, the tumor binding affinity may counter-balance the faster clearance of HMW HA when compared to the

clearance of LMW HA-based nanocarriers [13]. For example, 175-350 KDa HA-coated liposomes displayed accelerated clearance from blood, whereas 5-8 KDa HA-coated liposomes remained in circulation longer. This faster clearance may be explained by the high affinity of HMW HA to HARE-1 receptors expressed in the liver, which results in a faster elimination when compared to the elimination of LMW HA-coated nanoparticles [27].

Overall, these data suggest that the optimal response may be achieved when there is a balance between the clearance and the targeted biodistribution of HA-based nanocarriers (**Figure 2**).

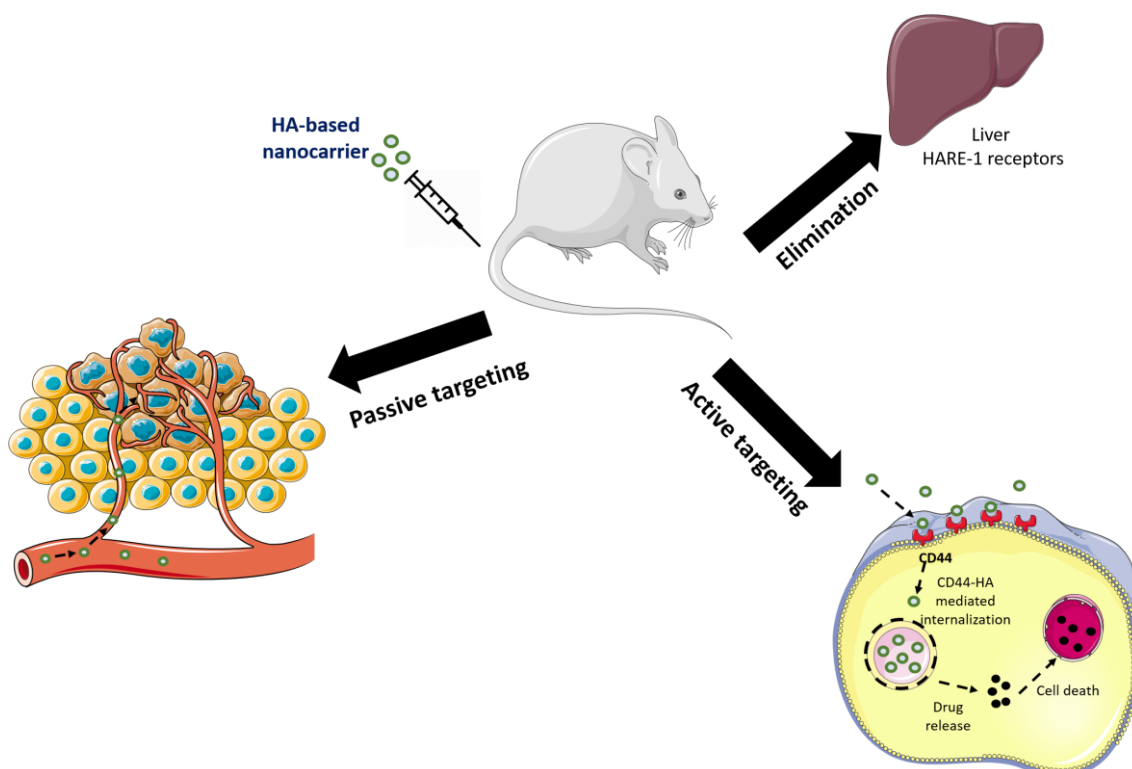


Figure 2. Design of HA-based nanocarriers to achieve an optimal antitumor efficacy. The optimal response must be achieved by a balance between the clearance and the target affinity of HA-based nanocarriers.

4. HA-based Nanocarriers for the Delivery of Anti-Cancer Drugs

HA-based nanocarriers are being developed as suitable carriers for the delivery of diverse therapeutic molecules, such as cytostatic drugs, polynucleotides, immunostimulating molecules and imaging agents. HA can be conjugated directly to therapeutic drugs, self-assembled into micelles, form polymeric nanoparticles or

decorate lipid and magnetic carriers. **Figure 3** represents the leading HA-based nanocarriers for anticancer drug delivery.

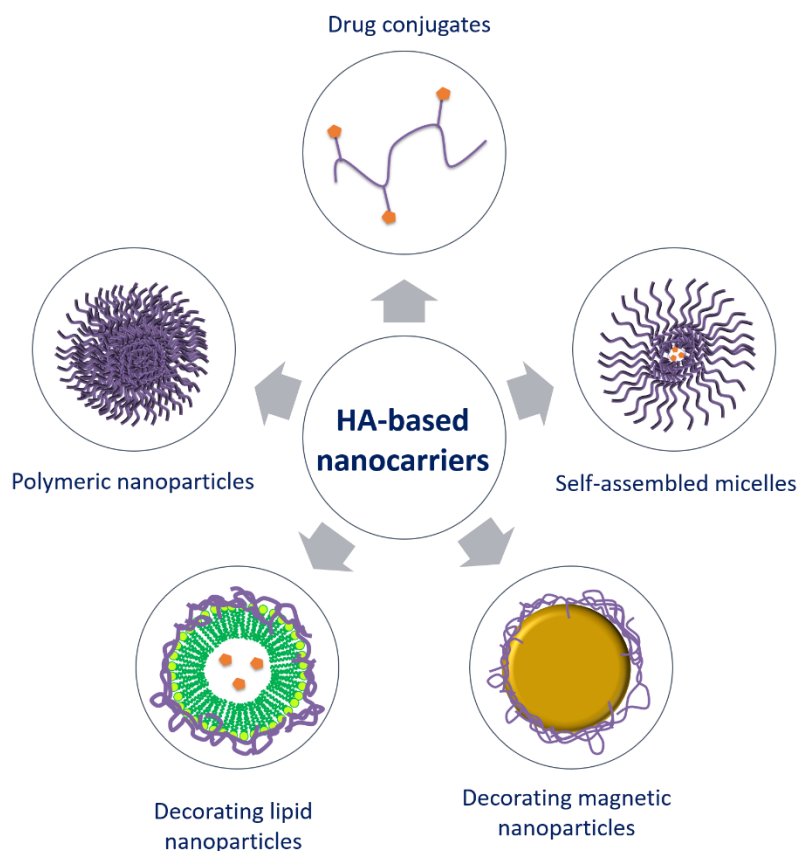


Figure 3. Leading HA-based nanocarriers for anticancer drug delivery. HA can be conjugated chemically with therapeutic drugs to form HA-based drug conjugates or with a hydrophobic molecule to self-assemble in micelles. HA can interact ionically with other polymers to form polymeric nanoparticles, and can be used to decorate the surface of lipid and magnetic nanoparticles.

4.1 Delivery of Cytostatic Drugs

Cytostatic molecules are known to be very efficient anticancer drugs, but, their poor water solubility and systemic toxicity have limited their clinical application. The conjugation, entrapment or encapsulation of cytostatic drugs within HA-based nanocarriers has led to their enhanced solubility/dispersability in aqueous media as well as to a reduction of their side effects thanks to their targeting behavior [28]. **Table 1** summarizes the most recent HA-based nanocarriers designed for anticancer drug delivery.

Table 1. In vivo results of the most recent HA-based nanocarriers designed for the delivery of cytostatic drugs and siRNA.

Carriers	Composition	Drugs	Results	Ref
Delivery of cytostatic drugs				
HA-drug conjugates	HA	Quercetin	2.5-fold enhanced plasma half-life. Inhibited 62% of the tumor growth comparing to 25% with the free drug.	[40]
		SN 38	Medium survival time increased from 66 days with the free drug to 71 days with ONCOFID-S.	[33]
		Paclitaxel	Medium survival time increased from 42 days with the free drug to 49 days with HA-paclitaxel.	[34]
		Paclitaxel	Significant accumulation in tumor. 4-fold reduced tumor volume comparing with the free drug.	[35]
Stimuli-responsive HA-drug conjugates		Paclitaxel	Improved biodistribution. 2.8-fold enhanced tumor uptake. 83% of tumor growth inhibition when compared to 51% with the free drug.	[39]
		Cisplatin	2.5-time higher accumulation in tumor than the free drug. HA-cisplatin allowed the administration of 3x 20mg/mL (compared with 3x 5mg/mL treatment for cisplatin alone) resulting in 95% of tumor inhibition without apparent toxicity.	[38]
Self-assembled micelles	HA - PLGA	Docetaxel	2-times enhanced plasma half-life. Inhibited 92% of the tumor growth comparing to 77% of the free drug.	[43]
	HA - cholic acid	Paclitaxel	Tumor growth inhibition was 3-fold higher than the free drug.	[45]
	HA - cholesteryl	Docetaxel	The degree of substitution (DS) of HA-cholesteryl influenced the in-vivo results. With a DS of 25%, HA-cholesteryl micelles improved 12.6-fold the plasma circulation time, 2-fold the tumor accumulation and significantly inhibited tumor growth, when compared with the free drug.	[47]
	HA - α -TOS	Docetaxel	3.7-fold enhanced tumor accumulation and a consequent 67% tumor inhibition when comparing to 57% with the free drug.	[52]

Stimuli-responsive self-assembled micelles	HA-ss-PLGA	Doxorubicin	Enhanced tumor accumulation.	[53]
	HA-ss-PCL	Doxorubicin	Enhanced tumor accumulation.	[54]
	HA-Lys-LA	Doxorubicin	20-fold higher tumor accumulation and improved half-life which resulted in remarkable tumor inhibition (after treatment, the relative tumor volume was 0.63 and 10.18 mm ³ for the mice treated with HA-Lys-LA loaded doxorubicin and free drug, respectively)	[55]
	HA-PDSMA-N3	Doxorubicin	2-fold enhanced accumulation in tumor and extended circulation time than HA-micelles. Bioreducible HA-micelles resulted in a tumor 60% and 40% lower than HA-micelles and free drug, respectively.	[56]
Nanogels	HA – Methacrylate	Doxorubicin	4-fold enhanced biodistribution and 3-fold higher plasma half-life for HA-nanogel when compared with the free drug, resulting in a 67% and 57% tumor inhibition, respectively.	[62]
HA-decorating nanocarriers	GAGs	Doxorubicin	23.5% of doxorubicin accumulated in the tumor when compared with 0.45% of the free drug. The encapsulated doxorubicin significantly attenuated the growth of the tumors relative to the free drug.	[67]
	Lipid NPs	Paclitaxel	HA-paclitaxel-NLCs exhibited the highest tumor inhibition rate (85%), followed by PTX-NLCs (73%) and PTX solution (25%).	[65]
	Silica NPs	5-fluorouracilo	Significant tumor growth inhibition.	[70]

Delivery of polynucleotides

Nanoparticles	HA – Protamine	miRNA 34-a	Suppressed tumor growth and induced tumor cell apoptosis when compared with the controls.	[80]
HA-decorating nanocarriers	Liposomes	anti-GGCT siRNA	PEG-HA-liposomes exhibited the most outstanding tumor inhibition effect, with a tumor volume 36.8%, 44.9%, 47.2%, and 60.4% smaller than free siRNA, naked liposomes, and HA-liposomes group, respectively.	[86]
		anti-Pgp siRNA	High tumor accumulation with a 34% P-glycoprotein downregulation.	[87]
		cpu-siRNA2	Highest silencing efficiency on the mRNA expression with 63.7% down-regulation.	[89]
	Calcium phosphate NPs	luc-siRNA	Significant accumulation into tumor.	[88]

	Lipid NPs	siPLK1	Knockdown of 80% for siPLK1 delivered via HA-lipid nanoparticles. The median survival was increased 60% when compared with the control.	[90]
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Abbreviations: HA, hyaluronic acid; PLGA, poly(lactic-co-glycolic acid); α -TOS, alpha tocopheryl succinate; PCL, polycaprolactone; Lys-LA, L-lysine methyl ester- lipoic acid; PDSMA-N3, azide-functionalized pyridyl disulfide methacrylate; GAGs, glycosaminoglycans; NPs, nanoparticles

4.1.1 HA-drug Conjugates and Complexes

As indicated, HA has reactive functional groups useful for its conjugation with, among others, small cytostatic drugs [29]. **HA-drug conjugates** need to be adequately designed in order to preserve the activity of the drug while maintaining the inherent properties of HA and its significant capacity to bind to CD44. For example, a high degree of substitution can result in HA-CD44 low binding affinity [30]. Moreover, the polymer can lose its aqueous solubility and, thus, change the system biodistribution [31]. The most recent studies report the conjugation of HA with different cytostatic drugs, such as docetaxel [32], camptothecin [31,33], doxorubicin [30] and paclitaxel [34–35] which have led to some promising results. For example, the locoregional administration of ONCOFID-S (HA conjugated with SN-38, an analog of camptothecin) dramatically reduced the tumor and metastatic spread of peritoneal carcinomatosis, when compared with the administration of the unloaded drug [36]. Nevertheless, the bioconjugate turned out to be ineffective after intraperitoneal and intravenous administration, a result that was attributed to the fast clearance of HA from circulation [37]. In another example, small LMW HA (5 KDa) grafted to paclitaxel was used to target brain metastasis by intravenous administration. The HA-paclitaxel conjugate was evaluated for *in vivo* efficacy in a preclinical model of brain metastasis of breast cancer. The results showed that the animals treated with the conjugate had an overall survival time longer than the controls (49 days for HA-paclitaxel compared to 42 and 37 days for paclitaxel or non-treated mice, respectively) and a significant reduction of the lesion burden in the brain [34].

In addition to covalently linked HA-drug conjugates, there are examples of complexes formed by ionic interaction between the negatively charged groups of HA with positively charged drug molecules. For example, the ionic complex formed between HA and

cisplatin was found to exhibit a pH-dependent release behavior [38]. Moreover, further studies show that the redox potential of a HA-drug conjugate formed by crosslinking HA with paclitaxel through disulfide bonds resulted in the rapid release of the drug in the presence of glutathione and a significant tumor suppression *in vivo* [39].

Considering the hydrophilic properties of HA and the hydrophobic character of most cytotoxic drugs, HA-drug conjugates are expected to self-assemble into micelles in aqueous solution. As expected, HA-conjugated with quercetin [40] and doxorubicin [41] formed self-assembled micelles when dissolved in water. HA-quercetin self-assembled micelles resulted in a 20-fold half-life increase and a 5-fold increase in the area under the curve, when compared to the free drug [40]. Although HA-drug conjugates can self-assemble into micelles, the majority of research articles on HA-based micelles describe the conjugation of HA with a hydrophobic molecule, as reported below.

4.1.2 HA-based Micelles

The chemical modification of HA with a hydrophobic molecule gives it an amphiphilic structure able to **self-assemble into micelles** in an aqueous environment. These structures, composed of a hydrophobic inner core, have shown the capacity to encapsulate lipophilic drugs and facilitate their delivery to the tumor site [42]. For example, HA has been grafted to poly(D,L-lactide-co-glycolide) acid (PLGA) [43–44], 5 β -cholic acid [45], copoly(styrene maleic acid) [46] and cholesteryl [47–48] for the delivery of several cytostatic drugs. Overall these micelle systems, a different approach is the one involving the assembling of HA-ceramide with docetaxel-loaded PLGA nanoparticles, which resulted in improved tumor targetability when compared with plain nanoparticles [49]. Furthermore, HA can be conjugated with α -tocopheryl succinate (α -TOS) and D-alpha-tocopheryl polyethylene glycol succinate (TPGS) to build multifunctional systems, for example, by taking advantage of the properties of these components in the inhibition of the P-gp pump and the overcoming of multi-drug resistance [50–51]. As a result, a multifunctional nanoparticle composed of HA- α -TOS (HT) and TPGS, and loaded with docetaxel in its hydrophobic core demonstrated, *in vivo*, a higher accumulation in tumor tissue and a pronounced anti-resistance tumor efficacy

in resistant breast cancer xenograft tumor compared with the commercial formulation, Taxotere® [52].

Finally, self-assembled conjugates can also be tailored to promote the release of the drug under redox conditions. For example, HA modified with disulfide bounds (ss) was cross-linked with PLGA [53], polycaprolactone (PCL) [54] and lipoic acid [55] for the delivery of doxorubicin. *In vitro*, the drug release was delayed under physiological conditions (pH 7.4), but was liberated from the conjugates by the addition of glutathione. In another study, doxorubicin was encapsulated in core-crosslinked HA functionalized azide-pyridyl disulfide methacrylate (PDSMA-N3) micelles, aimed to promote an intracellular release of the drug triggered by the high levels of glutathione. This micelle system was very stable in circulation, resulting in a 30-fold increase in the concentration of the drug in plasma over the drug inoculated on its own, and a subsequent increase on its accumulation in the tumor, which resulted in a 60% tumor growth inhibition [56].

4.1.3 HA-based Nanoparticles

Polymeric nanoparticles, consisting of a matrix of HA and counter ion polymers, have been proposed mainly for the delivery of polynucleotides (as described in section 4.2) [57], however, there are a few examples of their use for the delivery of cytotoxic drugs. For example, HA-chitosan nanoparticles were evaluated *in vitro* for their capacity to deliver curcumin to C6 glioma cells [58] and doxorubicin to hepatocyte HepG2 cells [59]. Curcumin loaded HA-chitosan nanoparticles had a strong dose dependent cytotoxicity and a high uptake efficiency in C6 cells [58]. The same kind of nanoparticles were also evaluated for the targeted delivery of 5-fluorouracil, following oral administration. In this experiment, the targeting affinity of HA to colon cancer cells was combined with the mucoadhesive properties of chitosan [60].

A different nanoparticle composition was the one made of HA-methacrylate copolymerized with di(ethylene glycol) diacrylate [61]. These nanoparticles, also called **nanogels**, were loaded with doxorubicin and the resulting composition led to an enhanced drug efficacy in a H22 hepatocarcinoma xenograft mice model [62].

4.1.4 HA-decorated Nanocarriers

HA can be used to decorate the surface of nanocarriers either by electrostatic interactions or covalent grafting. Recent studies have described the ionically-driven association of HA to the surface of cationic lipid nanoparticles and liposomes for the delivery of cytostatic drugs [63–64]. The most remarkable *in vivo* data were obtained with paclitaxel-loaded HA-coated cationic lipid nanoparticles, which resulted in an 85% tumor growth inhibition when compared with the control (25% tumor inhibition for free paclitaxel) [65].

On the other hand, HA can be chemically linked to phospholipids, and the resulting conjugate incorporated into the liposomes during its preparation [66], or after their formation by simple incubation [67]. Liposomes have also been decorated with HA conjugated with PEG in order to enhance their blood circulation time [25]. Although the PEGylation of HA nanocarriers can effectively reduce liver uptake and increase the circulation time, it can also affect the binding affinity of HA to the receptors on the cancer cells. In this context, 5% PEG coating was found out to be the optimal density to achieve a better cellular uptake *in vitro* and anticancer effect *in vivo* [68].

Glycosaminoglycan particle nanoclusters, known as GAGs, are hyaluronan coated phospholipid-based particles. The authors of this work suggested that the coating of these carriers with HA contributed to their steric stabilization and a substantial amount of doxorubicin was still detected in the plasma of mice 72h post-administration. Twenty-four hours after i.v. injection, about 25% of the dose injected via GAGs was accumulated inside the tumor, a substantial increase over the less than 0.5% accumulation detected when free doxorubicin was administered. As a consequence, the encapsulated drug significantly attenuated the growth of the tumors when compared to the growth reported with the free drug and without clinical toxicity. The authors justify these results by three main reasons: the hydrophilicity of the HA shell which allowed long blood circulation times, the affinity of HA for CD44 receptors overexpressed on the tumor cells, and the capacity of doxorubicin-GAG to bypass the P-gp-mediated drug resistance in NAR cells (P-gp-overexpression human ovarian adenocarcinoma resistant to doxorubicin) [67].

HA has also been described as a coating agent for inorganic nanoparticles and was recently conjugated onto the surface of silica nanoparticles for the delivery of curcumin and 5-fluorouracil [69–70]. For the coating procedure, the HA was chemically conjugated onto the surface of pre-formed silica nanoparticles. *In vivo* results in colon xenograft model showed that the coating of silica nanoparticles with HA enhanced the target ability of the system, resulting in a significant tumor reduction when compared with the naked particles and the free drug [70].

4.1.5 Functionalization with Tumor Targeting Molecules

Some authors have suggested that the inherent targeting capacity of HA-based nanocarriers could be further enhanced by functionalizing the polymer with tumor targeting moieties such as peptides, aptamers and antibodies [71]. As such, HA has been conjugated with folic acid [72–73] and, recently, with MUC-1 binding DNA aptamer [74]. In both cases, the cellular uptake of the functionalized nanocarriers was similar to that of the original nanocarrier. In a recent study, the tumor homing penetrating peptide tLyp-1 was conjugated with PEG-TOS and assembled with HA-grafted TOS, resulting in a multifunctional nanoparticle for the delivery of docetaxel. *In vivo*, this multifunctional nanoparticle resulted in a 74% of tumor growth suppression when compared to the 50% reduction obtained with plain HA nanoparticles. This higher efficacy was attributed to a combination of the HA target affinity for CD44 receptors with the tumor tissue penetration conferred by the peptide [75].

4.2 Delivery of Polynucleotides

The growing interest in small and micro interfering RNAs (siRNA and miRNA) in cancer therapy has encouraged studies on the drug delivery field to search for methods that would allow these nucleic acid-based molecules to overcome critical biological barriers and reach their target. Among the obstacles siRNA and miRNA must overcome are: the mononuclear phagocytic system (MPS), their limited access to the tumor cells, and their degradation both throughout this pathway and once inside the cells. To overcome these barriers, different delivery strategies have been designed to improve siRNA delivery *in vivo* [76]. Among them, the use of cationic lipids and polymers has shown a great

potential to promote intracellular delivery of siRNA/miRNA. By condensing anionic nucleic acids into the cationic chain, these positively charged polyplexes protect genetic material from enzymatic degradation and enhance cellular penetrance. On the other hand, the high positive charge density contributes to the cytotoxicity, particle aggregation and recognition by the mononuclear phagocyte system [77]. In an attempt to address these hurdles, HA has been successfully used to modify the surface of cationic complexes, either by entrapment of the material into a polymeric/lipidic matrix or by decorating the surface of polynucleotide loaded nanocarriers (**Table 1**). As described in the next sections, the results obtained with some of the nanocarriers have led to encouraging data [78].

4.2.1 HA-based Nanoparticles

HA-based nanoparticles have been prepared for the encapsulation of siRNA. In a recent study, HA was covalently grafted to polyethylenimine (PEI) (positive charge) and to PEG (negative charge) and the multi-drug resistance 1 (MDR1) siRNA was loaded within the nanoparticles. The PEG was added to the nanoparticles in order to mask their positive charge and to provide a hydrophilic PEG corona. *In vitro* studies demonstrated the potential of HA-PEI/HA-PEG/MDR1 siRNA to knockdown the gene expression of MDR1 in SKOV-3TR ovarian cancer cells. Mice treated with siRNA loaded nanoparticles and paclitaxel had a 3-fold smaller tumor volume than mice treated with paclitaxel alone. These results suggest an increase in the chemosensitivity to paclitaxel in mice treated with HA-PEI/HA-PEG/MDR1 siRNA and the system ability to deliver siRNA *in vivo* [79]. A simpler composition was succeeded by combining HA and protamine, a cationic polypeptide, with miRNA 34-a. The resulting nanostructures were evaluated in a breast cancer model in mice and resulted in a remarkable decrease in the tumor size. Moreover, the expression of miR-34a increased 200-fold for the mice treated with encapsulated miRNA [80].

4.2.2 HA-based Nanocomplexes

The conjugation of HA with lipophilic molecules is not limited to the delivery of hydrophobic drugs. Recently, **self-assembled** HA-cholesterol [81] and HA-5 β -cholanic

acid [82] nanocomplexes were described as suitable reservoirs for the delivery of siRNA. To this end, two strategies were assessed: (i) the modification of siRNA with hydrophobic 2b-protein, which neutralized the siRNA charges and favored its encapsulation within the hydrophobic core [81], and (ii) the conjugation of HA-5 β -cholanic acid micelles with a RNA receptor, the DPA/Zn, which promoted the incorporation of siRNA into the self-assembled carrier [82]. Upon consideration of the positive effect of the PEGylation on the stability of siRNA molecules in physiologic conditions [83], siRNA was also grafted with HA, and the resulting HA-siRNA conjugates were complexed with either cationic PEI [83] or lipid nanoparticles [84]. HA-siRNA conjugates were mixed with cationic lipid nanoparticles *via* electrostatic interactions, and the resulting system was evaluated for *in vitro* cytotoxicity and gene silencing efficacy in HeLa-cells. When compared to commercialized transfection reagents, HA-siRNA/cationic nanoparticles were remarkably safe as a delivery vehicle for siRNA, and had a 10-fold higher therapeutic index (LC50/IC50), confirming they were a possible choice for future *in vivo* studies [84].

4.2.3 HA-coated Nanocarriers

Shielding cationic nanocarriers with HA has been described as a successful strategy to mask the positive charge of polymeric nanoparticles, lipidic complexes or liposomes [85]. HA has been electrostatically attached to the surface of positive liposomes [86–87] and calcium phosphate nanoparticles [88], as well as chemically bound to lipids present on the nanocarrier's surface [89–91]. In a specific study, the second approach was found to be more effective than the first one. For example, the direct conjugation of HA on the surface of a cationic lipid-siRNA complex resulted in a greater *in vivo* stability and tumor targeting ability compared to the complex in which HA was physically adsorbed [89]. In a different study, it was reported that HA grafting to cationic lipoplexes resulted in a multilayer system, with the siRNA entrapped within the multilamellar structures, surrounded by the polymer. The binding affinity to CD44 receptors of non-coated versus coated HA-lipoplexes was determined using surface plasmon resonance, which revealed a preferential affinity of HA-lipoplexes compared to uncoated ones [91]. Finally, the use of HA-grafted lipid-based nanoparticles loaded with polo like kinase 1 (PLK1) siRNA led

to very promising data upon local delivery to an orthotropic glioblastoma mouse model. The results showed a drastic reduction in the PLK1 mRNA levels and an increased survival rate of mice treated with this nanocomposition [90].

4.3 Co-delivery of Multiple Drugs

The delivery of multiple therapeutic agents in a drug carrier has been motivated by two main reasons: (i) the combination of chemotherapeutic drugs can generate synergistic anticancer effects without overlapping toxicity, and (ii) the delivery of multiple drugs with different targets or mechanisms of action can suppress the cancer chemoresistance, which is responsible for the most frequent causes of failure in cancer therapy [92]. HA-based nanocarriers have been studied as multidrug containing platforms for the co-delivery of cytostatic drugs or cytostatic drugs together with siRNA therapy. **Figure 4** shows examples of multifunctional HA-based nanocarriers for the delivery of cytostatic drugs and cytostatic drugs together with siRNA therapies.

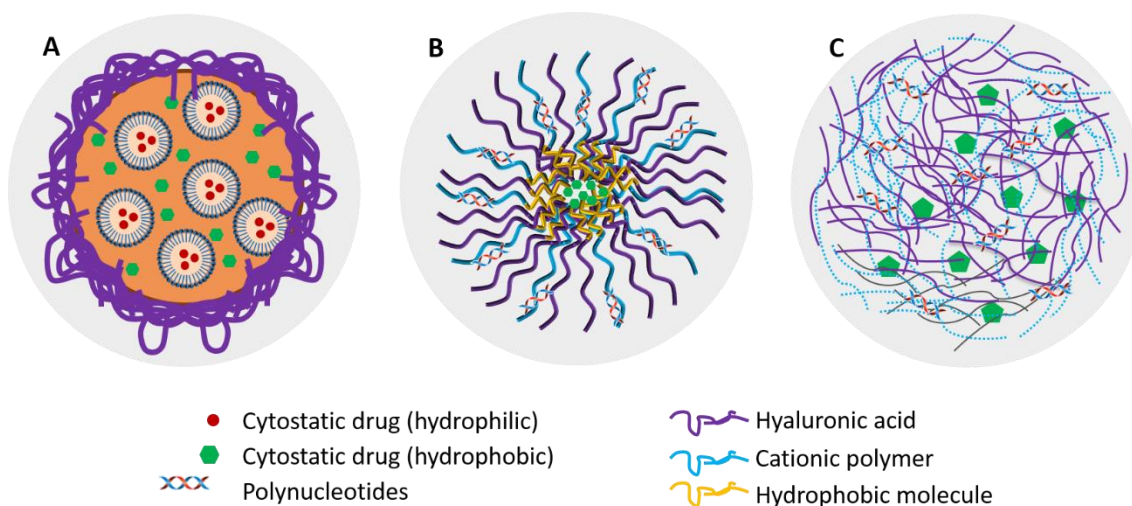


Figure 4. Multifunctional HA-based nanocarriers for the co-encapsulation of different drugs. A) HA coating w/o/w nanoparticles for the co-delivery of hydrophilic and hydrophobic drugs. B) Self-assembled HA-micelles for the delivery of a hydrophobic drug (inner core) and siRNA (ionically attached to a cationic polymer). C) Polymeric nanoparticles prepared from electrostatic interactions between negatively charged HA and siRNA and a positively charged polymer and drug.

4.3.1 Co-delivery of Cytostatic Drugs

The use of HA-based nanocarriers has been proposed for the development of combination therapies because they are able to avoid drug incompatibility, achieve appropriate pharmacokinetics profiles and overcome multidrug resistance [92–93]. This combinatorial effect can be achieved by mixing different HA-drug conjugates [94–95], or by associating different drugs to HA-based nanocarriers [93, 96–98]. Among the various combination strategies explored so far, it is worthwhile to emphasize HA-ss-PLGA nanoparticles loaded with doxorubicine and cyclophosphamide. The dual-drug loaded particles were prepared by double emulsion, allowing the incorporation of doxorubicin (hydrophilic) and cyclophosphamide (hydrophobic) within the same carrier. *In vivo*, the combined therapy demonstrated a remarkable synergistic anti-tumor effect, which was confirmed by the absence of tumor after the treatment, in an orthotopic mammary fat pad tumor model [99].

4.3.2 Co-delivery of Chemotherapeutics and Polynucleotides

The co-delivery of cytostatic drugs alongside with siRNA/miRNA has been described using two strategies: the co-encapsulation within the same nanocarrier or the co-administration of the cytostatic drug and the siRNA in different carriers. When the aim was the co-encapsulation within the same nanocarrier, the co-delivery of both therapeutic drugs was achieved by designing (i) self-assembled micelles with HA-octadecanoic acid and PEI, which resulted in a system in which the paclitaxel was entrapped into the oil core and the siRNA ionically attached to the PEI branch [100], and (ii) the preparation of nanoparticles by ionotropic gelation between HA and chitosan for the entrapment of doxorubicin and miR-34a [101]. The delivery of both drugs was intended to achieve a synergistic effect against triple negative breast cancer and overcome drug resistance, which was successfully achieved. As an example, doxorubicin/miR-34a loaded HA-chitosan nanoparticles resulted in a 2-fold and 4-fold reduction in the size of the tumor, compared with the size of the tumor in mice injected with the drug or the miRNA carried alone, respectively [101].

In another study, HA-PEI/HA-PEG nanoparticles were developed for the co-encapsulation of two siRNA against pyruvate kinase M2 (PKM-2) and multidrug

resistance gene-1 (MDR-1) to sensitize multidrug resistant ovarian cancer to paclitaxel. Along with paclitaxel, the co-delivery of siRNA within HA-nanoparticles resulted in the downregulation of gene expression in paclitaxel resistant SKOV-3 tumors, which resulted in a 20% more inhibition of the tumor growth compared to the single administration of each carried siRNA [102].

4.4 HA-based Nanocarriers for the Delivery of Anti-Cancer Antigens and Immunostimulatory Molecules

One of the strategies currently explored in cancer immunotherapy involves the stimulation of the immune system using specific antigens and immunostimulatory molecules, such as cytokines or interferons [103]. Within this field, the use of HA in a variety of formats has led to interesting data (**Figure 5**). For example, HA was chemically conjugated with ovalbumin (OVA, used as a model antigen) [104], and to cytosine-phosphate-guanidine (CpG), an immunostimulant epitope [105]. While the administration of the free OVA to immunized mice did not have a significant therapeutic effect, the intravenous administration of HA-OVA to the same murine model enhanced the production of cytotoxic T cells against the tumor, leading to the inhibition of the tumor growth [104]. In another study, HA-CpG was complexed with PLL by electrostatic interactions to form PLL/HA-CpG nanocomplexes. *In vitro*, the immunostimulating activity of PLL/HA-CpG resulted in an increase of cytokine IL-6 levels in blood, 77-times higher than after the administration of free CpG. *In vivo*, the i.v. administration of PLL/HA-CpG nanocomplexes in EG7-OVA-tumor-bearing mice resulted in a drastic inhibition of tumor growth and in the generation of a tumor specific memory response, as shown by the significant inhibition of a secondary tumor growth in mice vaccinated with PLL/HA-CpG complexes [105]. A different study explored the inhibition of TGF- β , an immune-suppressive cytokine, using TGF- β siRNA loaded HA-nanoparticles, which were administered in combination with CpG and Trp2 tumor antigen peptides loaded manose-modified nanoparticles into a skin melanoma xenograft murine model. The use of HA-nanoparticles loaded with siRNA resulted in about a 50% TGF- β reduction in the late stage tumor microenvironment. This nanotherapy helped to boost the vaccine

efficacy and to inhibit the tumor growth by 52% when compared with the results obtained using the vaccine treatment alone [106].

Several recent studies have focused on the evaluation of the immunomodulatory properties of HA “per se”. For example, LMW (MW 50-200kDa) HA has been shown to stimulate the activation of a pro-inflammatory M1-like macrophage phenotype, with anti-tumoral properties [107], while HMW HA (MW > 800 kDa) has been reported to promote the polarization of macrophages towards an anti-inflammatory, M2-like phenotype with pro-tumoral properties [108]. Although more research is required to propose a detailed mechanism of action, it should be noted that these studies suggest that LMW HA has an inherent capacity to favor the conversion of anti-inflammatory, pro-tumor M2-like tumor associated macrophages (TAM) into pro-inflammatory, anti-tumor M1-like macrophages [107–108]. In a recent study, HA-coated (MW 40 kDa) mannan-conjugated manganese dioxide nanoparticles (HA-Man-MnO₂ NPs) were used as a multifunctional platform to enhance the chemotherapeutic response of doxorubicin in a 4T1 murine breast cancer model. Although we believe that the mechanism of action need to be elucidated with more detail, the experiments showed that HA-Man-MnO₂ NPs were efficiently taken by macrophages, which suggests that HA is responsible for reprogramming anti-inflammatory M2-like into pro-inflammatory antitumor M1-like macrophages via a TLR2-MyD88-IRAK1-TRAF6-PKC ζ -NK- κ B-dependent pathway. The promotion of M1 macrophages results in a higher cytokine secretion and H₂O₂ generation. H₂O₂ reacts with MnO₂ NPs and forms O₂ and Mn²⁺, which results in decreased tumor hypoxia. These *in vitro* data agree with the *in vivo* response observed after administration of the HA-Man-MnO₂ NPs in combination with doxorubicin into 4T1 tumor-bearing mice. The reduction of tumor hypoxia by HA-Man-MnO₂ NPs could contribute to the enhancement of the chemotherapy response, resulting in the improved efficacy of doxorubicin and consequent tumor inhibition [109]. **Figure 5** shows a schematic representation of the possibilities of using HA for immunomodulatory purposes. One possibility is the stimulation of the adaptive immune system (dendritic cells and consequent activation of a T cell response). Another is the modulation of the macrophage polarization favoring the M1-like anti-tumoral phenotype that has the ability to kill tumor cells, inhibit angiogenesis and promote adaptive immune responses.

These are some of the most relevant studies performed in the last years regarding the use of HA with immunotherapeutic purposes. We strongly believe that meaningful research on the mechanisms of action and the possibilities of a clinical application of these studies will be published in the near future.

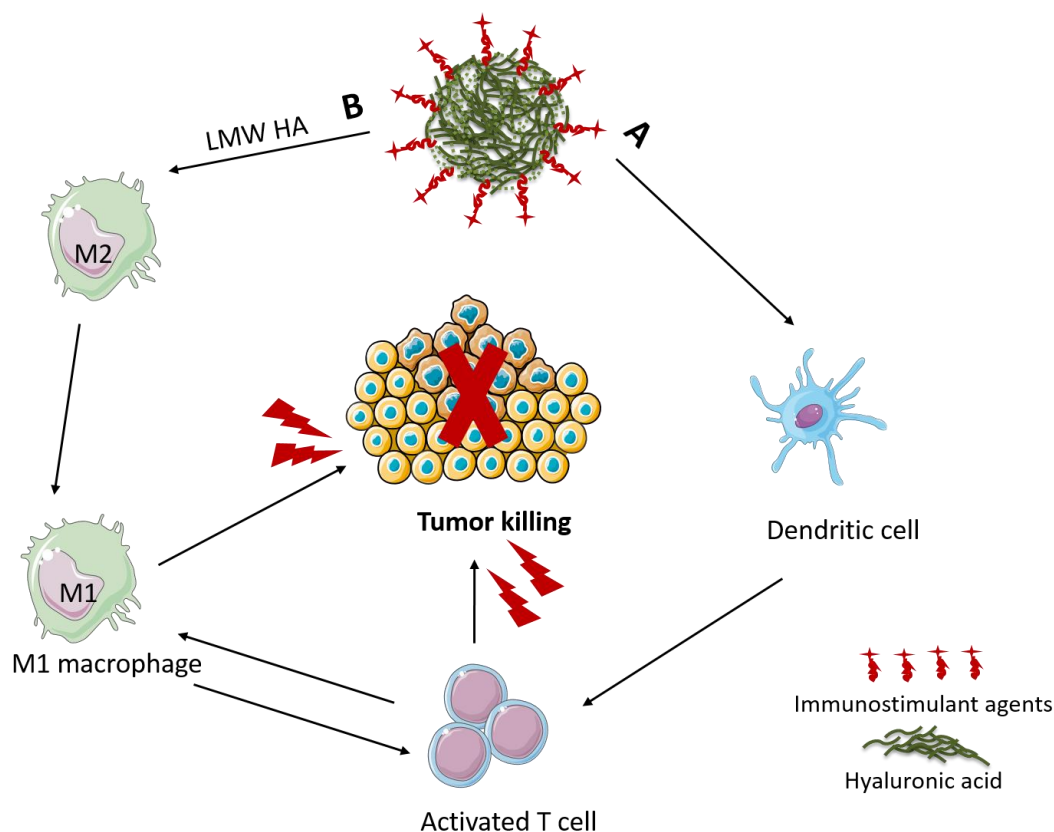


Figure 5. Immunotherapeutic possibilities for the use of HA-based nanocarriers in cancer. In addition to the immunomodulatory properties of HA “per se”, HA-based nanocarriers can be designed by association of antigens or immunostimulatory molecules to A) promote an adaptive immune response through the induction of dendritic cells to activate T cells or either B) by the polarization of anti-inflammatory M2-like macrophages into pro-inflammatory, M1-like macrophages with anti-tumoral properties.

4.5 HA-based Nanocarriers for Anti-Cancer Theranostics

Nanotheranostics refers to a treatment strategy that includes the combination of diagnostic and therapy entities within the same nanocarrier [110]. Because of its targeting ability, HA has received increasing attention in this field [111]. The most recent studies describe the use of HA to decorate theranostic nanoparticles using different strategies including: (i) the electrostatic attraction between HA and superparamagnetic IONS [112], (ii) the chemical conjugation of HA onto the surface of tantalum oxide

nanoparticles [113], and (iii) the self-assembling between amphiphilic HA-oleic acid and superparamagnetic IONS [114], HA-hydrocaffeic acid with gold nanoparticles [115] and HA-cholesteryl anchored reduced graphene nanosheets [116]. In one study, the imaging capacity and the antitumor efficacy of doxorubicin loaded HA-coated tantalum oxide nanoparticles were evaluated in a breast cancer xenograft tumor model. The coating of tantalum oxide nanoparticles with HA resulted in a higher accumulation in tumor than when non-coated nanoparticles were used, showing a bright computed tomography (CT) signal 24 h after administration. Moreover, the nanoparticles resulted in an 88% of tumor growth inhibition, compared with the tumor growth when using the free drug [113]. Photothermal therapy (PTT) takes advantage of electromagnetic radiation to treat cancer, without causing thermal injury to normal tissues. As such, fluorescent Cy5.5-conjugated HA nanoparticles were loaded with copper sulfide to combine optical imaging and PTT. *In vivo* the biodistribution of these nanoparticles in a subcutaneous SCC7 tumor model showed a highly accumulation in the tumors. Moreover, mice treated with copper sulfide loaded Cy5.5-conjugated HA nanoparticles and laser irradiation presented a remarkable 10-fold tumor growth inhibition, over the control [117].

5. Conclusion and Future Perspectives

The interest on HA-based nanocarriers has increased exponentially in the field of oncology. A few years ago the majority of research articles on HA-based nanocarriers referred to their use for the delivery of cytostatic drugs. Nowadays there is an extended interest on their application to emerging therapies, including immunotherapies, polynucleotide-based therapies, combined therapies and theranostics. This new interest is the result of new discoveries of HA biological properties, including its well-known CD44 targeting ability and, above all, to a deeper understanding of its chemical versatility. Both as a simple polymeric chain or as a nanostructure, HA has been shown to protect drugs from degradation and to target them to cancer cells. In the specific field of cancer immunotherapy, HA has been recognized for its ability to co-deliver antigens and immunostimulating agents as well as for its effect on reverting to TAM pro-tumor profile. Motivated by the success in therapy, HA has also been explored as a diagnostic

vehicle and it is evident its use as a theranostic tool by combining stealth and targeted properties with image guided diagnosis and treatment.

In the last few years, hundreds of publications and patents have been written on the development of HA-based nanocarriers for cancer therapy. This interest is also evident in the industry area, where many companies are moving towards the development of HA into possible clinical products. Currently, two ongoing clinical trials use this technology: (i) ONCOFID™-P, a phase II clinical trial that includes a HA-paclitaxel conjugate for the treatment of refractory bladder cancer (EudraCT number 2009-012274-13) [118–119], and (ii) FOLF(HA)iri, a phase III clinical study that uses HA Chemotransport Technology (HyATC®), a “gel-like” structure for the delivery of irinotecan against metastatic colorectal cancer [120–121].

Therefore, it could be concluded that, overall, HA offers a wide array of possibilities as a drug carrier in cancer therapy. Based on the clinical and advanced preclinical data, it could be expected that HA-based targeted delivery of anti-cancer drugs will lead to successful therapies in the coming years. In addition, it could be expected that significant knowledge will be generated in the specific areas of nucleic acid-based and immunotherapies and, this knowledge might lead to more advanced therapies to fight cancer. In brief, the use of HA is in the front line and is undoubtedly a polymer to continue exploring in nano-oncology.

Executive Summary

Hyaluronic Acid (HA)

- Natural polysaccharide characterized by its biocompatibility, non-toxicity and biodegradability.
- Chemically versatile, HA has reactive functional groups that are useful for chemical modifications and functionalization. It has two carboxyl groups ionized at physiologic pH, is highly hydrophilic and is predisposed to be associated with counter ions.
- Produced mainly by microbial fermentation that yields a highly purified polymer in a broad range of molecular weight grades.

HA Nanocarriers

- HA can be used to formulate a multitude of nanocarriers, such as drug conjugates, polymeric or self-assembled particles, micelles, nanocapsules, liposomes, polyplexes, and inorganic systems.
- HA nanocarriers can incorporate a wide variety of molecules, such as cytostatic drugs, proteins, polynucleotides, immunomodulators, and imaging agents.

Cancer Selectivity

- Passive targeting: HA may help to prolong the blood circulation time of nanocarriers and hence their capacity to reach the tumor due to the EPR effect.
- Active targeting: the binding capacity of HA to specific cancer cell surface receptors, such as CD44, helps to actively target drugs to cancer cells

HA Nanocarriers and Their Application in Oncology

- HA nanocarriers can be used for the efficient delivery and co-delivery of therapeutic molecules and/or diagnostic agents to achieve combined effects, reduce side effects, overcome cancer cell resistance, and modulate the immune system.
- Combined therapy: HA nanocarriers can co-incorporate different therapeutic molecules, generating a synergistic effect while suppressing multi-drug resistance.
- Immunomodulation: HA nanocarriers can be engineered with immunotherapeutic payloads to elicit an immune response against tumor cell antigens.
- Imaging and theranostic: to simultaneously deliver an imaging agent for diagnostic and/or an anticancer drug for therapy. HA nanocarriers are becoming important theranostic tools.

Challenges

- The binding affinity of HA to specific cells receptors is both an advantage and a disadvantage. HA has the ability to target receptors that are overexpressed in cancer cells, but, at the same time, it has the capacity to interact with receptors expressed in healthy cells. Thus, the main challenge is to find ways to enhance the binding affinity of HA to cancer cells receptors (i.e. CD44) but not its affinity to receptors in healthy cells, (i.e. HARE-1, which is involved in the elimination of HA from the blood circulation).
- Even though HA is commonly considered non-toxic and biocompatible, recent studies have revealed the immunogenicity of LMW HA and its role in macrophage polarization. Although in a preliminary stage, these results must be carefully

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evaluated to allow for a better understanding of the potential of HA in cancer immunotherapy.

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Background, hypothesis and objectives

Background

1. Progress in nanomedicine made possible the development of engineered nanoparticles aimed to treat cancer more effectively. These nanocarriers can be tailored regarding to size, charge and surface properties in order to improve cancer target capacity and therapeutic efficacy [1]. Moreover, nanoparticles can be designed to incorporate different types of anticancer drugs, either hydrophobic or hydrophilic, such as small molecules, peptides, proteins or polynucleotides [2]. These multifunctional platforms can change the solubility and release profile of therapeutic agents, prolong their circulation half-life, improve their biodistribution, cellular uptake and decrease the systemic toxicity of the free drug [3].

2. Spontaneous emulsification is a low-energy method used for the preparation of nanoemulsions without the need of organic solvents and heat [4]. This technique has important advantages, such as: (i) ease of preparation, (ii) allows the incorporation of different therapeutic molecules, such as small cytostatic drugs or sensible biomolecules and, (iii) reduces the environmental impact of nanoformulations [5].

3. Polymeric nanocapsules have been widely studied for anticancer drug delivery. These systems are composed of an oil core, able to highly encapsulate hydrophobic drugs, and a polymeric shell suitable for the association of different biomolecules [6]. Coating nanocapsules with hyaluronic acid (HA) has been described as a promising strategy to enhance the accumulation of anticancer drugs into the tumor by passive and active targeting [7]. HA can protect the carrier, promote long circulation times and increase the stability in plasma. In addition, HA can recognize and bind to CD44 overexpressed receptors in various tumor types, which results in enhanced drug accumulation and reduced cytotoxic side effects [8]. The modification of HA with a hydrophobic molecule provides the polymer with an amphiphilic character. Hydrophobically-modified HA can self-assemble into nanoparticles, consisting of a hydrophobic core surrounded by a hydrophilic shell [9].

4. During the last years, there has been a focus on the discovery of many intracellular cancer proteins, which are characterized by its nuclear or cytosolic localization and usually associated to cancer progression [10]. Without expressing a cell surface receptor, those proteins are usually targeted with small cytostatic molecules, protein kinase inhibitors, polynucleotides or small antibody fragments. Unfortunately, these approaches are ineffective and intracellular oncoproteins still lack from valid treatment options [11–13].

5. Monoclonal antibodies are used as one of the best therapies against cancer [14]. Up to now, the development of antibodies has been focused on the target of cancer cell surface proteins rather than intracellular targets, because antibodies are too large and hydrophilic to cross the cell membrane on their own. However, its target ability and specificity are pushing researchers to look at monoclonal antibodies as promising agents against intracellular oncoproteins [15].

Hypothesis

1. The development of a spontaneous emulsification method can result in a valuable strategy to formulate nanocapsules without the use of organic solvents.
2. The use of an amphiphilic hyaluronic acid (HA) can lead to the preparation of nanocapsules without the need of a cationic surfactant as the polymer counterion. The absence of the cationic surfactant should result in safer formulations.
3. The structure of HA nanocapsules can be used as a multifunctional platform for the intracellular delivery of different drugs: the oil core can encapsulate hydrophobic drugs whereas the polymeric shell can entrap and protect high molecular weight macromolecules, such as monoclonal antibodies.
4. Monoclonal antibodies associated to HA nanocapsules can overcome the cell membrane and reach the intracellular compartment. Once inside the cell, the monoclonal antibody must block the specific oncoprotein and inhibit cancer cell progression.

Objectives

Considering the previous background information and exposed hypothesis, the main objective of this thesis has been the development of a spontaneous emulsification method for the preparation of HA nanocapsules intended for the encapsulation of docetaxel, a hydrophobic cytostatic drug, and the association of a monoclonal antibody to achieve intracellular delivery. This goal will be covered through the following steps:

Preparation of HA-based nanocapsules using a spontaneous emulsification method

1. Components choice, formulation design and optimization of the spontaneous emulsification technique, firstly adapted for a nanoemulsion.
2. Preparation of HA-based nanocapsules using the settled up self-emulsification method and optimization of the formulation for the HA and hydrophobically-modified HA.

These results are presented in Chapter 2.

Evaluation of the capacity of HA-based nanocapsules to encapsulate the hydrophobic drug docetaxel

3. Formulation and characterization of HA-based nanocapsules loaded with docetaxel.
4. Study the release of docetaxel by using an original drug transfer method.
5. *In vitro* cytotoxicity of docetaxel-loaded HA-based nanocapsules in A549 lung cancer cell line.

These results are presented in Chapter 2.

Study the ability of HA-based nanocapsules to associate the monoclonal antibody, anti-gasdermin B, and to promote its intracellular delivery

6. Formulation design, optimization and physicochemical characterization of both HA-based prototypes containing anti-gasdermin B.
7. *In vitro* cytotoxicity assays in HCC1954 cells under different conditions for both HA-based prototypes

- 8.** Evaluation of the capacity of HA-based nanocapsules to effectively deliver anti-gasdermin B to the cytoplasm and to escape lysosomal digestion.
- 9.** Study the capacity of anti-gasdermin B-loaded HA-based nanocapsules to decrease the migration and invasive behavior of HCC1954 cancer cells.

Corresponding results are presented in Chapter 3.

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Chapter 2

Preparation of hydrophobically modified hyaluronic acid nanocapsules using a spontaneous emulsification technique for cancer therapy

Preparation of hydrophobically modified hyaluronic acid nanocapsules using a spontaneous emulsification technique for cancer therapy

This work was done in collaboration with: Ana Olivera¹, Magnus Besev², Pradeep Dhal², Lúdia Gonçalves³, Carmen Abuin Redondo⁴, Guillaume Bastiat⁵ and María José Alonso¹.

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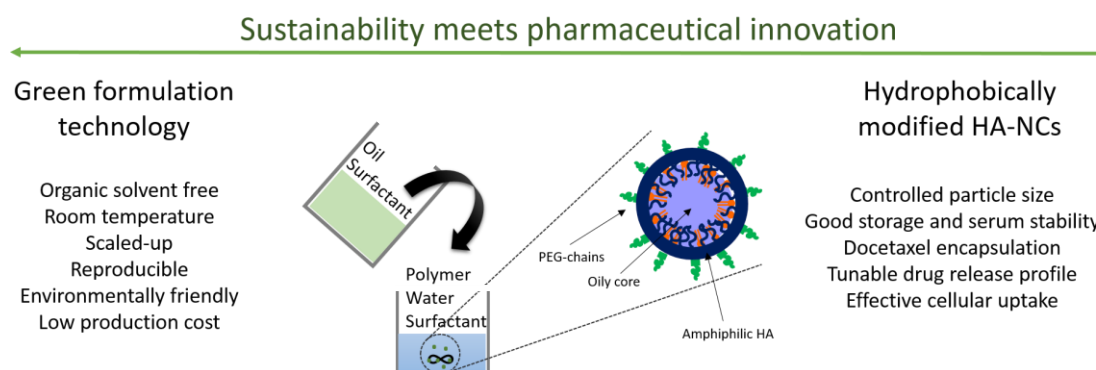
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Graphical abstract



Abstract

In this study, self-assembled nanocapsules were prepared under mild conditions and without the need of cationic surfactants or organic solvents by using a new spontaneous emulsification method and a hydrophobically-modified hyaluronic acid. The nanocapsules prepared with the amphiphilic hyaluronic acid derivative exhibited improved cytotoxic profile compared to the nanocapsules formulated with hyaluronic acid (HA) and cationic surfactants. Both HA-based nanocapsules demonstrated improved stability in human plasma, have higher capacity for the encapsulation of docetaxel and ability to release the drug in a controlled manner. Furthermore, docetaxel loaded into the nanocapsules showed improved uptake and cytotoxic activity towards A549 lung cancer cells. These results suggest that self-emulsifying HA-based nanocapsules have the potential for anticancer drug delivery while reducing the impact of organic solvent waste.

Keywords: nanocapsules, self-emulsifying, hyaluronic acid, anticancer, drug delivery

1. Introduction

During the last twenty years, cancer nanotechnology was established as a fundamental tool to improve conventional anti-cancer therapy. Diverse nanovectors, such as nanoparticles, micelles or liposomes have been engineered and loaded with cytostatic drugs to successfully target tumors [1]. Likewise, nanocapsules have gained special attention due to their versatile structure and physical properties for anticancer drug delivery [2]. Nanocapsules are vesicular systems composed of a liquid oil core stabilized by a surfactant layer and a surrounding polymeric shell. This core-shell structure has been proven to be advantageous for the delivery of diverse therapeutic molecules [3]. For example, the oil core has the capability to efficiently encapsulate hydrophobic molecules, while the polymeric shell endows the carrier with desirable characteristics, such as drug protection, extended blood circulation time and target ability [3–4]. One of the key challenges for creating effective nanocarriers has been to engineer them with the optimal physicochemical characteristics to guide them to the tumor [5]. As such, the development and optimization of nanocapsules can be achieved by tailoring the carrier with adequate properties, such as size, shape and surface characteristics [2]. Besides, it is desirable to prepare the nanocarrier through industry-friendly techniques and without organic solvents [6]. In general, the majority of publications report the preparation of nanocapsules using organic solvents [7–9]. However, in recent years, increased attention has been paid towards “green technology” and the development of chemical and material processes with less organic solvents [10]. Accordingly, the same principles can be applied in nanomedicine for the development of formulation techniques without organic solvents. This reduction must lead to a positive impact in the environment, as well as on the final production costs [11].

Self or spontaneous emulsification is a low energy method mostly described for the preparation of nanoemulsions [12–15]. Using this process, the formation of nanosized droplets is mainly dependent on the modulation of the interfacial phenomenon and the intrinsic physicochemical properties of oils and surfactants [16]. As such, nanoemulsions can be prepared without the need of organic solvents, heat or mechanical stirring, providing advantages from the manufacturing and scale-up standpoint. Furthermore,

the absence of heat makes it attractive to incorporate thermosensitive molecules, such as proteins, peptides or antibodies [17]. Recently, Hossein *et al* has shown that nanocapsules can be prepared by self-emulsification in a two-step process by coating self-emulsifying droplets with an anionic biopolymer [18].

The design of nanocapsules with a polymeric shell made of hyaluronic acid (HA) is an attractive approach to achieve active targeting. HA is an anionic, naturally occurring glycosaminoglycan polymer [19]. In addition to its biocompatibility, non-toxicity and biodegradability, HA can effectively recognize CD44 receptors that are overexpressed in many tumor types and direct the delivery of drugs to the tumor site [20]. Previously, we have prepared HA-based nanocapsules by electrostatic interactions between negatively charged HA and a cationic surfactant [21]. The modification of HA by adding a hydrophobic chain to the structure could be an interesting alternative to prepare self-assembled nanocapsules, which by passes the need for cationic surfactants and, consequently, must reduce the inherent toxicity associated to these surfactants [22]. Regarding the hydrophobicity of the functional group and the degree of substitution, HA derivatives can be tailored accordingly to desired requirements, without changing its target capacity [23]. Earlier research have demonstrated the potential of amphiphilic HA nanocarriers for the delivery of anticancer drugs [24–29]. Nevertheless, the main published work report the preparation of self-assembled HA nanoparticles using the sonication method followed by dialysis to incorporate the drug. As such, a milder and facile procedure to prepare such nanosystems is highly desired.

In the present study, we aimed to prepare HA nanocapsules by a one-step solvent-free emulsification process by utilizing amphiphilic HA precursors. By using docetaxel, we evaluate the capacity of HA nanocapsules to encapsulate hydrophobic anticancer drugs and further improve its therapeutic efficacy. This formulation process may constitute a green nanotechnology for drug delivery applications.

2. Materials and methods

2.1 Materials

Sodium hyaluronate (Mw = 200 KDa) was provided by Sanofi Genzyme, USA. Caprylic/capric triglyceride (Miglyol®812) was a kind gift from Cremer, Germany. Polyoxyethylene sorbitan monooleate (Tween®80), Hexadecyltrimethylammonium bromide (CTAB), Nile Red and DAPI were obtained from Sigma-Aldrich, Spain. Macroglol 15 Hydroxystearate (Solutol®HS15) was acquired from BASF, Germany. Centripure P10 columns were purchased from EmpBiotech, Germany, and Dulbecco's Modified Eagles Medium (DMEM) from Thermo Fisher Scientific, Spain. All other chemicals used were of reagent grade.

2.2 Synthesis of dodecylamide-functionalized sodium hyaluronate

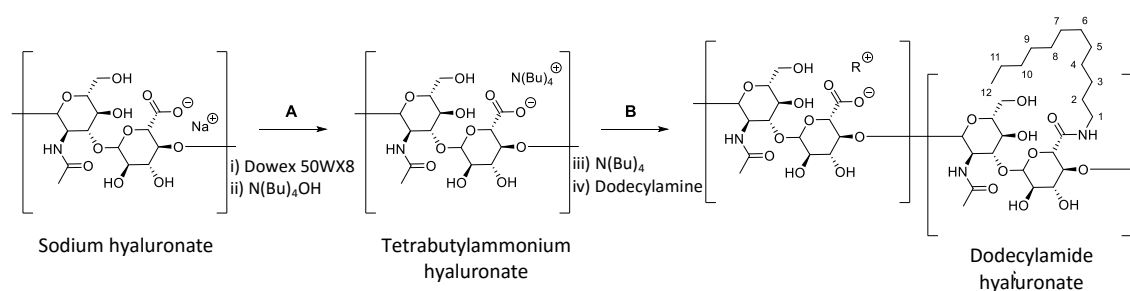


Figure 1. Synthetic scheme of dodecylamide functionalized sodium hyaluronate. **A)** Sodium hyaluronate was treated with **(i)** Dowex 50WX8-400 and **(ii)** tetrabutylammonium hydroxide. **B)** To the resulted tetrabutylammonium hyaluronate was added **(iii)** 2-bromo-1-ethyl pyridinium tetrafluoroborate followed by **(iv)** a solution of dodecylamine to form dodecylamide-functionalized hyaluronan.

Cation exchange

200 mg of **sodium hyaluronate (HA)** was dissolved in water (concentration below 10mg/mL) and treated with 5 mL Dowex 50WX8-400 (1.7 miliequivalents/mL, H⁺ form; freshly washed with water/methanol/water). The pH of the solution was <4. The resulting polymer solution was treated with tetrabutylammonium hydroxide (40wt solution in water) until the pH was 12.0 (**Figure 1 A**). The whole procedure was repeated twice and the final pH was subsequently adjusted to 7.5–8.0 by bubbling CO₂ followed by bubbling with N₂. The solution was concentrated by tangential flow using a 30 KDa

cut-off Pellicon XL Biomax filter cassette (EMD Millipore). The concentrate was lyophilized.

Synthesis of amphiphilic HA, 5% modification

To the above prepared tetrabutylammonium hyaluronate (400 mg, 0.64 milliequivalents) was added DMF (45 mL) and monomethyl formamide (4 mL). To this solution was added 2-bromo-1-ethyl pyridinium tetrafluoroborate (8.8 mg, 0.032 mmol, 0.05 equiv.) dissolved in 1 mL DMF. After aging the reaction for 1h, a solution of 1-aminododecane (12 mg, 0.064mmol, 0.1 equiv) and triethyl amine (150 mL, 1.08 mmol, 1.7 equiv.) in 1mL DMF was added to the reaction, and the mixture was left at ambient temperature for 48 hours (**Figure 1 B**). The reaction mixture was added drop-by-drop to 150 mL of a solution consisting of 1:1 acetone/tetrahydro-2-methylfuran. The precipitate was collected and redissolved in water and collected as an amorphous 50 mL of deionized water.

Purification and cation exchange

The above solution was treated with 5 mL of Dowex 50WX8-400 and stirred for 10 min. The resin was filtered off and washed with deionized water. The aqueous solution was treated with 1M NaOH until the pH was 12.0. The procedure was repeated two more times and the final pH was then adjusted to 7.5 – 8.0 by first bubbling CO₂ followed by bubbling with N₂. The solution was finally concentrated via tangential flow using a 30KDa cut-off Pellicon XL Biomax filter cassette and the concentrate was lyophilized. The **dodecylamide functionalized HA (C12-HA)** was analyzed by ¹HNMR spectroscopy to confirm its structure and degree of substitution.

2.3 Development of the self-emulsification method – primary emulsions

The self-emulsification method was initially optimized for the preparation of nanoemulsions, and subsequently adapted to the formulation of nanocapsules by the addition of HA.

Oil in water (o/w) nanoemulsions were prepared without organic solvents and heat using a one-step emulsification process. Briefly, spontaneous emulsification was

performed under magnetic stirring by the addition of an oil phase (containing Miglyol[®]812 and Tween[®]80) to an aqueous phase (composed of water and Solutol[®]HS15). Miglyol[®]812 and Tween[®]80 were firstly mixed together and then the mixture was poured into the aqueous phase, stirred at 900rpm over a 20min period. Nanoemulsion optimization was performed after analyzing the impact of the following variables in particle characterization:

2.3.1 Effect of Solutol[®]HS15 on the aqueous phase

An oil phase composed of Miglyol[®]812 and Tween[®]80 (1:1 ratio w/w) was added under magnetic stirring to an aqueous phase (oil/aqueous phase ratio 1:2 v/v) composed of increasing amounts of Solutol[®]HS15: 2.5, 5, 15 and 25 mg/mL.

2.3.2 Influence of Miglyol[®]812/Tween[®]80 ratio

An oil phase composed of different Miglyol[®]812/Tween[®]80 ratios (1:1, 1.5:1, 2:1, 3.5:1 w/w) was prepared and poured into an aqueous phase (oil/aqueous phase ratio 1:2 v/v) with 2.5 or 25 mg/mL of Solutol[®]HS15.

2.3.3 Influence of oil/aqueous phase ratio

The oil phase, composed of Miglyol[®]812/Tween[®]80 (1:1 ratio w/w) was added to the aqueous phase, with 2.5 mg/mL of Solutol[®]HS15, in a range of different ratios between 1:2 and 1:30 (v/v).

2.4 Preparation and optimization of HA-based nanocapsules

Using the optimized self-emulsification process, two types of HA-based nanocapsules were prepared by dissolving sodium hyaluronate (HA) or dodecylamide functionalized HA (C12-HA) in the aqueous phase. HA nanocapsules (HA NCs) and C12-HA nanocapsules (C12-HA NCs) were prepared using the same procedure. Nevertheless, to prepare HA NCs the cationic surfactant CTAB was dissolved into the oil phase at different

concentrations: 0.05, 0.10 and 0.15 mg/mL. For both prototypes, increased concentrations of HA or C12-HA at 0.25, 0.5 and 1 mg/mL were dissolved in water.

2.5 C12-HA and nanocapsules characterization

The amphiphilic C12-HA was characterized by ^1H NMR spectroscopy using Varian Mercury Plus 400 MHz spectrometer.

Nanocapsules were characterized regarding mean particle size, polydispersity index (PDI) and zeta potential (ZP) using dynamic light scattering (DLS) (Zetasizer Nano-ZS, Malvern Instruments). Morphological analysis was carried out by transmission electron microscopy (TEM, CM12, Phillips).

2.6 Physical stability studies

Physical stability of HA NCs and C12-HA NCs was performed under storage conditions and in the presence of human plasma. For long term stability, samples were kept undiluted at 4°C and stored for up to 6 months. The stability in biological fluids was performed by diluting the samples 1:10 (v/v) in human plasma for a period of 24h, at 37°C. At predetermined time intervals, samples were taken and particle size evaluated as described above.

2.7 Solubility of docetaxel in Miglyol®812

The solubility of docetaxel (DCX) in Miglyol®812 was determined following the procedure of Saliou *et al*, with slight modifications [30]. Briefly, an excess (2.5 mg) of DCX was poured in 0.5 mL of Miglyol®812 and stirred for 24h at room temperature. The mixture was centrifuged 45min at 20,000g to separate the solution from the undissolved drug. The supernatant was collected, filtered and the concentration of DCX analyzed by HPLC.

2.8 Preparation of docetaxel-loaded HA nanocapsules

DCX was solubilized in Miglyol®812 at 1.8 mg/mL and DCX-loaded HA-based nanocapsules were prepared as described before at a concentration of 112 µg/mL. Briefly, for DCX-loaded HA NCs, the oil phase was prepared by mixing Miglyol®812 with DCX (1.8 mg/mL) and Tween®80 (ratio 1:1 w/w) containing 0.15 mg/mL of CTAB. The aqueous phase was prepared by dissolving 2.5 mg/mL of Solutol®HS15 and 0.25 mg/mL of HA in water. The NCs were formed by pouring the oil phase into the aqueous phase (ratio 1:8 v/v) under magnetic stirring. DCX-loaded C12-HA NCs were prepared using the same procedure but without the cationic surfactant CTAB and by dissolving 0.5 mg/mL of C12-HA in water.

2.9 Nanocapsules isolation and docetaxel encapsulation

All formulations (blank and DCX-loaded HA-based nanocapsules) were isolated by size exclusion chromatography (SEC) using CentriPure®P10 columns. Column preparation and equilibration were performed as described in the manufacturers' protocol. One mL of nanocapsules was transferred to the column and eluted with water by simple gravity. The first 1.4 mL were discharged and the opalescent fraction, corresponding to 1.2 mL of the formulation, was collected and characterized as described before. Nanocapsules yield (%) was determined after lyophilizing and weighting 1 mL of the initial formulation and the collected elute. Encapsulation efficiency (EE%) was calculated by quantifying the drug concentration in the collected elute and in the initial formulation.

2.10 Docetaxel quantification

DCX was quantified by HPLC (Elite LaChrom, VWR-Hitachi) using a reverse phase Zorbax® Eclipse XDB C8- 5µm column (Agilent technologies) at room temperature as reported by Rivera-Rodriguez *et al* [31].

2.11 *In vitro* release assays

In vitro release (IVR) assays were assessed using a drug transfer method adapted from Bastiat et al [32]. This method was optimized for the IVR profile of DCX from self-emulsifying HA-based nanocapsules under sink conditions. Using 50 mL falcon tubes, DCX-loaded HA-NCs and C12-HA NCs were diluted in phosphate buffer saline (PBS) at 37°C and placed inside a water bath incubator. At specific time points, 15min, 3h, 6h and 24h, 500 µL of sample were collected to an eppendorf, mixed 1:1 (v/v) with an external oil compartment composed of Miglyol®812, vortex for 15sec and placed into a centrifuge for 30 min at 4000 rpm and 20°C. After centrifugation, the oil and aqueous phase were separated, the nanocapsules suspension characterized by DLS and the amount of drug in each phase quantified by HPLC. The release pattern of drug was calculated respect to the total amount of DCX in the release medium.

2.12 *In vitro* cytotoxicity assays

In vitro cytotoxicity of HA NCs and C12-HA NCs was evaluated by using the cell viability AlamarBlue® assay in A549 human lung adenocarcinoma cell line for both blank and DCX-loaded HA nanocapsules, using a similar experimental set-up from Ferreira *et al* [33].

2.12.1 *In vitro* toxicity of blank nanocapsules

The day before the experiment, A549 cells were cultured in sterile 96-well flat bottom plates in DMEM supplemented with 10% fetal bovine serum, 100 units of penicillin, 100µg of streptomycin sulfate and 2mM L-glutamine, at a cell density of 5×10^3 cells/well. Cells were incubated at 37°C and 5% CO₂. On the first day, medium was replaced by fresh medium containing the different formulations and each concentration was tested in six wells per plate. Cells were incubated for 24, 48 and 72h and after each time of exposition, medium was replaced by fresh medium containing 5 mM AlamarBlue® and incubated for 3h at 37°C. Fluorescence was measured at 530 and 590 nm (excitation and emission, respectively) in a microplate reader (Fluostar Omega, BMG Labtech,

Germany). The relative cell viability (%) compared to control cells was calculated as the percentage of the fluorescence of the samples divided by the control.

2.12.2 In vitro toxicity of docetaxel-loaded HA-based nanocapsules

A549 cells were exposed to serial dilutions of free DCX, blank nanocapsules and DCX-loaded HA-based nanocapsules (DCX concentrations of 0.625, 1.25, 2.5, 5, 10 and 100nM) and incubated for 24 and 48h. After each time, cells were incubated with AlamarBlue® and analyzed as described before.

Statistical evaluation of data was performed using one-way analysis of variance (ANOVA). A Tukey–Kramer multiple comparison test (GraphPad Prism 5, GraphPad Software, USA) was used to compare the significance of the difference between the groups. Differences were considered statistically significant at $P < 0.05$.

2.13 Fluorescently labeled HA-based nanocapsules

Nile red (NR) loaded HA NCs and C12-HA NCs were prepared as described before and the fluorescent probe was incorporated into the oil core. Encapsulated NR was separated from free NR by SEC following the defined protocol. The pink elute was collected, dissolved in acetonitrile and analyzed by spectrophotometry at 552 nm with DU 730 spectrophotometer (Beckman Coulter).

2.14 Cell uptake of HA-based nanocapsules

Cellular uptake of NR-loaded HA-NCs was studied on A549 cells. 60,000 cells/well were seeded in a cover glass and incubated with the volume of formulation equivalent to 50 ng of fluorophore, diluted in DMEM, for 4h. Then, cells were fixed, stained with DAPI and visualized by confocal microscopy (Leica, TCS SP5).

3. Results

3.1 Synthesis and characterization of dodecylamide-functionalized HA

Synthesis of the C12-HA was carried out by following a designed procedure. The reaction scheme is illustrated in **Figure 1** (materials and methods section). The reaction yield for each synthetic step was between 50-70%. The final product was characterized by ^1H NMR spectroscopy and the spectrum of the compound is shown in **Figure 2**. The degree of substitution (DS) of the dodecylamide group was determined from the peak area ratio of the methyl groups of the acetamide group of HA and the methyl group of dodecylamide substituent. The degree of substitution of the different lots of this compound was in the range of 2.5% to 5.0%.

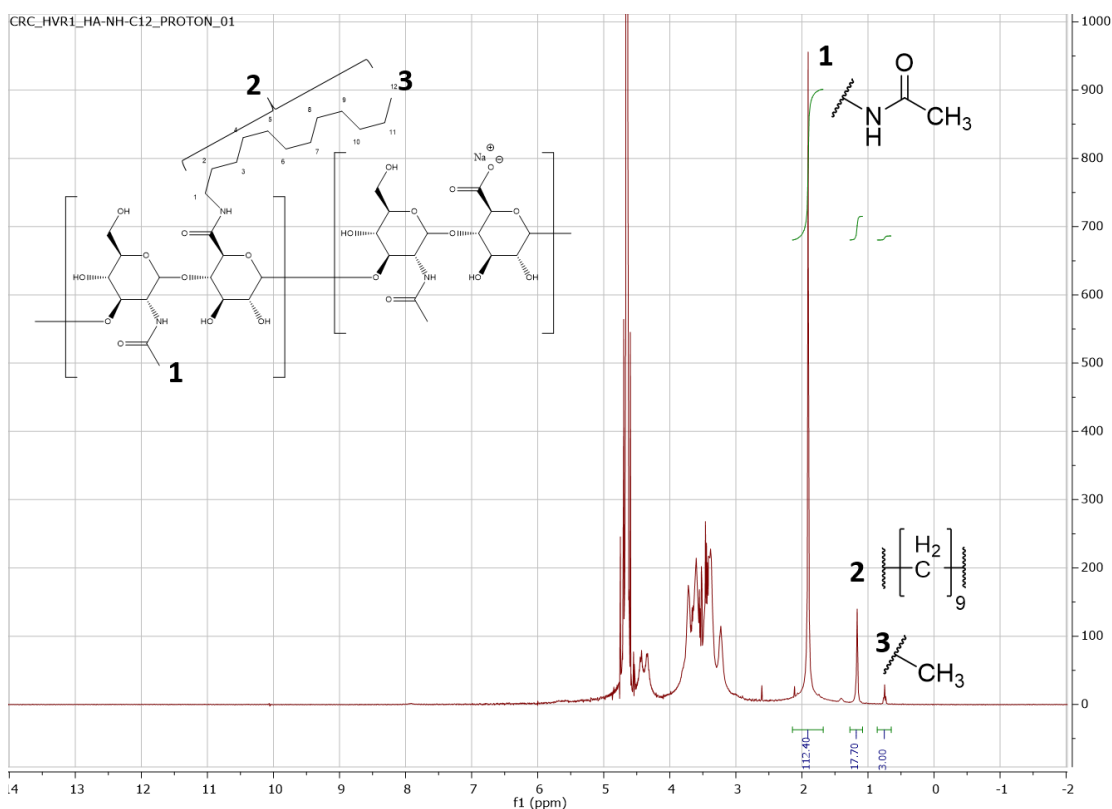


Figure 2. ^1H NMR of dodecylamide functionalized sodium hyaluronate in D_2O

Notes: The degree of substitution (DS) of the dodecylamide group was determined from the peak area ratio of the methyl groups of the acetamide group of HA (1) and the methyl group of dodecylamide substituent (2).

3.2 Optimization of the self-emulsification method – characterization of the nanoemulsions

The organic-solvent free, room temperature and low energy self-emulsification method was initially optimized for a nanoemulsion. The oil phase composed of Miglyol®812 (oil) and Tween®80 (surfactant) was added under magnetic stirring to an aqueous phase composed of water and Solutol®HS15. Formulation optimization was performed based on the effect of the amount of Solutol®HS15 in water, the ratio between Miglyol®812 and Tween®80 in the oil phase and the ratio between the oil and the aqueous phases. Nanoemulsions with a mean particle size ≤ 150 nm and a PDI ≤ 0.2 were selected for further optimization. **Table 1** summarizes the composition and respective granulometric characterization of the different nanoemulsions.

Table 1. Optimized parameters and physicochemical characterization of the nanoemulsions (NEs) prepared by self-emulsification.

Formulation parameters			NEs characterization	
Solutol®HS15 (mg/mL)	Miglyol®812/T80 ratio (w/w)	Oil/aq. phase ratio (v/v)	Size (nm)	PDI
2.5	1:1	1:2	138 ± 3	0.2
5			138 ± 2	0.2
15			149 ± 3	0.2
25			140 ± 1	0.2
2.5	1:1	1:2	138 ± 3	0.2
	1.5 :1		147 ± 3	0.2
	2 :1		164 ± 1	0.2
	3.5 :1		159 ± 3	0.3
2.5	1:1	1:3	139 ± 2	0.2
		1:4	144 ± 1	0.2
		1:5	152 ± 3	0.2
		1:8	138 ± 3	0.2

Notes: Results are presented as mean value \pm standard deviation (n=3)

Abbreviations: T80, Tween®80; PDI, polydispersity index.

3.2.1 Effect of Solutol®HS15 in the aqueous phase

The increased concentration of Solutol®HS15 into the aqueous phase resulted in very similar systems, without considerable changes in the globule size and in the polydispersity index.

3.2.2 Influence of Miglyol®812/Tween®80 ratio

The best Miglyol®812/Tween®80 ratio (w/w) was found to be 1:1. Smaller amounts of Tween®80 up to 3.5:1 resulted in an increase in particle size that led to polydisperse formulations.

3.2.3 Influence of oil/aqueous phase ratio

Maintaining the Miglyol®812/ Tween®80 ratio at 1:1 (w/w), nanoemulsions were prepared by varying the ratio of oil phase added to the aqueous phase. By reducing the oil/aqueous phase ratio (v/v) from 1:2 to 1:8 the droplet size of the nanoemulsions was as small as in the case of formulations based on high amounts of oil phase, resulting in nanoemulsions with a mean particle size of 140 nm and a monomodal distribution. It was possible to decrease the oil/aqueous phase ratio up to 1:30 (v/v) without affecting the physicochemical properties of the nanoemulsions (results not shown).

Based on the above findings, the following conditions were employed for the formulation of nanoemulsions: the oil phase was composed of Miglyol®812/ Tween®80 in a ratio 1:1 (w/w) and the aqueous phase of 2.5 mg/mL of Solutol®HS15 in water. The oil phase was poured into the aqueous phase in a ratio 1:8 (v/v) and stirred at 900 rpm during 20min.

HA-based nanocapsules were prepared using the optimized self-emulsifying process by dissolving HA or C12-HA in the aqueous phase. HA NCs were prepared in the same way but the cationic CTAB was added to the oil phase.

3.3 Characterization of HA NCs – effect of CTAB and HA concentration

Cationic nanoemulsions were initially prepared by varying the concentration of CTAB in the oil phase. The cationic surfactant promoted an inversion in the negatively charged nanoemulsion to positive values. Also, increasing amounts of CTAB resulted in a high zeta potential, without influencing the mean droplet size (**Table 2**). Since no further zeta potential increase was observed, 0.15 mg/mL of CTAB was used for HA NCs formation. The coating of cationic nanoemulsions with CTAB resulted in a shift of the zeta potential from +10 mV to -19 mV, regardless the polymer concentration.

3.4 Characterization of C12-HA NCs – effect of C12-HA concentration

Table 2 shows the characterization of HA-based nanocapsules prepared with C12-HA. The amphiphilic HA did not change the physicochemical properties of the system. However, the zeta potential was dependent on the concentration of the hydrophobically-modified HA. At least 0.5 mg/mL of C12-HA was needed to give the appropriate negative charge to the NCs.

Table 2. Influence of the coating material in the size and zeta potential of the different formulations: anionic nanoemulsion, cationic nanoemulsions, HA NCs and C12-HA NCs.

Formulation	Coating material (mg/mL)		Size (nm)	PDI	ZP (mV)
Anionic NE	-	-	145 ± 1	0.2	-15 ± 2
Cationic NE	CTAB	0.05	156 ± 2	0.2	-1 ± 1
		0.10	154 ± 2	0.2	+5 ± 1
		0.15	146 ± 3	0.2	+10 ± 1
HA NCs	HA	0.25	137 ± 11	0.2	-19 ± 1
		0.50	154 ± 2	0.2	-19 ± 2
		1.0	153 ±	0.2	-22 ± 4
C12-HA NCs	C12-HA	0.25	133 ± 11	0.2	-10 ± 1
		0.50	126 ± 5	0.2	-20 ± 2
		1.0	133 ± 3	0.2	-22 ± 3

Notes: Results are presented as mean value ± standard deviation (n=3)

Abbreviations: NE, nanoemulsion; PDI, polydispersity index; ZP, zeta potential

Based on the above results, further experiments were performed using HA NCs prepared with 0.15 mg/mL of CTAB and 0.25 mg/mL of HA, and C12-HA NCs prepared without CTAB and 0.5 mg/mL of C12-HA. **Table 3** shows the composition of each formulation.

Table 3. Composition (mg/mL) of HA NCs and C12-HA NCs prepared by self-emulsification.

Composition	HA NCs (mg/mL)	C12-HA NCs (mg/mL)
Miglyol®812		59
Tween®80		58
Solutol®HS15		2.5
CTAB	0.15	-
HA	0.25	-
C12-HA	-	0.5

TEM images (**Figure 3**) confirmed the proposed morphology of HA-based nanocapsules i.e. a structure consisting of an oil core surrounded by a polymeric shell.

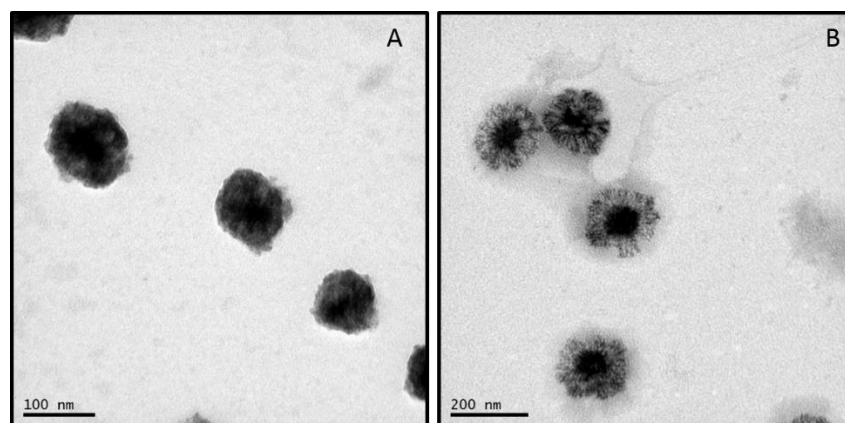


Figure 3. TEM images of HA-based nanocapsules. **A)** HA NCs; **B)** C12-HA NCs

3.5 Stability of HA-based NCs

The stability of both HA-based nanocapsules was tested under storage conditions at 4°C for 6 months, and in human plasma at 37°C for 24h.

Under storage conditions, both formulations were very stable, without significant change in particle size, PDI or zeta potential for up to 6 months (**Table 4**).

Table 4. Physical stability of self-emulsifying HA-based nanocapsules in storage conditions.

Time period	Size (nm)		PDI		ZP (mV)	
	HA NCs	C12-HA NCs	HA NCs	C12-HA NCs	HA NCs	C12-HA NCs
First day	134 ± 12	122 ± 3	0.2	0.2	-21 ± 1	-18 ± 1
1 month	138 ± 8	124 ± 6	0.2	0.2	-21 ± 1	-18 ± 1
4 months	136 ± 7	127 ± 6	0.2	0.2	-20 ± 1	-19 ± 1
6 months	137 ± 6	123 ± 1	0.2	0.2	-20 ± 1	-18 ± 1

Notes: Results are presented as mean value ± standard deviation (n=3)

Abbreviations: PDI, polydispersity index; ZP, zeta potential

When incubated in human plasma at 37°C, there was an increase in the size of both types of nanocapsules. However, the increase was less than 20% of the initial size and no aggregation of particles was observed (**Figure 4**). Thus, self-emulsifying HA-based nanocapsules can be regarded as physically stable under storage conditions and after incubation with human plasma up to 24h.

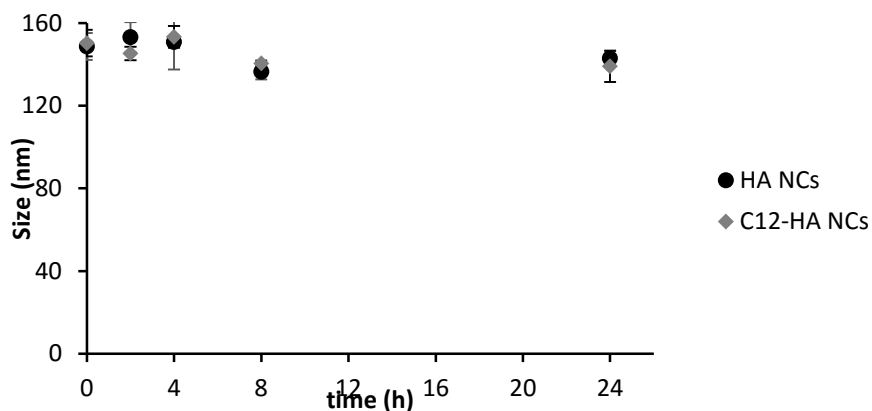


Figure 4. Size distribution of HA NCs and C12-HA NCs incubated with human plasma, at 37°C for 24h.

Notes: Results are presented as mean value \pm standard deviation (n=3)

3.6 Characterization of docetaxel-loaded HA-based nanocapsules

At first, the solubility of DCX in Miglyol[®]812 was assessed, which was found to be 2.03 ± 0.2 mg/mL. The stock solution of DCX in Miglyol[®]812 was always prepared at a concentration of 1.8 mg/mL, and the resulting solution was clear all the time. This was needed to ensure the complete dissolution of DCX in the oil core and to avoid its precipitation, as it could lead to formulation instability [34].

Encapsulated DCX was separated from the free drug by SEC. This method was initially validated for the free drug and the blank formulation by the quantification of DCX and the physicochemical characterization of the nanocapsules of consecutive eluted volumes (400 μ L). **Figure 5** presents the elution profile of free DCX and HA-based nanocapsules. Being a small molecule (Mw = 808 Da), DCX gets entrapped within the column matrix and is eluted after the nanocapsules, without an overlapping interference.

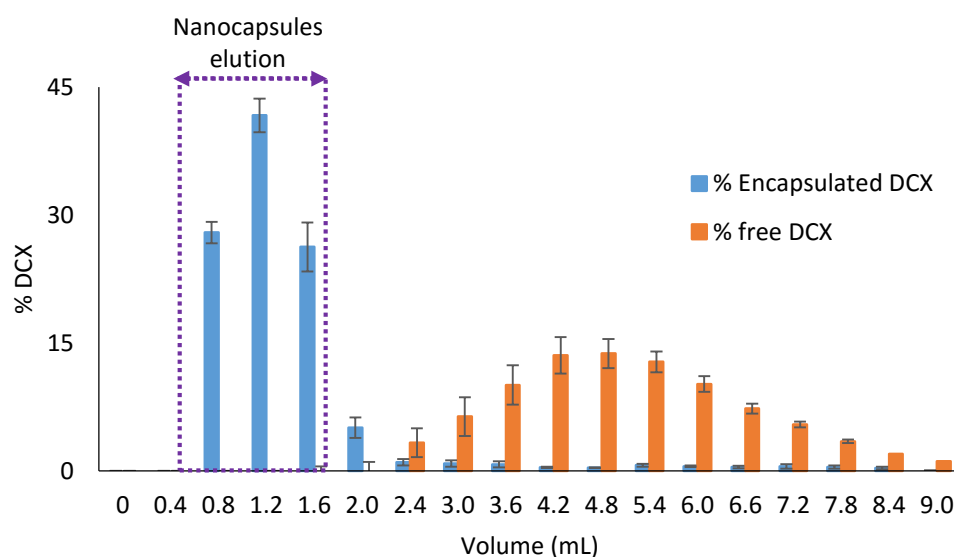


Figure 5. Elution profile of free DCX and DCX-loaded HA-based nanocapsules by SEC.

Notes: Results are presented as mean value \pm standard deviation ($n=3$)

DCX was successfully encapsulated into the oil core of both HA-based nanocapsules, without changing their properties. **Table 5** demonstrates the physicochemical characterization of both systems and the respective DCX encapsulation efficiency and nanocapsules yield.

Table 5. Characterization of DCX-loaded HA NCs and C12-HA NCs after isolation by SEC.

Formulation	Size (nm)	PDI	ZP (mV)	EE%	Yield %
HA NCs	140 \pm 5	0.2	-18 \pm 2	88 \pm 9	93 \pm 2
C12-HA NCs	145 \pm 6	0.2	-20 \pm 1	86 \pm 3	88 \pm 8

Notes: Results are presented as mean value \pm standard deviation ($n=3$)

Abbreviations: PDI, polydispersity index; ZP, zeta potential; EE%, encapsulation efficiency.

3.7 *In vitro* release assays

The release profile of DCX was evaluated using a drug transfer process [32]. Using this method, DCX-loaded HA NCs and C12-HA NCs were diluted in PBS under sink conditions, mixed with an external oil compartment composed of Miglyol[®]812 and centrifuged. After phase separation, the upper oil compartment acted as a drug reservoir where the

free DCX was solubilized, whereas encapsulated DCX was kept inside the nanocapsules suspension. After separation, nanocapsules suspension maintained the same physicochemical characterization (size, PDI and Derived Count Rate (DCR) as described by Bastiat *et al* [32]) (Results not shown).

Figure 6 displays the release behavior of DCX encapsulated into HA-based nanocapsules when compared with the free drug. As observed, 100% of the free DCX was transferred to the oil compartment, evidencing the ability of Miglyol®812 to solubilize all the free drug in solution. The release behavior of DCX from HA NCs and C12-HA NCs exhibited an initial burst release of 55% and 40%, respectively, followed by a continuous release for 24h.

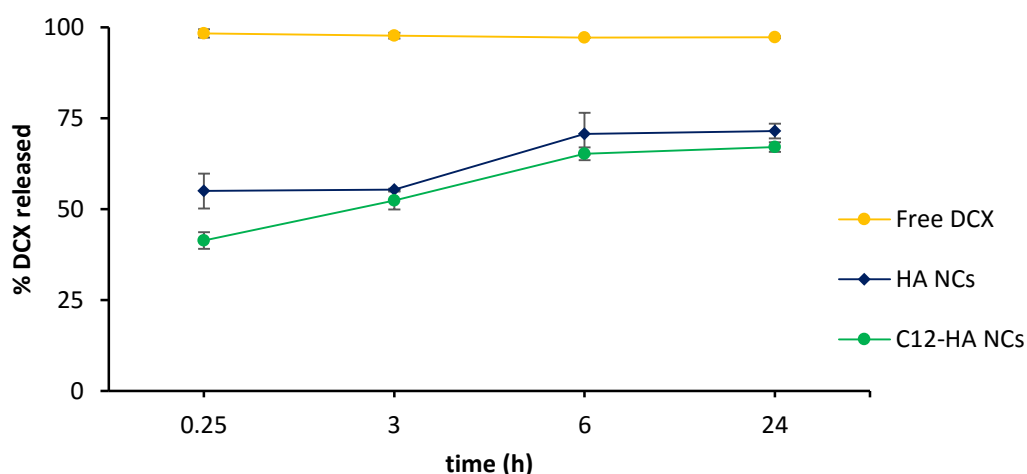


Figure 6. Release profile of DCX from HA NCs and C12-HA NCs.

Notes: Results are presented as mean value \pm standard deviation (n=3)

3.8 *In vitro* toxicity of empty HA-NCs

Cytotoxicity of both HA-based nanocapsules was assessed in A549 cells at different concentrations. Additionally, two surfactant solutions were prepared at the same concentration required for the formulation of nanocapsules, and they were used as controls. As can be seen from **Figure 7**, neither type of nanocapsules affected cell viability when tested at concentrations up to 350 $\mu\text{g}/\text{mL}$. On the other hand, C12-HA NCs showed absence of toxicity even when tested at the highest concentration (1000

$\mu\text{g/mL}$). The highest cytotoxicity was observed for the free surfactant mixture with CTAB, where only 20% of cells survived at 350 $\mu\text{g/mL}$ after 72h. It appears that by eliminating the use of a cationic surfactant, there is a possibility of preparing nanocapsules with improved biocompatibility and safer profiles.

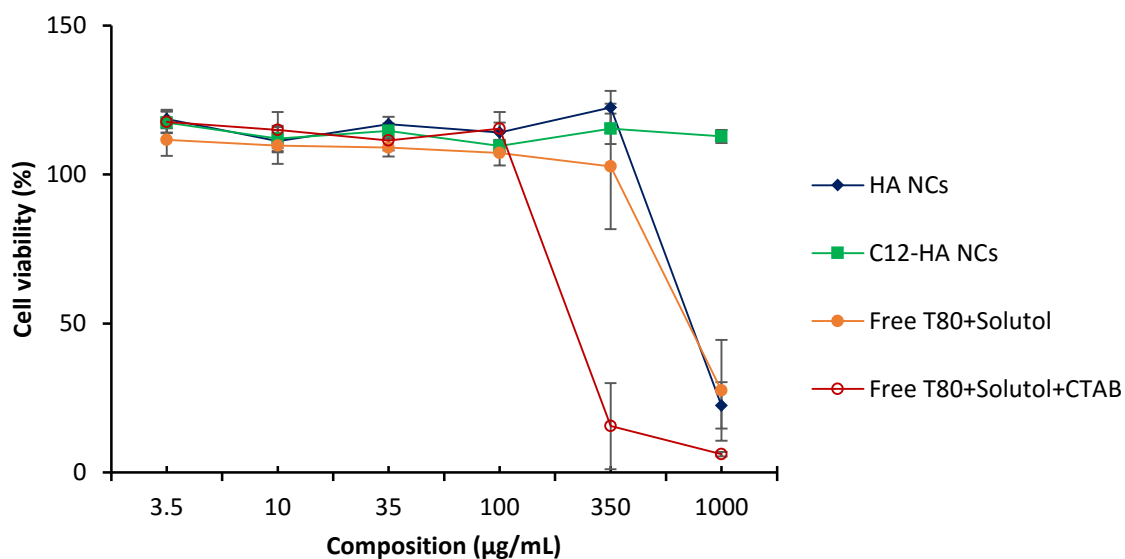


Figure 7. Cell viability of A549 cells after exposition to different concentrations of blank HA NCs, C12-HA NCs and free surfactants mixture for 72h.

Notes: Results are presented as mean value \pm standard deviation (n=6)

3.9 *In vitro* toxicity of docetaxel loaded HA-based nanocapsules

Both free and DCX-loaded HA-based nanocapsules showed a dose dependent cytotoxicity against A549 cells in the concentration range from 0.625 to 100 μM (**Figure 8**). The half minimal inhibitory concentration (IC₅₀) was reached only by the drug-loaded into the nanocapsules at 10 μM concentration after 48h. The free drug did not reach IC₅₀ for the concentrations tested after the same time. Blank NCs showed negligible toxicity, indicating that this exacerbation of drug cytotoxicity was not induced by a toxic effect of the vehicle itself. The cell viability of blank HA NCs and C12-HA NCs was statistically different from the formulations containing DCX (*P<0.05).

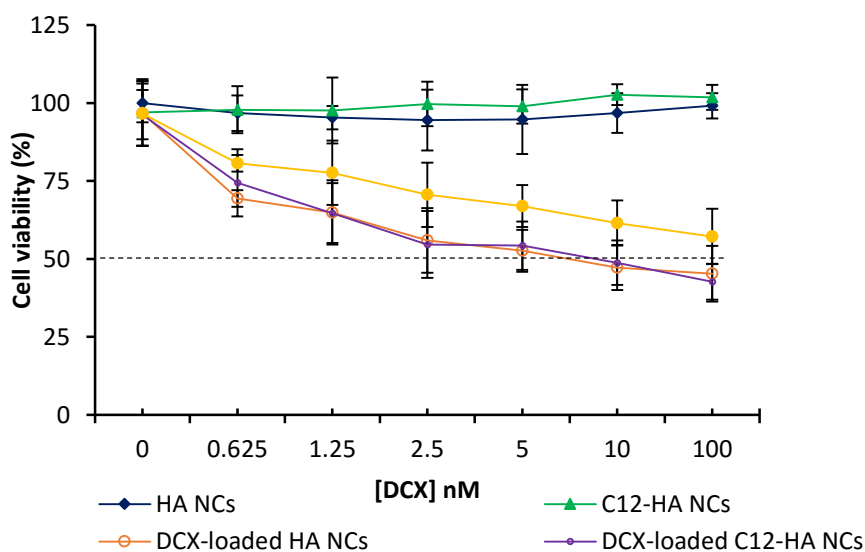


Figure 8. Cell viability of A549 cells after exposition to different concentrations of free DCX, DCX-loaded HA-based nanocapsules and blank HA-based nanocapsules.

Notes: Results are presented as mean \pm standard deviation (n=3).

3.10 Intracellular uptake of HA-based nanocapsules

To evaluate the intracellular uptake of HA NCs and C12-HA NCs, NR was loaded into both nanocapsules and their uptake observed in A549 cells overexpressing CD44 receptors by confocal microscopy. As a control, cells were exposed to a solution of NR, which was not internalized by the cells (**Figure 9A**). On the other hand, a high fluorescence (red color) was seen when both NR-loaded nanocapsules were taken by the cells (**Figure 9B, C**).

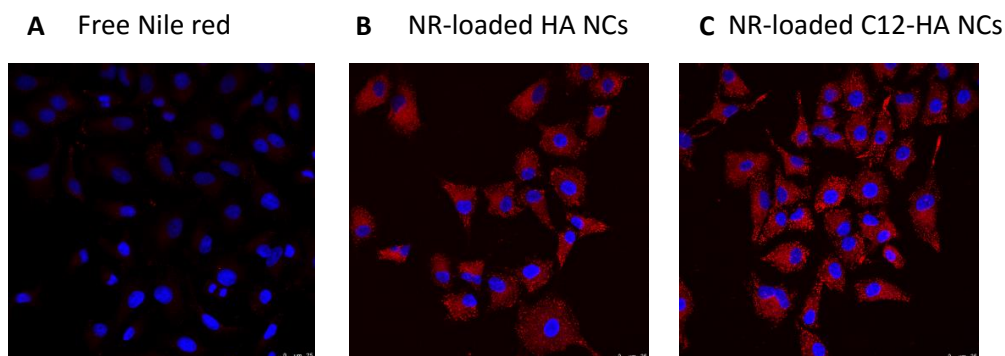


Figure 9. Intracellular uptake of NR-loaded HA-based nanocapsules in A549 cells.

Notes: The nuclei were stained with DAPI (blue). Nile Red (NR) exhibits red fluorescence.

4. Discussion

4.1 Synthesis and characterization of dodecylamide-functionalized HA

Hydrophobically dodecylamide-functionalized HA was synthesized by chemical modification of sodium hyaluronate by 1-aminodecane via an amide bond. The degree of substitution was kept in the range 2.5 to 5.0%. The modified polymer was characterized by ^1H NMR spectroscopy. Aqueous solubility of HA was not affected as a result of this chemical modification. Moreover, it has been reported that rheological and biodegradation characteristics of HA should not be affected by such low degree of modification [35].

4.2 Optimization of the self-emulsification method

The assessment of a green technology was achieved by the development of a self-emulsification method for the preparation of HA-based nanocapsules. Without organic solvents and heat, the self or spontaneous emulsification process is mainly determined by the system composition and their physicochemical characteristics [36]. Thus, components selection was based on their ability to formulate self-emulsifying systems, in such a way that small droplets form spontaneously when the phases are brought into contact. Miglyol[®]812, being a medium chain triglyceride, is described to reduce the interfacial tension, and shows better water solubility and partitioning ability to nanoemulsify when compared to long chain triglycerides [37–38]. In addition, it has the ability to solubilize hydrophobic drugs, such as docetaxel, which is relevant when the aim is to develop a process without organic solvents [39]. For the surfactant selection, non-ionic surfactants with a hydrophilic/lipophilic balance (HLB) between 12-18 are preferable as they have better hydrophilicity and can rapidly spread from the oil phase to the aqueous environment and provide good dispersion performance [37]. Among them, Tween[®]80 is one of the most used surfactants in self-emulsifying systems. Although it is associated to potential side effects [40], it was reported that Tween[®]80 showed remarkably less toxicity than other solvents such as Labrasol[®] or Cremophor[®] RL [41]. For example, Ma *et al* [42] prepared DCX-loaded poly- ϵ -caprolactone (PLC)-Tween[®]80 nanoparticles and demonstrated that the carrier showed less *in vitro* toxicity

than commercial Taxotere® at the same surfactant concentration. In fact, we assume that its localization at the interface of the particle surrounded by the polymeric shell might decrease its free circulating exposure. We decided to include Solutol®HS15 in the formulation for two main reasons. First, Solutol®HS15 has a PEG chain in its structure, which may provide stability and prolonged circulation time to the nanocarrier [43]. Additionally, it possesses the required high HLB (HLB = 14-16), along with an ability to inhibit p-glycoprotein pumps. The inhibition of this membrane pump must result in higher intracellular drug accumulation [44].

The optimized nanoemulsion was composed by 2.5 mg/mL of Solutol®HS15 dissolved in water and an oil phase composed of Miglyol®812/Tween®80 in a 1:1 ratio (w/w). Increased amounts of Solutol®HS15 up to 25 mg/mL did not improve the physicochemical characteristics of the system and we considered that 2.5 mg/mL was the minimum required to formulate and stabilize the nanoemulsion due to the greater partition extent between the oil/water interface [45]. Miglyol®812/Tween®80 ratios, with lower surfactant amount, promoted an increase in particle size and PDI. It has been described that at high oil/surfactant ratios (high oil content) the amount of surfactant is too small to microemulsify the large quantity of oil. However, once the surfactant concentration increases, the amount of Tween®80 became enough to perform its emulsifier function effectively [46].

Knowing the importance of the oil/surfactant ratio and, on the other hand, the possible toxicity associated to high amounts of Tween®80, the decrease in the surfactant amount was done by decreasing the oil/aqueous phase ratio. We found that by reducing the oil/aqueous phase ratio to 1:8 (v/v) we were able to produce nanoemulsions equally small than those obtained with a 1:2 ratio because the Tween®80 did not lose its surfactant capacity even when diluted in water [47]. We decreased up to 1:30 (v/v) the oil/aqueous phase ratio, however, those formulations were limited by a very low amount of oil which constrained consequently the amount of drug incorporated into the system.

Concerning the formulation of HA NCs, the effective attachment of the HA to the outer shell of the particle was achieved by an electrostatic interaction between the polymer

and the lipid core surrounded by the cationic CTAB. Due to the balance between the positive charge of the nanoemulsion and the possible toxicity associated with high amounts of CTAB [22], we chose a concentration of 0.15 mg/mL of CTAB for the nanocapsules preparation. The chosen concentration was previously demonstrated to be enough for polymer attachment and nanocapsules stabilization [21]. A HA concentration of 0.25 mg/mL was enough to promote the attachment of the polymer to the particle surface, resulting in a negative zeta potential. By shielding the nanocapsules with HA and rendering to the particles a negative charge, HA NCs must promote a longer half-life in the blood stream [48].

In order to simplify the process and to avoid the use of a cationic surfactant, HA was replaced by C12-functionalized HA. The hydrophobic dodecyl chains of HA facilitated the self-assembly of the polymer within the oil/surfactant nanoemulsion interface through hydrophobic interactions, resulting in an increased stability of the hydrophobic core [49]. For this formulation, 0.5 mg/mL of C12-amide HA was required to achieve the same negative zeta potential as that of HA prototype.

Figure 10 exemplifies the structure of a HA-based nanocapsules formulated with HA or C12-HA and its respective composition.

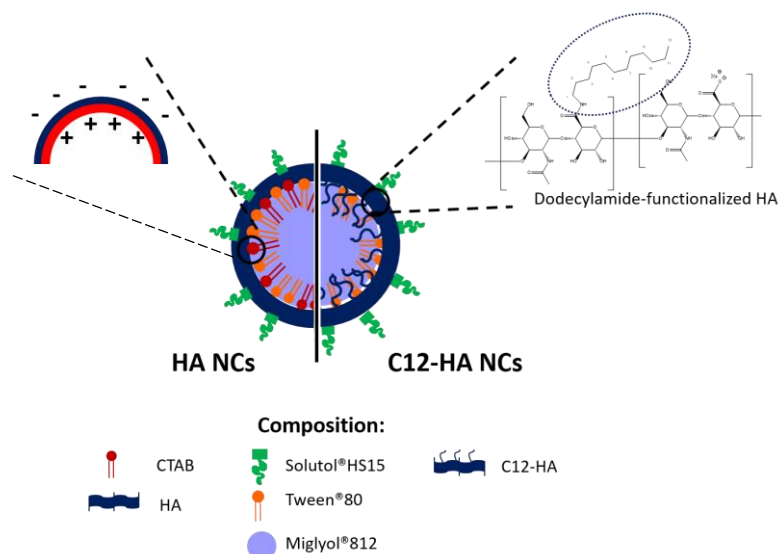


Figure 10. Structure and composition of HA-based nanocapsules formulated with HA (right) or C12-HA (left). HA NCs were formulated based on the ionic interaction between the positively charged CTAB and the negatively charged HA. On the other hand, the amphiphilic C12-HA self-assembled with the surfactant interphase, without the need of a cationic surfactant.

4.3 Stability assays

The stability of both HA-based nanocapsules was assessed thorough storage conditions and in human plasma. No significant differences in size, PDI and zeta potential were observed for both prototypes after storage for 6 months, at 4°C. The stability could be attributed to the high negative charge that prevents particle aggregation, due to a charge-charge repulsion. Moreover, the presence of Tween®80 should also add steric stability to the system [50]. The stability of the nanocapsules in plasma was determined by their physical integrity, mainly the particle size [51]. The observed increase in particle size after 24h at 37°C might be attributed to protein deposition. Nevertheless, this increase was less than 20% compared to the initial particle size, which means that these nanocapsules must be suitable for IV administration [52].

4.4 *In vitro* release assays

Nanocapsules formulated with HA and C12-HA showed a biphasic drug release profile, with an initial burst release of 45% and 55%, respectively. The release was sustainable up to 24h, with 70% of DCX being released from both systems. This biphasic release profile has been typically observed in other HA-based nanocapsules, which presented an initial burst release between 45-65%. The initial burst release has been justified by the own structure of the nanocapsules, favoring the partition of the drug between the oil core and the aqueous external medium [53]. Interestingly, the release was not affected by the ionic or hydrophobic forces that drove the formation of nanocapsules with HA or C12-HA, respectively. With a $pK_{a1}=2.82$ and $pK_{a2}=3.42$, HA is negatively charge at pH above 4, thus maintaining its ionic strength when in PBS at pH 7.4 [54]. Regarding the amphiphilic structure, the hydrophobic chain may enhance the hydrophobicity of the particle core, which helps DCX to be entrapped [55]. While this data provides us information about mechanistic details, it is important to highlight two important points: (i) the limitation of the method, where the external oil phase may force the release of DCX from the oil core of the nanocapsules to the external oil compartment and, (ii) the *in vitro* release is not necessarily expected to correlate with

the *in vivo* behavior, as the presence of macromolecules and ions in circulation could significantly influence the release profile [21].

4.5 *In vitro* cytotoxicity assays

A decrease in the cytotoxicity of C12-HA NCs was expected since the formulation of these nanocapsules with a hydrophobically functionalized HA eliminated the need for cationic surfactants. The results showed that irrespective of their composition, both prototypes did not affect cell viability when tested at concentrations up to 350 µg/mL. However, only self-emulsified nanocapsules prepared with C12-HA did not cause any toxicity when tested at the highest concentration (1000 µg/mL). The higher toxicity for HA NCs must be correlated to the presence of the cationic surfactant CTAB, which is in agreement with previous reports [56]. In addition, the marked difference in viability between HA NCs and the surfactant solution composed of Tween®80/Solutol/CTAB at 350 µg/mL effectively denotes the beneficial effect of HA surrounding the surfactant layer as well as to the correct isolation of the system from the free surfactants [57].

DCX-loaded HA NCs and C12-HA NCs presented an inhibition in the cell viability when compared with the free drug. The IC₅₀ was reached only by the drug-loaded into the nanocapsules, at a concentration of 10 µM and after 48h. On the other hand, the free drug did not reach the IC₅₀ for the concentrations tested for the same time period. Since the drug became more efficacious when loaded into HA-based nanocapsules, which in turn did not express any inherent cytotoxicity themselves, it is fair to assume that these nanocapsules must be taken up by cancer cells either via receptor mediated (CD44) endocytosis or simultaneous interaction with the cancer cell membrane followed by endocytosis and release in the endosome [58].

4.6 *In vitro* cellular uptake

In order to monitor the cellular uptake of NR-loaded HA-based nanocapsules, both prototypes were incubated with A549 cells overexpressing CD44 receptors. As seen by confocal microscopy, strong fluorescent signals were detected in the cells cytoplasm for

both nanocapsules prototypes when compared to the free fluorophore, further suggesting an intracellular uptake mediated by CD44 receptors [59]. Additionally, the fluorescent intensity was similar for both NR-loaded HA NCs and C12-HA NCs, which suggests that the functionalization of HA with the dodecylamide chain did not affect its binding affinity through CD44 receptors. In fact, this might suggest the localization of the lipophilic chain into the interface, and the hydrophilic branch turned to the outside [60].

5. Conclusions

In conclusion, here we report the application of a green methodology for the preparation of HA-based nanocapsules. A self-emulsification method was developed for the preparation of HA nanocapsules without the aid of organic solvents and heat, which offers a promising and sustainable approach to prepare nanoformulations for therapeutic molecules. The preparation of HA-based nanocapsules with an amphiphilic functionalized HA derivative led to the development of nanocarriers with low toxicity and the potential to efficiently encapsulate and deliver cytostatic drugs, such as docetaxel, into cancer cells.

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Chapter 3

**Hyaluronic acid nanocapsules as a platform for the
intracellular delivery of monoclonal antibodies:
when the target is inside the cell**

Hyaluronic acid nanocapsules as a platform for the intracellular delivery of monoclonal antibodies: when the target is inside the cell

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Abstract

Monoclonal antibodies (mAbs) are one of the most selective therapies against cancer. However, their high molecular weight and hydrophilicity have hampered their application for the targeting of intracellular oncogenic proteins. The main object of this work has been the development of a new nanotechnology adapted for the intracellular delivery of mAbs. For the validation of this technology, we have chosen anti-GSDMB, a mAb whose target is confined to the intracellular compartment. The selected technology consists of hyaluronic acid nanocapsules, which enabled the packing of anti-GSDMB by physicochemical interaction with the polymeric shell. Indeed, the results showed that it was possible to efficiently associate (70% association efficiency, AE) anti-GSDMB to tiny HA NCs (130 nm). *In vitro* assays performed in HCC1954 breast cancer cells showed that the anti-GSDMB carried by the nanocapsules was efficiently internalized while preserving their immunological determinants. Moreover, immunohistochemistry analysis demonstrated the capacity of the mAb to escape the endosomal compartment, thus avoiding their premature intracellular degradation. The preservation of the mAb activity upon its association to the nanocapsules was also confirmed using a wound healing migration assay. The results of this study showed that the anti-GSDMB-loaded into the nanocapsules could effectively interact with GSDMB, and that the result of this interaction was a decrease in the cell migration and invasion of HCC1954 breast cancer cells. Taken together, these results represent the first preliminary evidence of the capacity of hyaluronic acid nanocapsules as a new mAb-based therapeutic platform against intracellular cancer proteins.

Keywords: antibody, intracellular targeting, cancer, nanocarrier, nanocapsules, hyaluronic acid, intracellular delivery

1. Introduction

Monoclonal antibodies (mAbs) have become an important class of oncological biomolecules due to their ability to target tumor associated cell surface antigens and promote cytotoxicity through several mechanisms of action [1]. This targeting capacity has also been the basis for their use as targeting ligands, when conjugated to cytotoxic drugs [2] or decorating the surface of nanoparticles [3]. Indeed, antibody-conjugated drug-loaded nanoparticles were developed to selectively bind to a cancer cell receptors, promote the internalization of the nanoparticles, release its payload, and elicit cytotoxicity while decreasing undesired side effects [3]. An important consequence of this research activity has been the development of 13 prototypes of nanoparticles targeting cell surface receptors, which are currently under clinical development [4]. Nevertheless, onco-protein receptors are not always presented to the cell membrane, but they are exclusively restricted to the cytosol compartment [5]. In fact, hundreds of intracellular proteins have been associated to cancer progression, for example, RAS (GTPases), non-receptor tyrosine kinases, BRAF or heat shock proteins. Unfortunately, these oncoproteins could not be targeted with mAbs, due to the inability of these complex molecules to cross the cell membrane [6]. Rather than this, other strategies including silencing therapy, cytostatic molecules or protein kinase inhibitors have been explored until now. However, the complexity of these pathways, the off-target effects and cellular barriers, specially associated to polynucleotide molecules, hold these therapies far from their clinical use [6–7].

To our knowledge, so far, no one has reported scientific evidence of the intracellular targeting of mAbs against onco-proteins, which are exclusively restricted to the cytosol compartment and that lack from a surface region. Within this context, it is worthwhile to mention that two companies released recently news about the development of mAbs for intracellular targeting [9]. However, the way to achieve this targeting has not been disclosed in a scientific journal.

Taking advantage of the potential of mAbs as a disruptive therapy against intracellular proteins, our goal has been to engineer a nanocarrier with the capacity to overcome the cell membrane barrier and carry the mAb into the intracellular compartment [10]. To achieve this goal we started our formulation study with hyaluronic acid (HA)

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nanocapsules, previously disclosed by our group for the intracellular delivery of anticancer drugs [11] and adapted in this study to the loading of mAbs. To assess the efficacy of the delivery technology, we chose the mAb anti-Gasdermin-B (GSDMB), based on the discovery of Moreno-Bueno et al, who identified Gasdermin-B (GSDMB) as an intracellular marker for breast cancer [12]. These authors found that the over-expression of GSDMB was responsible for cell motility, tumor progression and metastasis in the MCF7 breast carcinoma cell line. Although the role of GSDMB in cancer is not completely understood, we believe that its intracellular targeting using anti-GSDMB may lead to a great opportunity to stop cancers over-expressing this protein.

In addition to its biocompatibility, non-toxicity and biodegradability, HA can bind to CD44 receptors, which are overexpressed in many cancer cells [13–14]. Different techniques have been used for the preparation of nanocapsules and, recently, our group have developed HA-based nanocapsules using HA and amphiphilic dodecylamide-functionalized HA by a spontaneous emulsification method. This method is characterized by the absence of organic solvents, heat and strong mechanical stirring, which makes it suitable for the association of proteins.

Therefore, these premises led us to the design of self-emulsifying HA-based nanocapsules specifically adapted for the association of mAbs. The resulting prototypes were characterized with regard to their physicochemical properties, mAb association efficiency, integrity and specificity. Finally, anti-GSDMB-loaded nanocapsules were evaluated in HCC1954 breast cancer cell line for their capacity to enter the intracellular compartment, achieve the intracellular delivery of anti-GSDMB and inhibit cancer cell migration and invasion.

2. Materials and methods

2.1 Materials

Sodium hyaluronate and dodecylamide-functionalized HA, both with 200KDa, were a kind gift from Genzyme Sanofi, USA. Caprylic/capric triglyceride (Miglyol®812) was provided by Cremer, Germany. Polyoxyethylene sorbitan monooleate and monolaurate (Tween®80 and Tween®20, respectively) and hexadecyl trimethylammonium bromide (CTAB) were purchased from Sigma-Aldrich, Spain. Macrogol 15 Hydroxystearate

(Solutol®HS15) was obtained from BASF, Germany. The following antibodies were purchased: rabbit anti-EEA1, from Cell Signaling Technology, Spain, rabbit anti-LAMP2, from Sigma-Aldrich, goat anti-mouse and anti-rabbit conjugated with Alexa-488, Alexa-594 or Alexa-647 were from Molecular Probes®, Thermo Fisher Scientific, Spain. Alexa-647 coupled phalloidin and 4,6-diamidino-2-phenylindole (DAPI) were also purchased from Molecular Probes®. Phosphate Buffer Saline (PBS) in tablets was obtained from Medicago, Sweden. HRP-conjugated goat anti-mouse IgG and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from DAKO (Agilent Technologies, Spain) and Roche (Switzerland), respectively. Coomassie Blue solution was acquired from Thermo Fisher Scientific. All other chemicals were of analytical grade.

2.2 Anti-GSDMB preparation and purification

Mouse anti-GSDMB monoclonal antibody was generated as described by Hergueta-Redondo et al (submitted) accordingly to specified techniques. The antibody was purified with a Hi-trap Protein G column (GE Healthcare, UK). After purification, anti-GSDMB solution was aliquoted in 2 mL Eppendorf's and kept at 4°C.

2.3 Anti-GSDMB characterization

2.3.1 Concentration and Integrity

Anti-GSDMB was quantitatively analyzed using a NanoDrop®2000 Spectrophotometer with the IgG reference at 280 nm (Thermo Fisher Scientific) and qualitatively analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed to identify IgG constant and variable fragments under non-reducing and reducing conditions, respectively. Anti-GSDMB samples were mixed with loading buffer composed of 10% SDS and 30% glycerol and reduced samples were prepared with the same loading buffer plus β -mercaptoethanol and boiled for 5, 7, 10 and 12 minutes in a Vortemp Shaking Incubator (UniEquip). Samples containing 20 and 40 μ g of anti-GSDMB were loaded into porous 10% SDS-PAGE gel under the effect of an electric field. The electrophoresis was run under constant voltage (100 V, 2h) and gels were stained with Coomassie Blue Solution.

2.3.2 Quantification and binding assay by ELISA

The specific binding activity and the quantification of Anti-GSDMB were also performed by ELISA. The ELISA plates (Nunc Medisorp™, Thermo Scientific) were coated with 5 µg/mL of GSDMB antigen diluted in water and incubated 1h at room temperature. Plates were washed three times with PBS containing 0.1% Tween®20 (v/v) and, then, blocked with PBS containing 2% (w/v) non-fat skim milk overnight at 4°C and washed again three times the day after. Anti-GSDMB solution was prepared from a stock solution at 10 µg/mL, sequentially diluted in PBS to obtain standard solutions with a concentration range between 0.125 and 10 µg/mL and incubated for 30 min at 37°C. The washing step was repeated and HRP-conjugated goat anti-mouse IgG (DAKO) was added (1:1000 in PBS), incubated 30 min at 37°C and plates washed as before. The reaction was revealed with substrate ABTS solution and the absorbance was read at 405 nm using a Multiskan FC Microplate Photometer (Thermo Scientific). All determinations were made at least in triplicate. The sigmoidal standard curves were set up using a sigmoidal dose-response (variable slope) model (GraphPad Prism 5) and were plotted in the form of absorbance at 405 nm against log of anti-GSDMB concentration.

2.4 Preparation of anti-GSDMB-loaded HA-based nanocapsules

Nanocapsules made of sodium hyaluronate (HA NCs) or dodecylamide-functionalized HA (C12-HA NCs) were prepared by a spontaneous emulsification method. Briefly, an oil phase composed of a 1:1 ratio (v/v) of Miglyol®812 and Tween®80 with CTAB, was added to an aqueous phase composed of HA and Solutol®HS15 under magnetic stirring in a 1:8 ratio (v/v). Both type of nanocapsules were prepared using the same protocol except that C12-HA NCs were prepared without the cationic surfactant CTAB. Formulations were developed using different HA concentrations: 0.25 and 0.75 mg/mL of HA and 0.5 and 1mg/mL for the C12-HA. All nanocapsules were isolated by size exclusion chromatography (SEC) using Centripure P10 columns (EmpBiotech, Germany).

Anti-GSDMB was adsorbed onto the surface of HA-based nanocapsules by a physical procedure. The process was firstly optimized for the HA NCs using 0.25 and 0.75 mg/mL of the polymer. First, 150 µL of HA NCs (corresponding to 10 mg) were incubated with

2.5, 5, 10 and 25 μg of anti-GSDMB per mg of nanocapsules, at 4°C under mild horizontal shaking (titramax 1000 platform shaker, Heidolph, Germany). The influence of anti-GSDMB's charge on its association to the nanocapsules was investigated for the original solution, at pH 7.4 (neutrally charged anti-GSDMB) or after protonating the mAb, at pH 4.5 (positively charged anti-GSDMB). Protonated anti-GSDMB (anti-GSDMB+) was prepared by acidification with sodium acetate/ acetic acid buffer solution (initial pH 3.8) until a final pH of 4.5.

Anti-GSDMB and protonated anti-GSDMB+ were associated to the surface of C12-HA NCs using the same methodology.

The pH of blank nanocapsules, anti-GSDMB and anti-GSDMB+, alone or after association to both types of HA-based nanocapsules were assessed using a pH meter (Docu-pH_{Meter}, Sartorius, Germany). Anti-GSDMB was concentrated using an antibody concentration kit (Abcam, UK) and associated to C12-HA NCs formulated with 1 mg/mL of C12-HA as described before.

2.4.1 Characterization of anti-GSDMB-loaded HA-based NCs

The size, polydispersion index (PDI) and surface charge of HA-based nanocapsules and anti-GSDMB coated HA NCs and C12-HA NCs were measured using dynamic light scattering (DLS) and laser Doppler anemometry (Nano-ZS instrument, Malvern, UK). Scanning electron microscopy (SEM) (EVO LS15 microscope, ZEISS, Germany) was utilized to obtain high-resolution images of blank and anti-GSDMB coated HA NCs.

2.4.2 Antibody association efficiency

The association efficiency of anti-GSDMB to HA NCs and C12-HA NCs was evaluated by ultra-filtration using Nanosep[®]300K membranes (Pall Corporation, Spain). 250 μL of anti-GSDMB-loaded nanocapsules were placed into the filter and centrifuged 4 min at 14,000 g and 4°C. The filtrate containing the free antibody was taken and analyzed by ELISA as previously described. The association efficiency was calculated as: $(T-F)/T \times 100$, where T is the total anti-GSDMB and F the free anti-GSDMB.

2.4.3 Stability of anti-GSDMB-loaded C12-HA NCs in human plasma

Blank C12-HA NCs and anti-GSDMB-loaded C12-HA NCs were diluted 1:10 (v/v) in human plasma for a period of 24h, at 37°C. At predetermined time intervals (0, 2, 4, 8 and 24h), samples were taken and particle size evaluated as described above.

2.5 In vitro cell assays

2.5.1 Cell culture

The HCC1954 cell line, endogenously expressing GSDMB, was obtained from the American Type Cell Culture (ATCC) (LGC Standards-SLU). Cells were cultured and authenticated using STR-profiling according to ATCC guidelines. Cells were maintained as monolayer cultures at 37°C in an atmosphere with 5% CO₂.

2.5.2 In vitro cytotoxicity assays

10x10³ cells were grown into 96-wells plate in 200 µL culture medium. Two experiments were performed: (i) after 24h, medium was replaced by fresh medium containing different concentrations (0.05 – 1 mg/mL) of each formulation and cells were incubated for 72h at 37°C, and (ii) after 24h, serial assays were performed with C12-HA NCs at different concentrations and incubation times at 37°C (**Table 1**). After 72h, cell proliferation was performed in order to test the cytotoxic effect of nanocapsules using the AlamarBlue® assay according to the manufacturer specifications. The data was fitted to a sigmoidal dose-response curve (variable slope) using GraphPad Prism (GraphPad Software).

2.5.3 Cell Immunofluorescence studies

For the microscopy analysis, cells were seeded on sterile glass coverslips in culture medium. Twenty-four hours after seeding, cells were incubated at 37°C for the periods and concentrations indicated with empty C12-HA NCs, anti-GSDMB alone or anti-GSDMB associated to C12-HA NCs. After incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. The coverslips were then incubated with the

appropriate primary antibody to intracellular organelles for 90 min at room temperature, followed by a secondary antibody for 45 min at room temperature. Primary antibodies were: rabbit anti-EEA1 (1:100) and rabbit anti-LAMP2 (1:100). Secondary antibodies were goat anti-mouse (1:1000) and anti-rabbit (1:1000) conjugated with Alexa-488, Alexa-594 or Alexa-647. Alexa-647-coupled phalloidin was used to stain F-actin and cell nuclei were stained using DAPI. After staining, coverslips were mounted in Prolong Gold Antifade (Molecular Probes®). Images were collected by confocal microscopy (Zeiss).

Table 1. Serial incubation steps of C12-HA NCs with HCC1954 breast cancer cells. At t0h, cells were incubated with C12-HA NCs and after 4h, 24h or 48h of incubation, medium was replaced by fresh medium or fresh medium containing C12-HA NCs at the specified concentration. Cell cytotoxicity was measured after 72h.

Serial incubation steps – C12-HA NCs					
time (h)					
Experiment	t0h	t24h	t48h	t72h	Final conc. (mg/mL)
E1	Add NCs 0.8 mg/mL	-----	-----	Measure	0.8
E2	Add NCs 0.8 mg/mL	-----	Replace medium	Measure	0.8
E3	Add NCs 0.8 mg/mL	Replace medium	-----	Measure	0.8
E4	Add NCs 0.8 mg/mL	Replace medium Add NCs 0.8 mg/mL	-----	Measure	1.6
E5	Add NCs 0.8 mg/mL	Replace medium Add NCs 0.8 mg/mL	Replace medium Add NCs 0.8 mg/mL	Measure	2.4
E6	Add NCs 0.8 mg/mL Replace medium 4h	Add NCs 0.8 mg/mL Replace medium 4h	Add NCs 0.8 mg/mL Replace medium 4h	Measure	2.4
E7	Add NCs 0.4 mg/mL	Replace medium Add NCs 0.4 mg/mL	-----	Measure	0.8
E8	Add NCs 0.26 mg/mL	Replace medium Add NCs 0.26 mg/mL	Replace medium Add NCs 0.26 mg/mL	Measure	0.8

2.5.4 Wound healing migration assay

For the migration capacity of cells, a wound healing assay was performed as previously described by Hergueta-Redondo et al [12]. Briefly, 10^4 cells were seeded in 6-well plates and maintained until 90-100% confluence. Subsequently, the artificial wounds were created on the confluent cell monolayer using 10 μ L pipette tips, and the detached cells were removed by washing twice with PBS. The media was then replaced with empty

C12-HA NCs (control) and anti-GSDMB-loaded C12-HA NCs at concentrations of 3.2 mg/mL of nanocapsules (corresponding to 80 µg of anti-GSDMB), for 2h and diluted in RPMI 1640 medium. Cells were cultured at 37°C/5% CO₂. Wound closure was monitored and photographed at time points 0, 24, 48 y 60h under an inverted microscope (Axio Vert, Zeiss).

3. Results and discussion

Up to now, a significant number of drug nanocarriers have been functionalized with mAbs with the final goal of targeting cancer cells that overexpress in their surface a receptor for the selected mAb [3]. The novelty of this work relies on the fact that the selected mAb, the anti-GSDMB, is not simply a targeting ligand, but a therapeutic entity. In addition, contrary to the mAb therapies developed so far, the targeted protein is not expressed on the surface of a cancer cell, but it is confined to the cytosol. The explanation for the lack of therapies based on mAbs targeting intracellular proteins is related to the fact that mAbs are not able to cross the cell membrane unless they have a surface receptor. Therefore, the idea of developing a nanocarrier as a platform for the intracellular delivery of mAbs is a challenging but promising strategy. The criteria for the design of such delivery technology was defined taking into account the expected properties for these nanocarriers, namely (i) stability in plasma; (ii) affinity towards the cancer cells; (iii) capacity to load mAbs and (iv) capacity to enter the cancer cells and release the mAb while preventing the potential lysosomal degradation. Our lab and others have already shown that HA-based nanocarriers fulfill the first two properties [11–15], however no one has shown the possibility to achieve the intracellular delivery of a mAb. In the next sections we will describe the necessary steps to achieve this goal. **Figure 1** illustrates the potential of HA-based nanocapsules described here as a technology to promote the delivery of a mAb, anti-GSDMB, into the intracellular compartment of HCC1954 breast cancer cells.

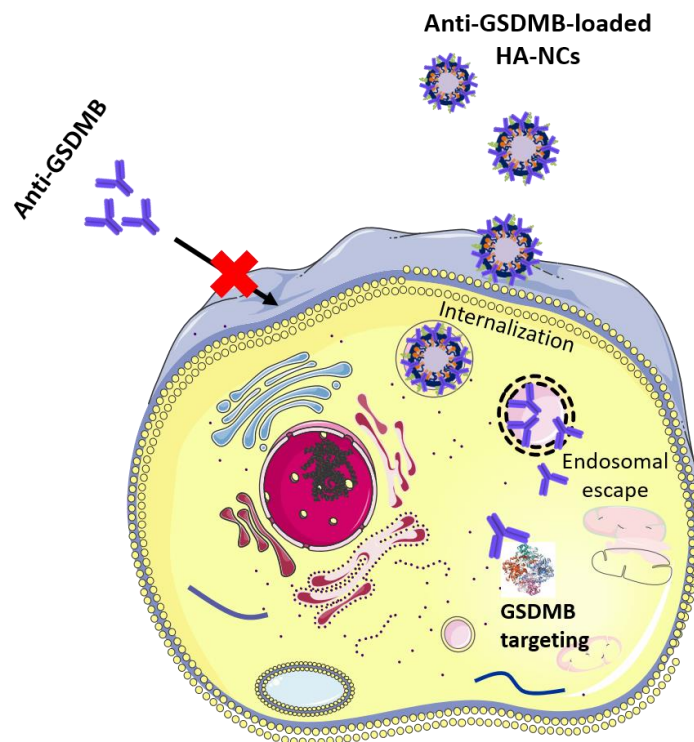


Figure 1. Intracellular transport of anti-GSDMB delivered by HA-based nanocapsules.

3.1 Characterization of anti-GSDMB

The concentration of anti-GSDMB was determined by nanodrop as 2.9 mg/mL.

The purity and integrity of anti-GSDMB was evaluated by SDS-PAGE under non-reducing and reducing conditions. Using this technique, intact antibodies must present a single band while denatured fragments two bands, the constant fraction (Fc) and the variable fraction (Fab), at 50 kDa and 25 kDa respectively [16]. **Figure 2** shows the stained gel containing the different bands. Lane 9 represents a molecular weight marker, lane 1 and 2 anti-GSDMB under non-reduced conditions at 20 and 40 $\mu\text{g/mL}$, lane 3 to 6 anti-GSDMB fragments after denaturing 5, 7, 10 and 12 min and lanes 7 and 8 the commercial DAKO horseradish peroxidase (HRP) antibody under non-reducing and reducing conditions, respectively. Under non-reducing conditions we observed the band of the whole antibody at 160 kDa as well as unspecific bands analogous to some impurities and protein crumbling. Protein fragments on non-reducing SDS-PAGE are a common feature and have been related to the breakage of inter chain disulfide bonds during sample preparation [17]. At reducing conditions, antibody fragmentation was confirmed by the presence of two bands corresponding to IgG fragments and some additional impurity

bands. The separation profile was the same regardless the boiling time. As a control, DAKO HRP IgG showed the same structural profile as anti-GSDMB for non-reducing and reducing conditions with an additional band corresponding to the HRP chain conjugated to the antibody. This study confirms the stability and integrity of anti-GSDMB and the presence of non-specific impurities lie behind normal method production and purification techniques [18].

The binding affinity of anti-GSDMB towards the GSDMB antigen was analyzed by ELISA. This assay was performed in order to assure the specificity of anti-GSDMB against the target protein, as well as to validate a method to quantify the amount of anti-GSDMB associated to HA NCs. A sigmoidal calibration curve was obtained in a concentration range from 0.125 to 10 $\mu\text{g/mL}$ and $r^2= 0.9969$ (results not shown).

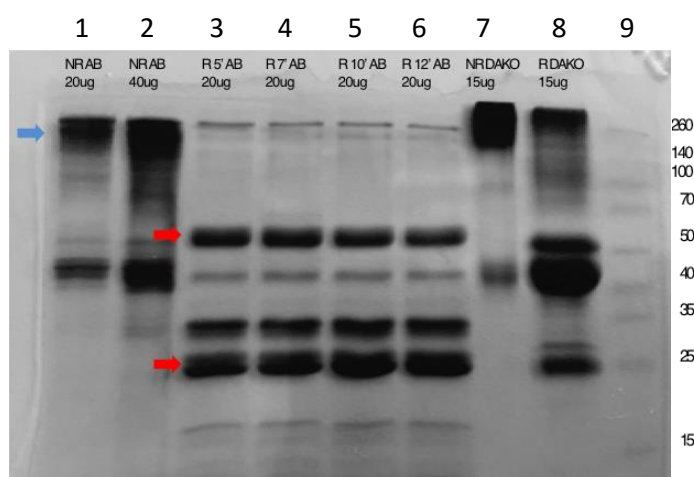


Figure 2. SDS-PAGE gel electrophoresis. Lanes 1 and 2, anti-GSDMB under non-reducing conditions at 20 and 40 $\mu\text{g/mL}$; lanes 3 to 6, anti-GSDMB under reducing conditions after boiling 5, 7, 10 and 12 min; lanes 7 and 8, control DAKO HRP IgG under non-reducing and reducing conditions, respectively; lane 9, MW marker (KDa). Blue arrow marks intact anti-GSDMB band and red arrows the Fc (50KDa) and Fab (25KDa) fractions

3.2 Development of HA-based nanocapsules containing anti-GSDMB

Nanocapsules made of sodium hyaluronate HA (HA NCs) or dodecylamide-functionalized HA (C12-HA NCs) were developed using a self-emulsification technique. These nanocapsules are constituted by an oily core composed of Miglyol[®]812 and Tween[®]80 and a shell made of Solutol[®]HS15 and HA or C12-HA. Also, the NCs formulated with HA have the cationic surfactant, CTAB, in the oil core. The self-

emulsification was achieved thanks to the addition of the oil phase composed of Miglyol®812 and Tween®80 (with or without CTAB at 0.15 mg/mL) in a ratio 1:1 (w/w) to the aqueous phase composed of Solutol®HS15 at 2.5 mg/mL and HA or C12-HA at concentrations between 0.25 and 1 mg/mL. The oil phase was added to the aqueous phase in a ratio 1:8 (v/v). The nanocapsules formed a homogeneous population ($PDI \leq 0.2$) with a mean size around 130 nm and a negative zeta potential (-20 mV). Both the small size and the negative zeta potential, together with the hydrophilicity of the polymeric shell, are attractive properties for preserving the stability of the nanocapsules in the blood stream and achieve a passive targeting to the tumor [18–19].

The association of anti-GSDMB to the surface of HA-based nanocapsules was done by physical adsorption by controlling the appropriate charge and hydrophobicity of the mAb and, hence, its ionic or hydrophobic interaction with the different components of the nanocapsules. The major advantages of using physical adsorption rather than chemical conjugation [21] relies on the use of a mild and easy process, without the need of aggressive reagents, which preserves the antibody activity [21–22]. Although the majority of publications refer to antibody-conjugated nanocarriers, the adsorption of antibodies to the surface of nanoparticles is a common approach successfully described in different studies [11, 21, 23–24].

3.2.1 Association of Anti-GSDMB to HA nanocapsules

Considering the negative charge of HA NCs, anti-GSDMB was protonated and associated to their surface by ionic interactions. Anti-GSDMB has an isoelectric point (pI) between 6.5 and 8.1, and therefore becomes positively charged at pH values below 6.5. Cationic anti-GSDMB (anti-GSDMB⁺) was prepared by acidifying the medium with a 15% (v/v) sodium acetate/ acetic acid buffer (pH 3.8) [24]. After acidification, the pH of the mAb solution changed from 7.4 to 4.5 and its zeta potential switched to a positive value ($+3$ mV) without changing its binding affinity to the GSDMB antigen, as tested by ELISA. (Results not shown).

In order to identify the most adequate association conditions, we prepared different formulations by associating the protonated mAb to the surface of nanocapsules using different ratios HA: anti-GSDMB⁺. Based on this, HA NCs prepared with 0.25 and

0.75mg/mL of HA, were incubated with increasing amounts of protonated anti-GSDMB+ (2.5, 5, 10 and 25 μg anti-GSDMB+/mg nanocapsules). The results present in **Table 2** show that, irrespective of the amount of antibody and HA, the association of anti-GSDMB+ to the nanocapsules did not have an influence on their size, which remained very similar to the one of blank nanocapsules. On the other hand, no aggregation of the nanocapsules was observed due to the association of the mAb [26].

Table 2. Influence of the concentration of hyaluronic acid (HA) and protonated anti-GSDMB+ on the size of nanoparticles.

Conc. HA (mg/mL)	μg anti-GSDMB+/ mg NCs	HA: anti-GSDMB+ ratio (w/w)	Size (nm)
0.25	Blank	-	115 \pm 3
	2.5	1.5 : 1	118 \pm 4
	5	1 : 1.3	115 \pm 6
	10	1 : 2.7	120 \pm 4
	25	1 : 6.7	119 \pm 5
0.75	Blank	-	110 \pm 1
	25	4.5 : 1	123 \pm 13
	5	2.25 : 1	122 \pm 14
	10	1.25 : 1	120 \pm 13
	25	1 : 2.2	117 \pm 6

Notes: Results are presented as mean value \pm standard deviation (n=3)

On the contrary, a significant change was observed in the charge of the particles. The zeta potential of the nanocapsules changed from highly negative to neutral values, as a consequence of increasing amounts of mAb associated to their surface (**Figure 3**). These results represent an evidence of the effective interaction between cationic anti-GSDMB+ and the negative HA. As expected, charge neutralization was more evident for the nanocapsules containing a lower amount of HA. On the other hand, the fact that the zeta potential remained negative indicates the prevalence of the polymer on the surface of the nanostructure under the tested conditions. Based on these results HA NCs prepared with 0.75 mg/mL of HA were used for further experiments.

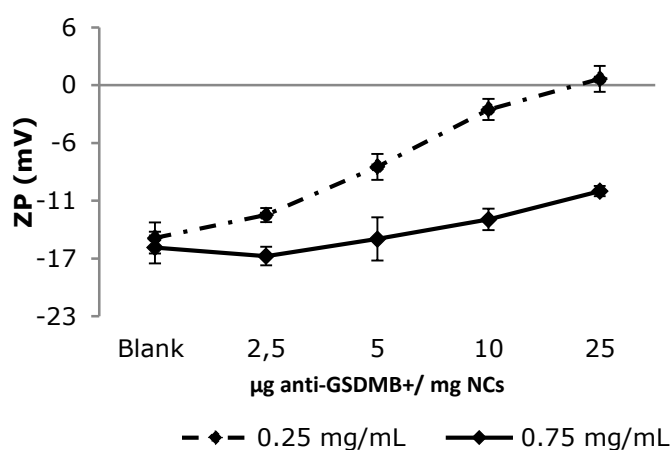


Figure 3. Zeta potential of anti-GSDMB-loaded HA NCs. HA NCs were prepared with 0.25 and 0.75 mg/mL of HA and associated with increased amounts anti-GSDMB+.

Notes: Results are presented as mean value \pm standard deviation (n=3).

Figure 4 illustrates the morphology and structure of HA NCs and anti-GSDMB+ loaded HA NCs. The image shows that the nanocapsules' size was uniform (≤ 200 nm), their morphology was spherical and, apparently, presented the typical reservoir-type structure (**Figure 4A**). **Figure 4B** shows the association of white dots around the nanocapsules loaded with the mAb, whereas these dots do not appear in blank formulations. As such, we hypothesized that these dots could represent the entrapment of anti-GSDMB into the polymeric shell [27].

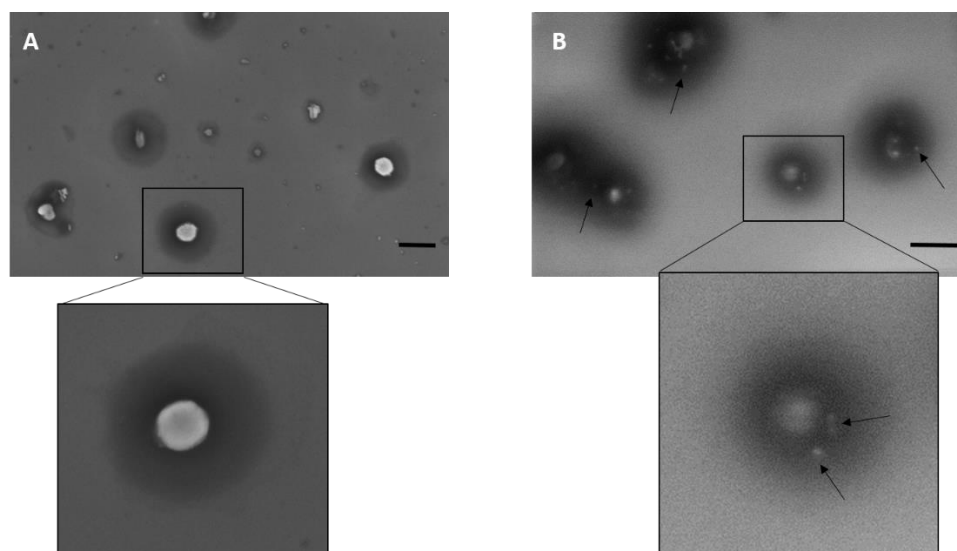


Figure 4. Scanning electron microscopy images. **A)** Empty HA NCs. **B)** anti-GSDMB+ coated HA NCs. Scale Bar=200nm.

Besides the electrostatic interaction indicated above, we also speculated that hydrophobic forces might play an important role in the association of the mAb to the nanocapsules [26]. In order to validate this hypothesis, we studied the association of anti-GSDMB in its neutral form to HA NCs. Upon this addition, the pH of anti-GSDMB-loaded nanocapsules was 7.4. At this pH, the mAb is neutral and its water solubility is reduced, which favored its hydrophobicity. In these conditions, neither the size or the zeta potential were significantly modified upon association of the mAb.

In order to better understand the mechanism of interaction between anti-GSDMB and HA NCs, we determined the association efficiency of anti-GSDMB, either protonated (pH 4.5) or in its neutral form (pH 7.4), to the nanocapsules. Interestingly, the association efficiency for all prototypes was around 80%. Consequently, these results indicate that the association of anti-GSDMB to the nanocapsules may occur by both, electrostatic and hydrophobic interactions. The electrostatic interaction between cationic antibodies and negatively charged particles has been described before. For example, cationic SMF-1 single-chain antibody was effectively adsorbed onto the surface of negatively charged poly(lactide-co-glycolic acid) (PLGA) nanoparticles [25]. On the other hand, it was also reported the adsorption of proteins in the pH region close to the pI. It was described that at pH values closer to the pI, protein molecules are neutrally charged and must attain a closer packing at the surface of particles than when carrying a net charged [28].

3.2.2 Association of anti-GSDMB to C12-HA nanocapsules

The HA NCs described in the previous section were formed based on the interaction between the cationic surfactant CTAB and the HA shell. As an alternative and in order to avoid the use of the cationic surfactant, nanocapsules were produced using dodecylamide-functionalized HA (C12-HA). Anti-GSDMB was associated onto the surface of C12-HA NCs prepared with 0.5 mg/mL of C12-HA. The effect of anti-GSDMB charge (protonated or neutral) on the association efficiency and physicochemical properties of the nanocapsules was also investigated using the formulation with 1:3.3 ratio of HA: anti-GSDMB.

Table 3. Physicochemical characterization and association efficiency of anti-GSDMB-loaded C12-HA NCs.

Formulation	pH	C12-HA: anti-GSDMB ratio (w/w)	Size (nm)	PDI	ZP (mV)	Association efficiency (%)
anti-GSDMB+	4.5	1 : 3.3	119 ± 3	0.2	-6 ± 1	84 ± 8
anti-GSDMB	7.4		116 ± 6	0.2	-10 ± 1	93 ± 3

Notes: Results are presented as mean value ± standard deviation (n=3)

Abbreviations: PDI, polydispersity index; ZP, zeta potential

The results expressed in **Table 3** show that the association of anti-GSDMB, either protonated (pH 4.5) or neutral (pH 7.4) to C12-HA NCs did not change its physicochemical properties. Moreover, a high association efficiency was observed for both systems. The high entrapment of neutral anti-GSDMB on C12-HA NCs can also be explained by the favorable affinity of uncharged peptides to the hydrophobic interphase, composed by the lipidic HA-chain and the surfactant layer [29].

In another experiment, the entrapment of concentrated anti-GSDMB (125µg mAb/ mg nanocapsules) was successfully achieved using 1 mg/mL of C12-HA (HA: anti-GSDMB ratio 1:8 w/w). This system had a mean size of 140 nm, a -10 mV surface charge and an association efficiency around 90%.

Although “at priori”, the hydrophilic HA shell might be thought to prevent the association of the neutral mAb, we have speculated the attachment of the mAb around

the polymeric chain based on three main reasons: (i) the 3D structure of the nanocapsule made possible the entrapment of the mAb, with the hydrophobic domain attached to the surfactant interface, especially when this interaction occurs at neutral pH [30]; (ii) the hydroxyl-carboxyl groups presented in the polymeric structure are important for protein adsorption by hydrogen bonds [28] and; (iii) the presence of PEG groups conferred by the Solutol®HS15 are not long enough to prevent anti-GSDMB adsorption [31].

The stability of blank C12-HA NCs and anti-GSDMB-loaded C12-HA NCs (25µg of anti-GSDMB/mg of NCs prepared with 0.5 mg/mL of C12-HA) was evaluated in human plasma at 37°C for up to 24h. We observed an increase in the particle size of both blank C12-HA NCs and anti-GSDMB-loaded C12-HA NCs less than 20% of the initial size while the PDI was maintained below 0.2, denoting the absence of aggregates (**Figure 5**). The increase in the nanocapsules size might be related to the deposition of plasma proteins around the nanocapsules shell without affecting its delivery properties [32].

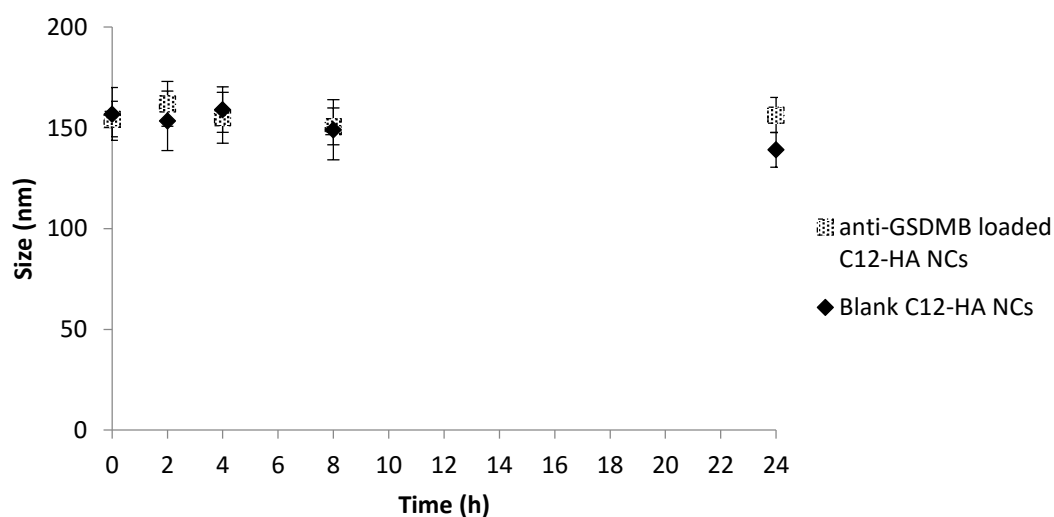


Figure 5. Size distribution of C12-HA NCs and anti-GSDMB-loaded C12-HA NCs incubated with human plasma, at 37°C for 24h.

Notes: Results are presented as mean value \pm standard deviation (n=3)

In order to study the influence of HA-based nanocapsules in the intracellular delivery of anti-GSDMB, the first step was the assessment of the *in vitro* cytotoxicity of HA NCs and C12-HA NCs in HCC1954 cells.

3.3 *In vitro* cell toxicity assays in HCC1954 breast carcinoma cell line

The cytotoxicity of blank HA NCs and C12-HA NCs was measured using the cell viability AlamarBlue® assay in HCC1954 breast carcinoma cell line (**Figure 6**). This cell line was treated with increased concentrations of nanocapsules during 72h. The results showed a marked cytotoxicity for HA NCs, with a decrease of 50% in cell viability (IC50) around 0.3 mg/mL. Contrarily, C12-HA NCs exhibited a low toxicity at concentrations as high as 0.8 mg/mL. These results were in agreement with our previous studies where the high toxicity of HA NCs was associated to the presence of the cationic surfactant, CTAB [33]. As such, C12-HA NCs were chosen as the leading formulation for future experiments.

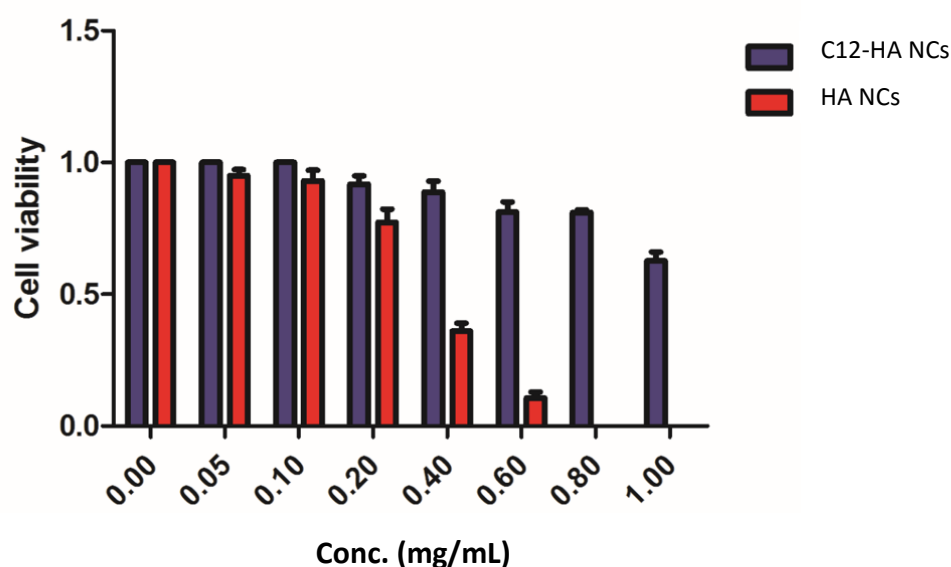


Figure 6. Cytotoxicity of HA NCs and C12-HA NCs after 72h of incubation with HCC1954 cells.
Notes: Results presented as mean value \pm standard deviation (n=6)

In a second set of experiments, we studied how the time of exposure influenced the cytotoxicity of nanocapsules. For this, we incubated the cells with C12-HA NCs according to the specifications described in **Table 1** (materials and methods section). The cytotoxicity of each serial treatment was evaluated in HCC1954 cells using the AlamarBlue® assay. Supporting previous cell viability studies (**Figure 6**), a high cellular

toxicity was achieved for 0.8 mg/mL of C12-HA NCs when the incubation time was superior to 24h (E1-E2) and to consecutive incubations of 0.8 mg/mL of C12-HA NCs after 24h and 48h (E4-E5) (**Figure 7**). On the other hand, an absence of toxicity was observed for a final concentration of nanocapsules at 0.8 mg/mL when cells were incubated at t0h and t24h with 0.4 mg/mL or at t0h, t24h and t48h with 0.26 mg/mL of C12-HA NCs and the medium replaced each 24h (E7-E8). Importantly, a higher final concentration of 2.4 mg/mL was possible to achieve without affecting cell viability when cells were incubated every day with 0.8 mg/mL of C12-HA NCs for 4h and the medium replaced by fresh medium until the next incubation step at t24h and t48h (E6). As a result, next experiments were performed using short incubation times, like the E6 experiment.

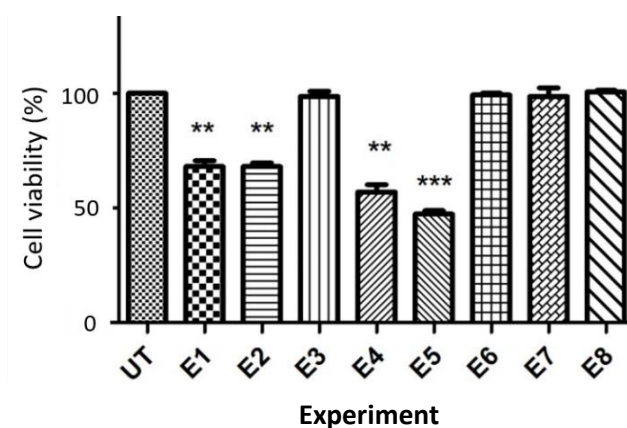


Figure 7. Cytotoxicity of C12-HA NCs under different serial incubations steps (E1 to E8). HCCT1954 were incubated with increased concentrations of C12-HA NCs at different time points and analyzed after 72h. Untreated cells (UT) represent the negative control, where cells were incubated with only fresh medium every day. In E1, E2 and E3, cells were incubated with 0.8 mg/mL of C12-HA NCs at time t0h and medium was replaced by fresh medium at time t72h, t48h and t24h, respectively. In E4 and E5, cells were incubated at time t0h with 0.8 mg/mL with C12-HA NCs and medium was replaced by fresh medium containing 0.8 mg/mL of NCs at t48 or t48h and t72h, respectively. In E6, cells were incubated with 0.8 mg/mL of C12-HA NCs at t0h, t24h and t48h; after 4h of incubation medium was replaced by fresh medium until the next incubation step. In E7, cells were incubated with 0.4 mg/mL of C12-HA NCs at time t0h and t24h and in E8, cells were incubated with 0.26 mg/mL of C12-HA NCs at time t0h, t24h and t48h.

Notes: UT means untreated cells and were used as negative control. **0.001<p<0.05; ***p<0.001 are significantly different from negative control. Results presented as mean value \pm standard deviation (n=6)

The intracellular delivery of anti-GSDMB was analyzed for three different prototypes: neutral anti-GSDMB and protonated anti-GSDMB+ associated to C12-HA NCs (0.5 mg/mL of C12-HA) both at 25 μ g mAb/mg NCs, and the concentrated prototype, anti-GSDMB-loaded C12-HA NCs (1mg/mL of C12-HA) using 125 μ g mAb/mg NCs.

3.4 Internalization of anti-GSDMB associated to C12-HA nanocapsules in HCC1954 breast cancer cells

As previously indicated, the major goal of this study was the design of a nanocarrier with the capacity to deliver mAbs at the intracellular level. The internalization of neutral anti-GSDMB (pH 7.4) associated to C12-HA NCs into HCC1954 cells was visualized by confocal microscopy (**Figure 8**). For this, cells were incubated with 0.8 mg/mL of C12-HA NCs, which corresponds to 20 μ g of anti-GSDMB, at different time points, 1, 2 and 3h. Blank C12-HA NCs and anti-GSDMB alone were used as negative controls (**Figure 8 A**). After incubation, cells were washed with PBS, permeabilized with Triton and anti-GSDMB visualized after being labeled with a secondary antibody conjugated with an Alexa-Fluor. The results showed that, as expected, anti-GSDMB on its own was not able to cross the cell membrane due to its high molecular weight and hydrophilicity [34]. On the contrary, C12-HA NCs were capable to penetrate the cell membrane and to carry the anti-GSDMB to the intracellular compartment of HCC1954 cells (**Figure 8 B**). Although 1h of exposition was enough to observe the mAb inside the cell, 2h were considered as the best exposition time to get the high internalization without exhibiting any toxicity.

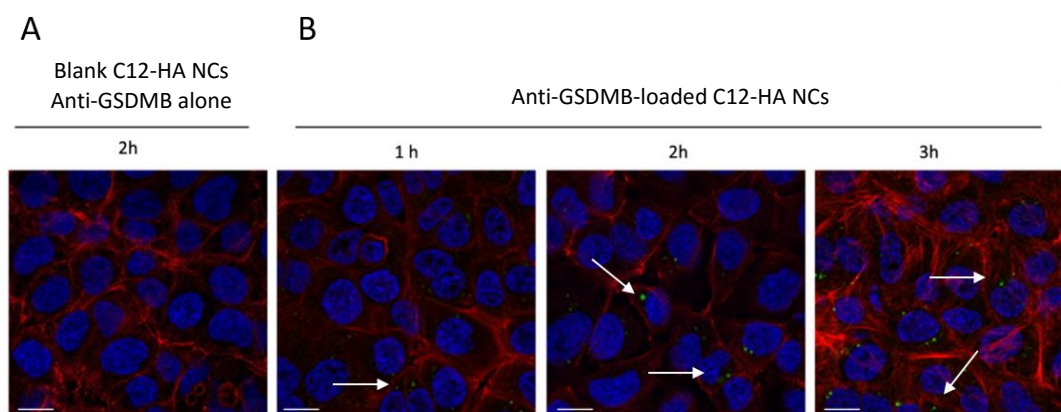


Figure 8. Cellular uptake of anti-GSDMB (green dots) in HCC1954 after 1, 2 and 3h of incubation. **A)** Anti-GSDMB alone and blank C12-HA NCs were used as controls. Without a cell surface receptor, the anti-GSDMB was not able to cross the cell membrane. **B)** Anti-GSDMB associated to C12-HA NCs permeated the cell membrane and was internalized inside HCC1954 cells.

Notes: Scale bar = 20 μ m. Cell nuclei were stained with DAPI (blue) and cell membrane with phalloidin (red).

Additionally, cell uptake assays were performed for protonated anti-GSDMB+ loaded C12-HA NCs and for C12-HA NCs carrying concentrated anti-GSDMB. This experiment was performed to understand if the supposed electrostatic or hydrophobic association of anti-GSDMB to the nanocapsules could impact their internalization by the cancer cells (**Figure 9 A**). Additionally, the influence of anti-GSDMB loading on the internalization mechanism was assessed (**Figure 9 C**). The selected prototypes were incubated in HCC1954 cells for 2h with 0.8 mg/mL of nanocapsules, which did not affect cell viability, and the internalization of anti-GSDMB was visualized by confocal microscopy as described before (**Figure 9**).

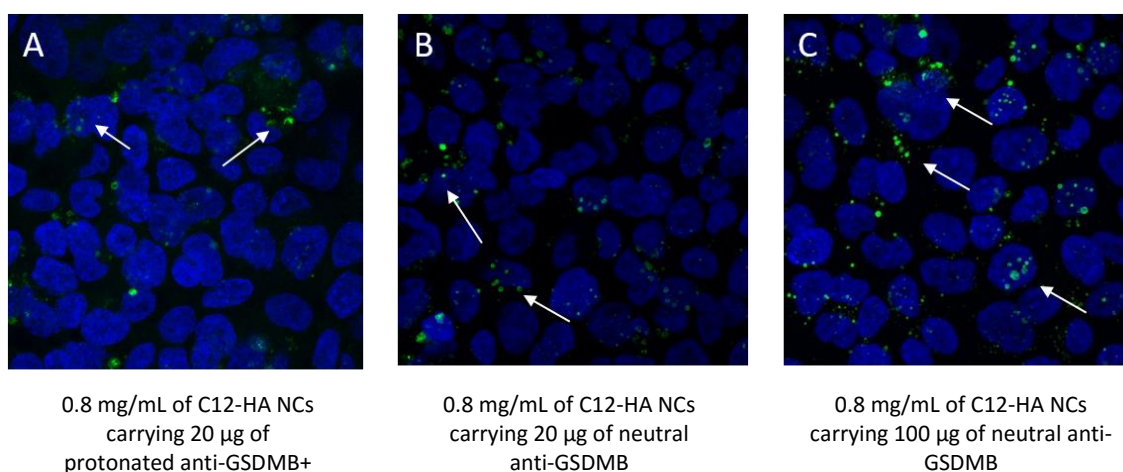


Figure 9. Internalization of anti-GSDMB (green) into HCC1954 cells carried by the different prototypes: **A)** 0.8 mg/mL of C12-HA NCs containing 20 µg of protonated anti-GSDMB+; **B)** 0.8 mg/mL of C12-HA NCs with 20 µg of neutral anti-GSDMB and **C)** 0.8 mg/mL of C12-amide HA-NCs with 100 µg of anti-GSDMB. Cellular uptake was performed for 2h of incubation.
Notes: Cell nuclei were stained with DAPI (blue).

The internalization behavior of protonated anti-GSDMB+ was similar to that of the neutral anti-GSDMB when associated to C12-HA NCs. From these results, we may conclude that the mechanism of interaction between the mAb and the nanocarrier, either through electrostatic or hydrophobic interactions, did not have an apparent impact on the internalization. On the other hand, the image in **Figure 9 C**, indicates that the amount of green dots was higher for the internalization of greater amounts of anti-GSDMB (0.8 mg/mL of C12-HA NCs carrying 100 µg of anti-GSDMB).

Based on these results, further experiments were done using C12-HA NCs carrying anti-GSDMB.

3.5 Intracellular trafficking and endosomal co-localization of anti-GSDMB in HCC1954 breast cancer cells

The analysis of the intracellular trafficking was performed in HCC1954 cells in order to determine the mechanism of uptake of the nanocapsules, as well as their potential co-localization within the endosomes and their endosomal escape. These assays were performed with 3.2 mg/mL of C12-HA NCs containing 80 µg of anti-GSDMB. This high dose was used to ensure the correct visualization of the antibody and the endosomes. The incubation time was reduced to 2h, which did not lead to any damage of the cells. **Figure 10** illustrates the trafficking of anti-GSDMB (green) through **(A)** early endosomes (red) and **(B)** late endosomes (yellow), as visualized by confocal microscopy. The observed co-localization of anti-GSDMB with the early endosome marker, EEA1 (**Figure 10 A**) indicates that the nanocapsules are taken up by endocytosis. This endocytic pathway might be mediated by the interaction with CD44 receptors [35–37] over-expressed on the surface of HCC1954 cells [37]. Alternatively, nanoparticles uptake could be mediated by a non-specific mechanism of adsorption onto the cell surface [39–40] as well as by the penetration enhancing nature of some of the compounds of the nanocarrier. For example, it was described that nanoparticles formulated with Tween®80 presented a higher cellular uptake than plain nanoparticles [41–42], which was associated to the capacity of Tween®80 to enhance cell permeability [43–44].

Once the endocytic mechanism of transport was visualized, it was important to ensure that the mAb was not degraded inside the endosomes and, thus, the endosomal escape of anti-GSDMB was investigated by labeling late endosomes with LAMP1 (**Figure 10 B**). Interestingly, in this late stage, anti-GSDMB did not co-localize with LAMP1, which provided an evidence of the endosomal escape of the mAb. We hypothesized that this endosomal escape could be explained by two main reasons: (i) a pH dependent mechanism (like a proton-sponge effect) [44] and (ii) the influence of the anionic surfactant Tween®80 in destabilizing the endosomal membrane [45]. The first one, must be related to the protonation of anti-GSDMB inside the acidic endosomes, the loss of

the hydrophobic interactions between anti-GSDMB and the nanocapsules and the consequent destabilization of the endosomal membrane [46]. As indicated in section 3.2, anti-GSDMB-loaded C12-HA NCs have a pH of 7.4. At this pH, anti-GSDMB is neutral and its interaction with the nanocapsules must be mainly driven by hydrophobic interactions. When the pH drops to acidic values (pH 5-6), like in the endosomes, anti-GSDMB becomes ionized and its hydrophobic interaction with the nanocapsules structure gets weaker [47]. Once protonated, cationic anti-GSDMB⁺ can form ion pairs with anionic lipids within the endosome and destabilize the endosomal membrane [46]. These results are in accordance with those described for doxorubicin-loaded HA-Poly-L-Histidine (pHis)/ D- α -Tocopherol polyethylene glycol 1000 succinate (TPGS) mixed micelles that showed a pH dependent drug release due to the protonation of pHis, with the highest release occurring at endosomal pH [48]. On the other hand, Tween[®]80 might also be responsible for endosomal escape. It is described that this anionic surfactant can interact with the endosomal membrane, thus disrupting the organelle and breaking the endosome [45]. In another study, Tween[®]80 was compared with dioleoylphosphatidylethanolamine (DOPE), which is one of the most commonly used helper-lipids in facilitating DNA-escaping from endosomes. The Tween[®]80, having a high hydrophilic lipophilic balance (HLB = 15) can be solubilized in the cytosol and promote a lamellar-to-inverted hexagonal phase transition, which can disturb the endosome membrane and promote endosomal escape [49].

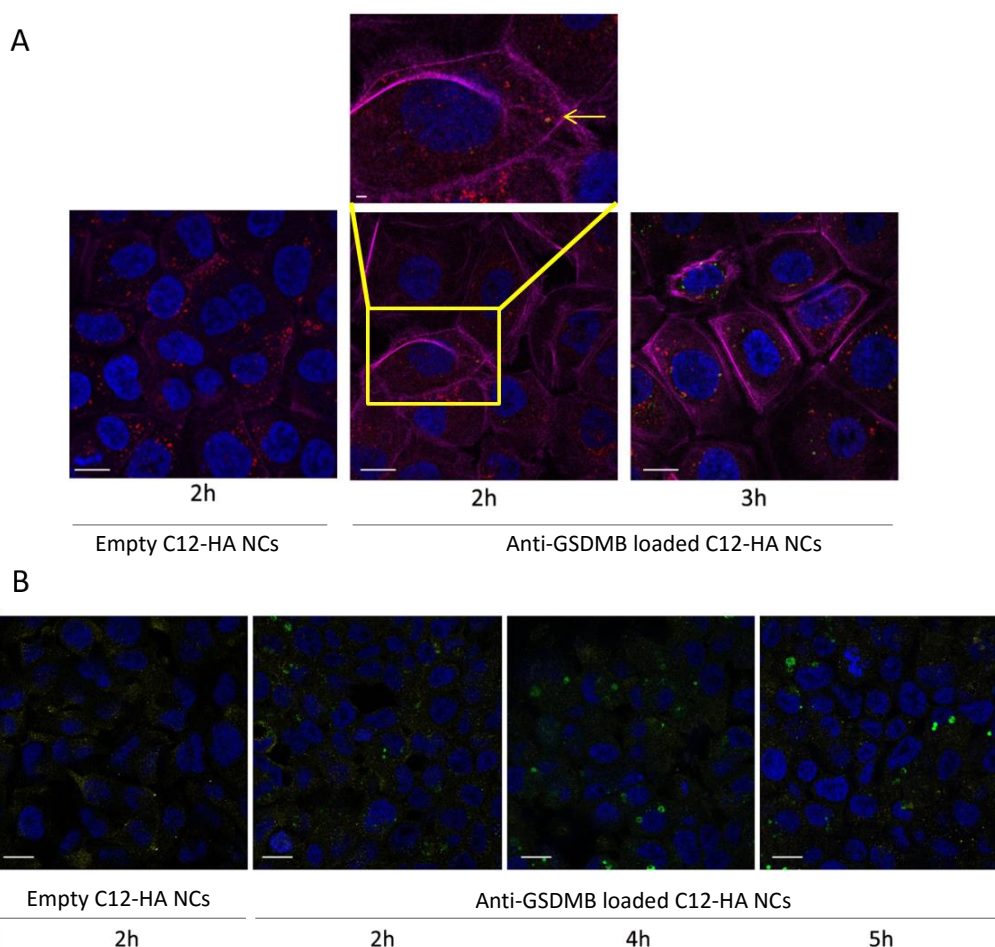


Figure 10. Trafficking of anti-GSDMB (green) through endosomes. HCC1954 cells were incubated with 3.2 mg/mL of C12-HA NCs with 80 μ g of anti-GSDMB for 2h, 3h, 4h and 5h. **A)** Early endosomes were detected by early endosome antigen 1 (EEA1) (red). **B)** Late endosomes were detected by lysosomal-associated membrane protein (LAMP1) (yellow).

Notes: Nuclei were stained with DAPI (blue) and the cytoskeleton with phalloidin (purple). Scale bar = 20 μ m.

3.6 Effect of anti-GSDMB-loaded C12-HA nanocapsules on the migration of HCC1954 cells

It is known that the overexpression of GSDMB is responsible for cell migration and invasion whereas its knockdown with shRNA results in a marked decrease of the migratory ability and the invasive capacity of HCC1954 cells [12]. Consequently, we performed wound healing assays in HCC1954 cells in order to assess the capacity of anti-GSDMB to block the GSDMB protein and inhibit its migration and invasive behavior (**Figure 11**). HCC1954 cells were incubated with empty C12-HA NCs (negative control) and C12-HA NCs carrying anti-GSDMB (3.2 mg/mL of NCs and 80 μ g of anti-GSDMB). The results in **Figure 11** show that cells treated with anti-GSDMB-loaded C12-HA NCs

Chapter 3

inhibited cell migration as compared to the negative control: after 60h, HCC1954 cells treated with empty NCs migrate and close the wound healing to 165 μm whereas, HCC1954 cells treated with anti-GSDMB-loaded C12-HA NCs maintain an opening heal of 291 μm , which prove the slow migration of cells. As such, once inside the cell, anti-GSDMB must maintain its original conformation and affinity to GSDMB, thus effectively blocking the protein and decrease its invasive behavior.

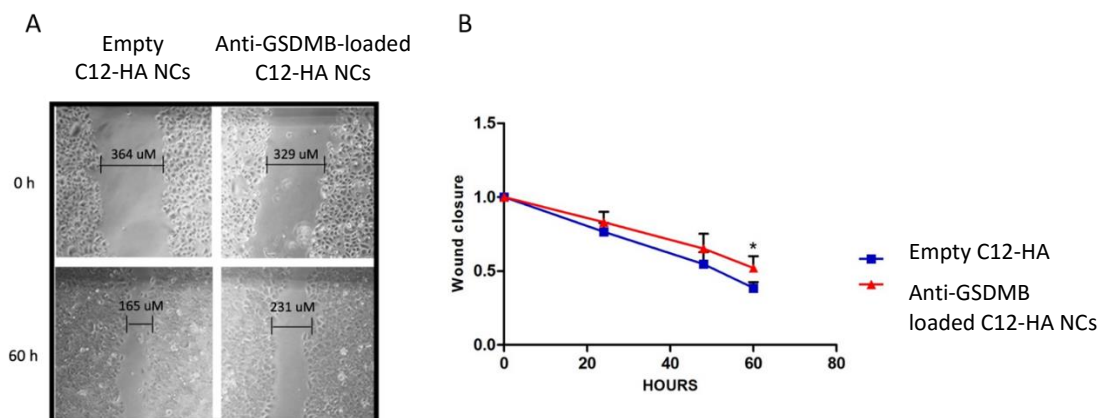


Figure 11. Effect of C12-HA NCs carrying anti-GSDMB on the migration of HCC1954 cells. **A)** Scratch wound healing assay was performed to examine the effect of C12-HA NCs entrapped anti-GSDMB on cell migration. **B)** Mobility rate histograms of each group across the selected times (hours). * $p < 0.05$

4. Conclusions

This work reports for the first time the use of HA-based nanocapsules as a platform for the intracellular delivery of a monoclonal antibody, anti-GSDMB. In this study we demonstrated that HA NCs or C12-HA NCs successfully associated the anti-GSDMB around the polymeric shell. Both systems resulted in high association efficiency of anti-GSDMB to the nanocapsules and allowed an incorporation of up to 25 μg of antibody per mg of NCs. Without a carrier, anti-GSDMB was incapable of penetrating the cell membrane. On the other hand, the association of anti-GSDMB to the nanocapsules resulted in its successful internalization into HCC1954 cells. Once inside the cell, anti-GSDMB escaped lysosomal degradation and maintained its original conformation to intracellularly target GSDMB. This interaction led to an effective inhibition of the protein, which resulted in a decrease of the migratory ability as well as the invasion

capacity of HCC1954 cancer cells. We hope that the association of anti-GSDMB to HA-based nanocapsules presents a new strategy for the delivery of antibodies against intracellular proteins that do not express a cell surface receptor. This approach has the potential to overcome the cell membrane barrier, and allowed the design of a platform to carry antibodies as therapeutic agents against intracellular proteins that are responsible for cancer invasion and progression.

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Overall discussion

Over the past decade, special attention has been paid towards the development of nanocarriers for anticancer drug delivery. These systems have been designed as an alternative to conventional chemotherapy and have resulted in more efficient and safe treatments [1]. Currently, the FDA has approved ten nanoparticle-based therapies in oncology and almost twenty are under clinical investigation [2].

Pushed by this innovation, polymeric nanocapsules have gained special attention as a delivery platform for cancer therapy [3]. Structurally, nanocapsules are vesicular systems with a typical core-corona architecture, consisting of an oily cavity surrounded by a polymeric coating, which confers several advantages for anticancer drug delivery [4]. First, the oil core is an ideal environment for the encapsulation of hydrophobic cytostatic drugs at high payloads and, secondly, the polymeric shell can be engineered with specific polymers in order to control drug release, improve the biodistribution profile and, ultimately, to enhance the tumor targeting ability of the nanocarrier [5]. Additionally, the polymeric shell can be designed to associate or entrap a variety of biomolecules, including peptides, proteins and polynucleotides, and to favor their intracellular delivery [6]. Nanocapsules should be designed with specified properties such as small size (100-200 nm), high stability and hydrophilic surface, which endows the system with appropriate characteristics for parenteral administration followed by long circulation time and enhanced accumulation into tumors [7].

In the present work, the technology and composition of nanocapsules was adapted to explore its potential as a multifunctional carrier to deliver conventional and complex biomolecules to cancer cells. The first step was the preparation of nanocapsules using a self-emulsification process, without organic solvents, heat or high energy input. By using this method, we aimed to find a compromise between an innovative formulation and the use of sustainable technologies [8]. The targeting capacity of nanocapsules was achieved by selecting hyaluronic acid (HA) as the coating agent. HA is a natural polysaccharide and is expected to carry the drug to the tumor tissue thanks to its recognition and binding affinity for CD44 receptors, overexpressed in many cancer cells [9–10]. Additionally, HA has a hydrophilic stealth character and a negative charge that may contribute to low protein adsorption and improved blood circulation time [11].

Overall discussion

Moreover, the chemical structure of HA makes possible its conjugation with other molecules [12]. In this work, we decided to explore the design of HA nanocapsules and a hydrophobically modified HA, the docecylamide functionalized HA (C12-HA), which is expected to provide some specific advantages. In detail, the use of a hydrophobically modified HA permits the formulation of HA-based nanocapsules by a self-assembly process, avoiding the use of a cationic surfactant, and therefore, leading to a decrease in the potential nanocarrier toxicity.

As a multifunctional drug carrier, HA nanocapsules were designed to encapsulate the cytostatic hydrophobic drug docetaxel into the oil core, and to associate the mAb anti-gasdermin B (anti-GSDMB) to the HA shell, for achieving an intracellular delivery. Small hydrophobic drugs, such as docetaxel, continue to be a challenge in nanomedicine. In fact, besides its toxic side effects, Taxotere® continues to be the only commercialized formulation for docetaxel. New delivery systems such as HA-based nanocapsules could improve the therapeutic efficacy of docetaxel while decreasing the cytotoxicity associated to the drug solvents used in the commercial formulation [13]. The development of mAbs has emerged as a potential therapy against cancer [14]. To date, mAbs have been designed to target cancer cell receptors and its application has been limited by their inability to reach proteins inside the cell. As therapeutic molecules, mAbs are considered too large and hydrophilic to cross the cell membrane on their own [15]. Likewise other biologic agents, the adequate delivery of mAbs could be achieved by the development of a carrier that overcomes these limitations. Accordingly, we have associated anti-GSDMB to the surface of HA nanocapsules and studied the potential of this platform for the intracellular delivery of mAbs.

In this study, self-emulsifying HA nanocapsules were designed as multifunctional nanocarriers for the delivery of different anticancer molecules, such as docetaxel and anti-GSDMB. **Figure 1** illustrates a summary of this work.

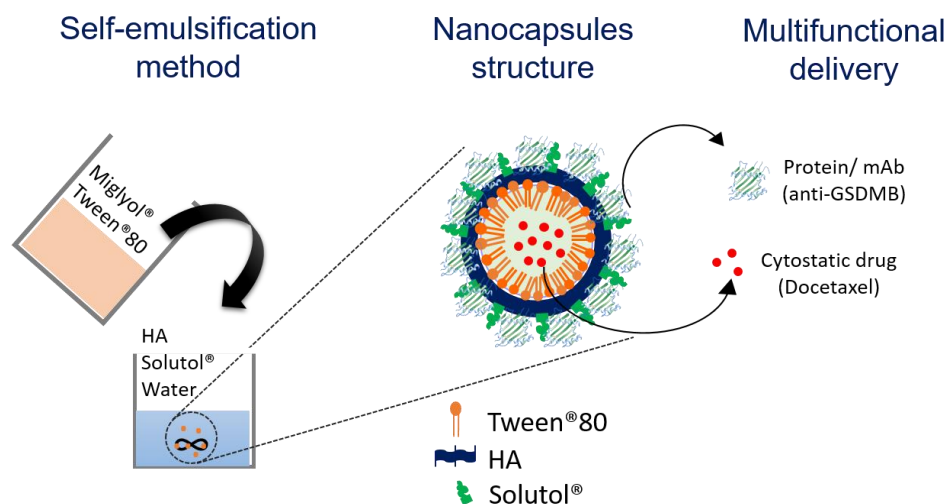


Figure 1. Schematic representation of the preparation of HA-based nanocapsules by self-emulsification and their structure as a multifunctional carrier for the delivery of cytostatic drugs and monoclonal antibodies.

1. The self-emulsification method as a green nanotechnology process

The idea of developing a “green nanotechnology” for formulation design started after we noticed that the pharmaceutical industry was getting more concerned about sustainability and the environmental impact of their drug discovery processes. Pharmaceutical companies are one of the largest users of organic solvents which impacts, for one side, the environmental “footprint” and, on the other hand, the production costs [16]. In an attempt to create sustainable methodologies, big pharmaceutical companies such as GlaxoSmithKline or Pfizer started a “green chemistry initiative” where they aimed to discover new medicines while reducing the impact of their manufacturing, for example, by reducing the amount of organic solvents or changing conventional organic reactions for environmentally friend ones [17]. In nanotechnology, the application of “green methodologies” have been mainly described in two ways: (i) the development of green synthesis processes, usually reported for the formulation of metallic nanoparticles, such as gold, zinc or cooper nanoparticles [18] and (ii) the preparation of biodegradable nanoparticles using organic solvent-free and mild methods, for example, ionotropic gelation [19], phase inversion temperature (PIT) [20] and spontaneous emulsification [8]. In this work, we decided to develop a green formulation process based on the spontaneous emulsification technique. Comparing

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with the ionotropic gelation, which has been reported for the preparation of polymeric nanoparticles intended for the delivery of hydrophilic drugs [21], the spontaneous emulsification technique is best suited for the formulation of oil in water (o/w) nanoemulsions, an advantageous system for hydrophobic drugs like docetaxel [22]. On the other hand, the main disadvantage of the PIT method is the use of heating-cooling cycles, which might compromise thermolabile drugs [23].

The self or spontaneous emulsification technique has been widely described for the preparation of nanoemulsions [24–27] and, recently, Hossein et al has shown that nanocapsules can be prepared in two steps by spontaneous emulsification and coated with an anionic biopolymer [28]. The preparation of polymeric nanocapsules using this method can combine the advantages of a sustainable methodology with the intrinsic advantages of the core/shell structure for anticancer drug delivery. To our knowledge, this was the first time HA nanocapsules were prepared in a one-step, organic solvent free self-emulsification method.

2. Spontaneous emulsification – preparation of self-emulsifying nanoemulsions

The self-emulsification technique was firstly optimized for the formulation of a nanoemulsion and the components choice was done based on the intrinsic properties of each element. As such, the oil phase was composed of Miglyol®812 and Tween®80 and the aqueous phase composed of water and Solutol®HS15. Miglyol®812 was chosen as the oil core because it is a medium chain triglyceride widely applied for the formulation of self-emulsification systems. Comparing to long chain triglycerides, medium chain triglycerides reduce the interfacial tension and have better partitioning ability to emulsify [29–30]. In addition, docetaxel can be effectively incorporated within Miglyol®812 with enhanced drug loading capacity [31]. Regarding the surfactant, Tween®80 is among the most used for self-emulsification. It has a hydrophilic-lipophilic balance (HLB) of 15, which assists the immediate formation of o/w droplets and lead to a rapid dispersion of the formulation into the aqueous medium [32]. On the other hand, the concentration of Tween®80 was kept at the minimum needed to formulate. Comparing with other surfactant options such as Labrasol®, Tween®80 was described as

safer, and it is approved for the intravenous route [33]. The selection of Solutol®HS15 to form the aqueous phase was related to its surfactant properties (HLB 14-16) that enhance the flexibility of the surfactant layer formed at the interface, resulting in stabilized nanoemulsions [14]. Moreover, its PEGylated chain has been described as responsible for the inhibition of the P-glycoprotein (P-gp) pump, providing a higher intracellular accumulation of the system [34–35].

After components selection, nanoemulsions were prepared step-by-step by varying: (i) the concentration of Solutol®HS15 in the aqueous phase, (ii) the Miglyol®812/Tween®80 ratio and, (iii) the oil/aqueous phase ratio. The final nanoemulsion formulation was prepared by the addition of the oil phase, composed of Miglyol®812/Tween®80 (1:1 ratio w/w) to the aqueous phase, composed of water and 2.5 mg/mL of Solutol®HS15. The oil phase was poured into the aqueous phase using a 1:8 ratio (v/v), under magnetic stirring and at room temperature. **Figure 2** represents the flow chart of the self-emulsification process. Under these conditions, spontaneous nanoemulsions were formed, showing a mean particle size around 140 nm, a polydispersity index of 0.2 and a zeta potential of -15 mV. This process was used as a basis for nanocapsules preparation.

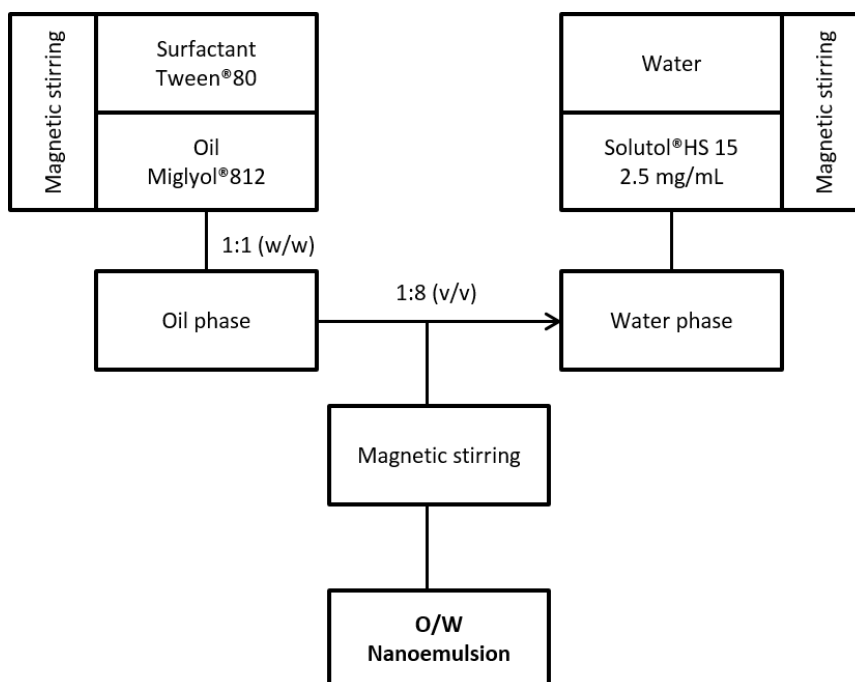


Figure 2. Flow chart of the preparation of self-emulsifying nanoemulsions.

3. Preparation of HA-based nanocapsules using HA and dodecylamide-functionalized HA

HA-based nanocapsules were prepared using the optimized spontaneous emulsification technique by dissolving the polymer into the aqueous phase. The nanocapsules were prepared using two types of HA: sodium hyaluronate (HA) and a hydrophobically functionalized HA with a dodecylamide chain (C12-HA) (**Figure 3**). C12-HA has a 2.0-5.0% degree of substitution, which is considered to be enough to confer to the polymer an amphiphilic behavior without changing its aqueous solubility over the concentration range required for formulation [36]. Additionally, it is not expected that this degree of substitution interferes with CD44 recognition, an important feature for active targeted delivery [37].

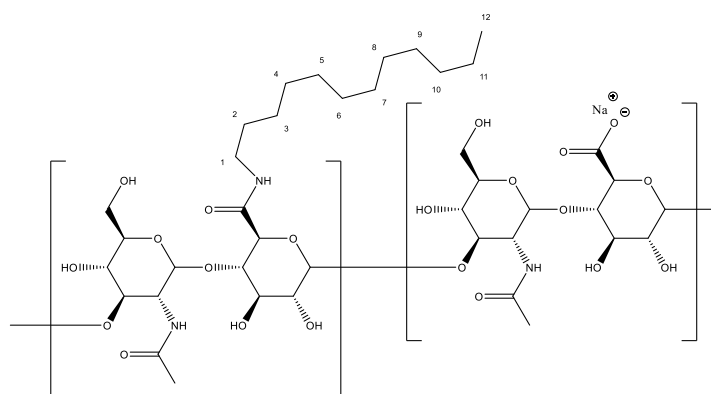


Figure 3. Chemical structure of dodecylamide-functionalized HA

The preparation of HA nanocapsules (HA NCs) has been reported by our group and consists on the interfacial deposition of negatively charged HA onto a positive charged surface [38]. Accordingly, self-emulsifying HA NCs were prepared by the addition of the cationic surfactant cetyltrimethylammonium bromide (CTAB) to the oil phase. The addition of CTAB to the nanoemulsion promoted an inversion of the zeta potential to positive values. Consequently, the attachment of HA to the cationic layer was achieved by electrostatic interaction between the positively charged CTAB and the HA, resulting in the shift of the zeta potential from +10 mV to -19 mV (**Figure 4**). Different CTAB and

HA concentrations were studied and the characterization of the optimized formulation (using Miglyol®812/ Tween®80 in a ratio 1:1 (w/w) and Solutol®HS15 solution at a concentration of 2.5 mg/mL; oil/aqueous phase ratio of 1:8 (v/v)) is shown in **Table 1**.

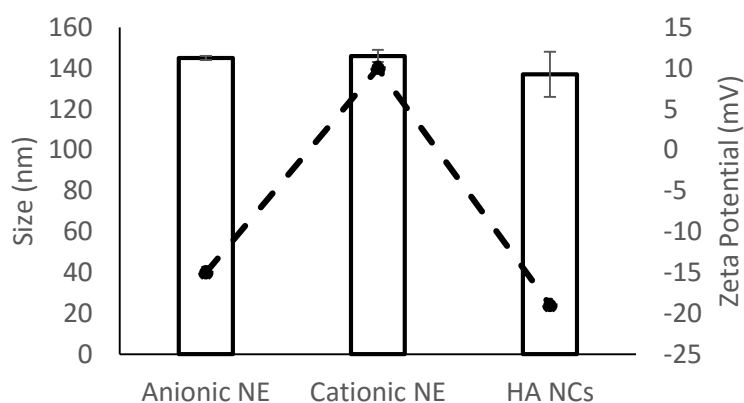


Figure 4. Size (bars) and zeta potential (line) values of the optimized anionic nanoemulsion, cationic nanoemulsion and HA NCs prepared by the spontaneous emulsification method. It was observed an inversion of the zeta potential after CTAB addition (from negative to positive values) and again after HA deposition (from positive to negative values).

Notes: Results are presented as mean value \pm standard deviation (n=3)

To simplify the process and avoid the use of the cationic surfactant, which is commonly associated to potential cytotoxic effects, HA was replaced by the C12-HA amphiphilic polymer, which made possible the preparation of nanocapsules through hydrophobic interactions. The use of amphiphilic HA derivatives is widely reported in the literature for the preparation of self-assembled nanoparticles or micelles by sonication [39–41]. Nevertheless, it is important to highlight that these self-assembled structures are formed upon application of a high shear force (sonication) and are dependent on the degree of substitution of the HA [42]. Moreover, HA can be grafted with phospholipids, like L- α -Dioleoylphosphatidylethanolamine (DOPE), and incorporated onto the surface of liposomes [43–45].

In our work, the use of C12-HA resulted in the formulation of nanocapsules with physicochemical properties very similar to those prepared with HA. Moreover, the absence of micelles formed by the C12-HA itself in water was confirmed using light-scattering measurements.

Table 1. Physicochemical characterization of HA NCs and C12-HA NCs.

Formulation	CTAB conc. (mg/mL)	HA conc. (mg/mL)	Size	PDI	ZP (mV)
HA NCs	0.15	0.25	137 ± 11	0.2	-19 ± 1
C12-HA NCs	-	0.5	126 ± 5	0.2	-20 ± 2

Notes: Results are presented as mean value ± standard deviation (n=3)

Abbreviations: PDI, polydispersity index; ZP, zeta potential

TEM images show the morphology of HA-based nanocapsules and the typical core/shell structure was visualized for both systems (**Figure 5**). These results confirmed the possible formation of C12-HA NCs by hydrophobic interactions, with the dodecyl chains of the amphiphilic-HA facilitating the entrapment of the polymer on the interface of the nanoemulsion. A similar mechanism has been described for the formulation of HA-DOPE-coated liposomes, where the DOPE molecule plays the role of the anchor in the lipid membrane [46].

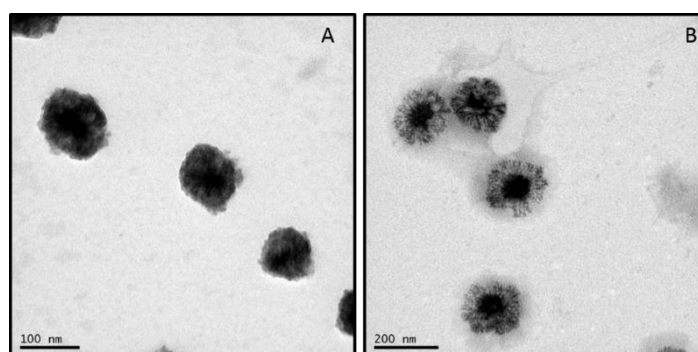


Figure 5. TEM images of **A)** HA NCs and **B)** C12-HA NCs.

Self-emulsifying HA NCs and C12-HA NCs were evaluated for stability under storage conditions at 4°C and after dilution in human plasma at 37°C. Under storage conditions, both prototypes were very stable, without significant changes in particle size, polydispersity index, and zeta potential for up to 6 months. This high stability may be a consequence of different factors: (i) the negative charge conferred by the HA shell must avoid particle aggregation [47], (ii) the PEGylated chains from Solutol®HS15 are described as a stabilizer [48] and, (iii) the presence of Tween®80 can also provide steric

stability [49]. When incubated in human plasma at 37°C we observed an increase in particle size; nevertheless, this increase was less than 20% of the initial size. Moreover, the polydispersity index of the system did not change, which denotes the absence of particle aggregates. This increase in size may be due to a small deposition of plasma proteins around the nanocapsules.

The cytotoxicity of HA NCs, C12-HA NCs and a mixture of free surfactants was compared using the AlamarBlue® assay. As presented in **Figure 6**, the survival curves of A549 cells showed a concentration-dependent profile in the range of 3.5 – 1000 µg/mL. Irrespective of their composition, both nanocapsules did not affect cell viability when tested at concentrations up to 350 µg/mL. Nonetheless, only C12-HA NCs did not cause toxicity at the maximum concentration tested (1000 µg/mL). These results must associate the presence of CTAB in HA NCs with its higher cytotoxicity, as revealed in other studies [50]. On the other hand, the free surfactant mixture composed of Tween®80, Solutol®HS15 and CTAB showed remarkable toxic effects, resulting in 85% of cell death. However, when encapsulated within the nanocapsules structure, this toxic profile changed and HA NCs were less toxic [51]. C12-HA NCs were also prepared by the solvent displacement technique and the anionic surfactant Tween®80 was replaced by lecithin [38] (results not shown). Regardless its composition, both nanocapsules showed the same cell viability profile, which demonstrated that the amount of Tween®80 needed to prepare HA-based nanocapsules by the self-emulsification method was not responsible for additional toxicity. This result must be related to the correct isolation of the nanocapsules and/or to the capacity of the polymeric shell to mask surfactant toxicity.

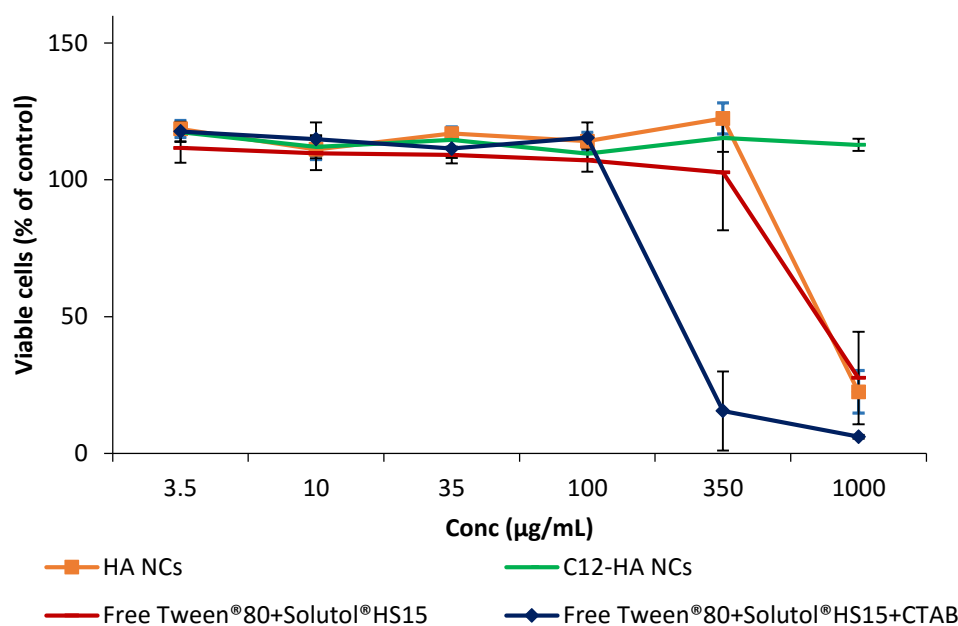


Figure 6. Viability of A549 cells after 72h of incubation with different concentrations of HA NCs, C12-HA NCs and free surfactant mixtures.

Notes: Results are presented as mean value \pm standard deviation (n=6)

4. Encapsulation of docetaxel into HA-based nanocapsules

Docetaxel was first solubilized in Miglyol®812 and then incorporated into HA and C12-HA NCs, following the protocol previously described. The drug was efficiently encapsulated in both prototypes, without changing their physicochemical characteristics. The solubility of docetaxel in Miglyol®812 allowed a final drug loading of 0.125 % (w/w), which corresponded to 100 µg of DCX per mL of nanocapsules. In order to achieve a high drug loading without compromising the composition and toxicity of the carrier, docetaxel was solubilized in a small amount of ethanol (<10%), followed by evaporation. Docetaxel-loaded HA NCs could be prepared with a loading up to 2.75% (corresponding to 2.5 mg/mL of docetaxel) without changing the physicochemical characteristics of the system. This higher drug loading would result, *in vivo*, in the administration of a lower amount of nanocapsules to deliver a therapeutic dose, thus reducing the potential adverse effects of Tween®80 [52]. The encapsulation efficiency of docetaxel was between 86-89% for both types of nanocapsules. The cytotoxicity of docetaxel-loaded HA-based nanocapsules was studied by using the AlamarBlue® assay. Both free and encapsulated docetaxel showed a dose dependent toxicity in A549 cells.

Nevertheless, the IC₅₀ was only achieved for the docetaxel delivered from nanocapsules, demonstrating the potential of HA-based nanocapsules for cytostatic drug delivery.

Commonly, the majority of *in vitro* release assays for hydrophobic drugs are performed by ultracentrifugation and dialysis, and less frequently by size exclusion chromatography or continuous flow filtration. Nevertheless, the drug and the carrier cannot always be easily separated using those methods. For example, ultracentrifugation cannot be applied to samples that aggregate under this separation conditions [53]. Regarding dialysis, the addition of surfactants to achieve the total solubility of docetaxel in the medium under sink conditions can interfere with the structure of colloidal particles and change the drug release [54]. Considering the limitations of those isolation methods, *in vitro* release assays of docetaxel from both nanocapsules prototypes were assessed using a drug transfer method adapted from Bastiat et al [55]. For that, docetaxel-loaded HA NCs and C12-HA NCs were diluted under sink conditions in PBS at 37°C, and at fixed time points (15minutes, 3h, 6h and 24h) a sample volume was taken, mixed with Miglyol®812 (in a 1:1 v/v ratio) and placed into a centrifuge for phase separation. The idea behind this technique is that the oil phase would act as an acceptor compartment for the free drug, whereas the encapsulated docetaxel would be kept into the nanocapsules suspension (**Figure 7**).

Overall discussion

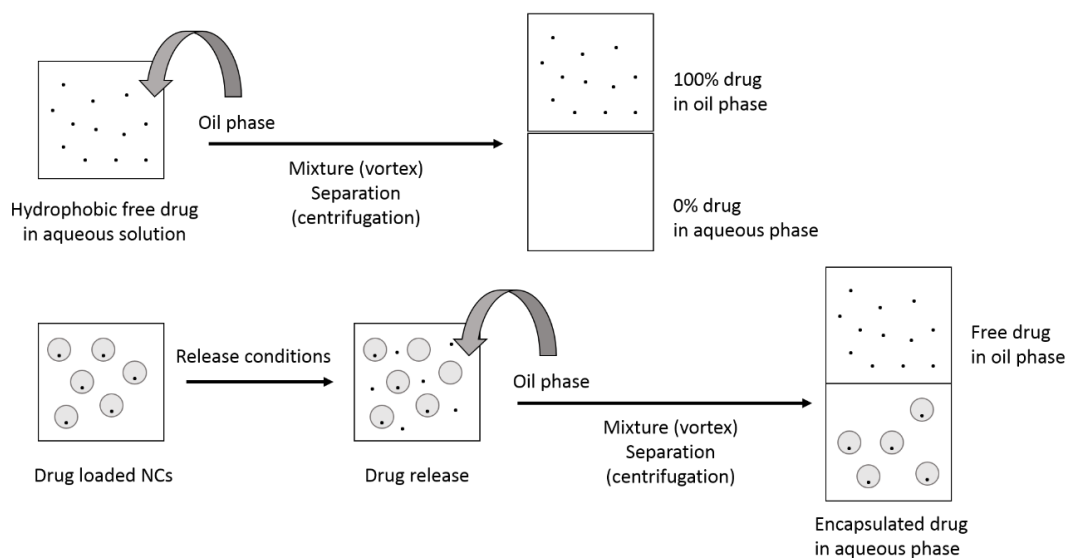


Figure 7. Schematic representation of the drug transfer method for the *in vitro* release of docetaxel from HA-based nanocapsules.

To validate the method, an initial experiment was performed with only the free docetaxel dissolved in PBS at sink conditions. As observed in **Figure 8**, all the free docetaxel was taken up by the oil phase, confirming the ability of Miglyol®812 to act as an acceptor phase for free DCX. Regarding the release of docetaxel from the nanocapsules, the results demonstrated an improved profile. Docetaxel was released from HA NCs and C12-HA NCs following a biphasic profile, showing an initial burst release of 55% and 45%, respectively, and then, a sustained release over 24h (**Figure 8**).

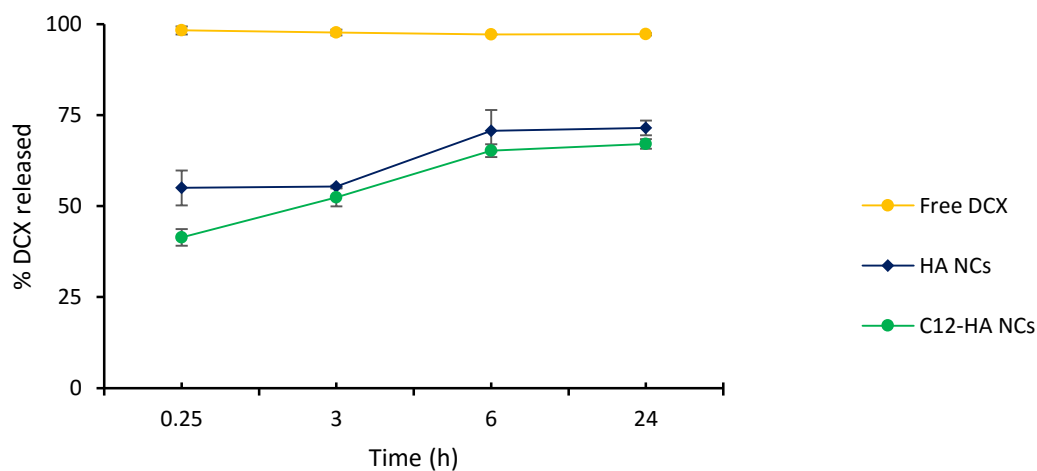


Figure 8. *In vitro* release profiles of docetaxel from HA NCs and C12-HA NCs in PBS.

Notes: Results are presented as mean value \pm standard deviation (n=3)

This release behavior can be justified by the structure of the nanocapsules and the partition coefficient of the drug between the oil core and the aqueous external medium [56]. Moreover, the oil phase in contact with the nanocapsules suspension can act as a “lipophilic attractor”, which means that it can generate a continuous transfer of the free drug to the oil compartment. In this way, the formulation is under continuous forced sink conditions. **Figure 9** illustrates both mechanisms that can justify the release of docetaxel from HA-based nanocapsules.

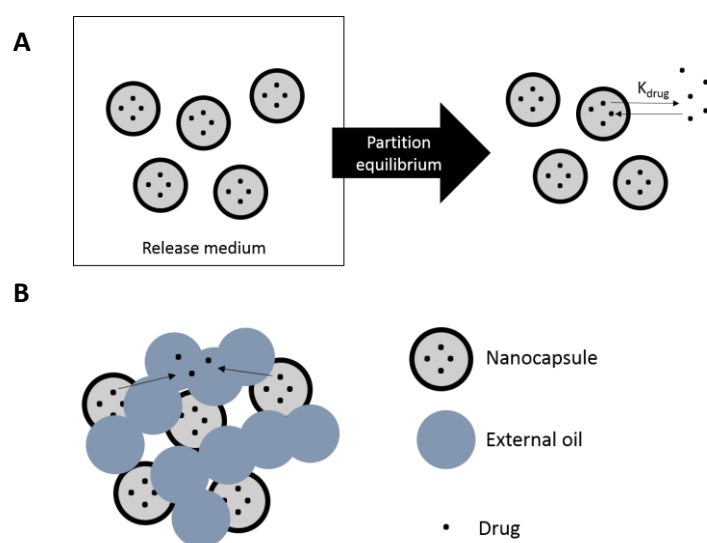


Figure 9. Schematic illustration of the mechanisms responsible for the release behavior of docetaxel from HA-based nanocapsules. **A)** Influence of the partition equilibrium in the drug release of nanocapsules; **B)** Drug transfer from the nanocapsules nucleus to an external oil phase

5. Intracellular delivery of anti-gasdermin B associated to HA-based nanocapsules

Contrarily to tumor associated cell surface antigens, some cancer-causing proteins are confined to the intracellular compartment and do not express an extracellular receptor [57]. Examples of those proteins are RAS (GTPases) [58], non-receptor tyrosine kinases (like Bcr-Abl) [59], BRAF [60] or heat shock proteins (like HSP90 that interacts and stabilize mutant p53) [61]. Recently, gasdermin B (GSDMB) was discovered as an intracellular protein whose overexpression was associated with cancer progression and invasion [62]. This protein, localized into the cytosol and without a specific cell membrane receptor, can join the group of “undrugable molecules” in oncology [63].

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The current approaches to target these proteins are by means of silencing therapy, cytostatic molecules or protein kinase inhibitors. However, the complexity of these pathways, the off-target effects and cellular barriers make these therapies unsuccessful [63]. Besides the use of mAbs to target cancer cell receptors, an emerging new strategy is their use to target intracellular cancer proteins. However, this therapeutic application has been limited due to the high molecular weight and hydrophilicity of mAbs, properties that prevent them to cross the cell membrane on their own [15]. It is important to note that the intracellular delivery of antibodies is a common approach in biochemistry, for example, antibodies are used to localize proteins after fixation and permeabilization of cells [64], or can be transfected into the cell by the use of cationic-lipids [65] or commercial transfection reagents. Nevertheless, those approaches are only intended for cellular processes validation and cannot be used for a therapeutic application. Additionally, intrabodies were also investigated for its ability to target and modulate intracellular proteins. Intrabodies are antibodies that bind intracellularly to their antigen after being produced in the same cell, in contrast to mAbs that are prepared on a bioreactor, administered to patients and exert their activity in the surface of a cell [66]. The inefficient delivery of the genetic material encoding the intrabody and its instability into the cytosol are the main reasons that have hampered the therapeutic development of this approach [67].

The strategy of using mAbs against intracellular cancer proteins has pushed the interest of the pharmaceutical industry and, recently, two companies have announced promising results in the transport of mAbs into cancer cells. Sorrento Therapeutics has developed a technology based on the chemical modification of mAbs that allows them to penetrate into the cell while maintaining their ability for binding specific target proteins [68]. BioCell Challenge has prepared lipid-based formulations to encapsulate a specific mAb directed against the RAS oncoprotein, without the need of a chemical modification. *In vivo* results demonstrated that mice survival was prolonged by up to 30% and complete recovery was observed in 33% of cases [69].

Knowing the potential of mAbs as therapeutic agents against intracellular proteins, Moreno-Bueno's group has developed a mAb against the intracellular protein GSDMB,

the anti-GSDMB. We have associated the anti-GSDMB to the polymeric shell of HA-based nanocapsules with the aim of facilitating its entry into the cancer cells. This platform was studied as a new technology for intracellular mAb-based therapies.

Anti-GSDMB was generated as described by Hergueta-Redondo et al (submitted). After purification, the concentration of anti-GSDMB was determined as 2.9 mg/mL. The purity and integrity of the mAb was evaluated by SDS-PAGE. The resulting gel electrophoresis demonstrated a single band at 160KDa (intact antibody) and two bands, at 50 KDa (constant fraction, Fc) and 25 KDa (variable fraction, Fab) [70], when non-reducing or reducing conditions were applied, respectively (**Figure 10**). Moreover, the additional bands that are observed might correspond to some additional impurities. For example, it has been described that protein fragments on non-reducing SDS-PAGE are a common feature and are related to the breakage of inter-chain disulfide bonds during sample preparation [71]. Therefore, these results confirm the stability and integrity of anti-GSDMB.

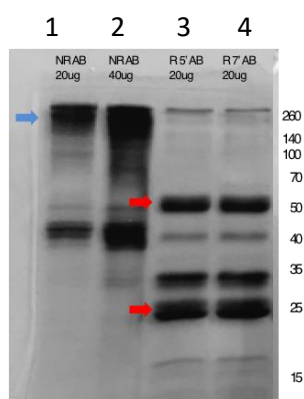


Figure 10. SDS-PAGE gel electrophoresis. Lanes 1 and 2, anti-GSDMB in non-reducing conditions at 20 and 40 μ g/mL; lanes 3 and 4, anti-GSDMB under reducing conditions after 5 and 7, 10 and 12 min. Blue arrow marks intact anti-GSDMB band and red arrows the Fc (50KDa) and Fab (25KDa) fractions.

The specific activity of anti-GSDMB was analyzed by ELISA. This assay allowed us to confirm its activity against the target protein, GSDMB, as well as to validate a method to quantify the mAb associated to the nanocapsules. **Figure 11** shows the sigmoidal calibration curve for the mAb in a concentration range from 0.125 to 10 μ g/mL.

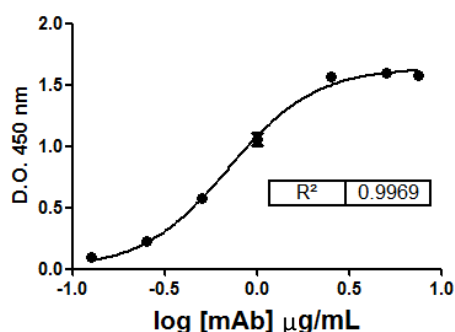


Figure 11. Calibration curve of anti-GSDMB, showing the optical density values vs log of mAb concentration.

Notes: Results are presented as mean value \pm standard deviation (n=3)

Anti-GSDMB was successfully associated to the polymeric shell of HA-based nanocapsules by physical adsorption. The advantage of using this technique is related to its mild and easy conditions, which avoids the need of aggressive reagents and preserves the mAb activity [72–73]. Moreover, this physical adsorption may provide an effective release of the mAb inside the cells, without the need for breaking covalent bonds. On the other hand, the lack of a chemical conjugation may facilitate the displacement of the adsorbed antibody when diluted in cell culture medium or human plasma [74].

The association of anti-GSDMB was carried out with both HA NCs and C12-HA NCs. First, HA NCs were incubated with increasing amounts of mAb, from 2.5 to 25 µg of anti-GSDMB per mg of nanocapsules. The interaction of anti-GSDMB with the nanocapsules was assessed using two strategies: (i) electrostatic interactions between protonated anti-GSDMB (anti-GSDMB+) and the negatively charged HA and, (ii) hydrophobic forces by simply incubating anti-GSDMB with the nanocapsules. Different studies report the efficient association of proteins to oppositely charged polymers. For example, the cationic thiolated branched polyethyleneimine was complexed with the anionic bovine serum albumin [75] and the anionic caspase-3 protein with positive methacrylate-crosslinked nanoparticles [76]. Moreover, it is widely described the association of anionic proteins or enzymes to positively charged lipids, liposomes or lipoplexes [15,

77]. In a recent study, the preparation of liposome/protein complexes through electrostatic interactions was achieved by mixing the cationic liposomes with a solution of albumin, as a model protein, or the mouse anti-F actin primary antibody, as a model antibody [78]. The majority of protein complexes are formed with cationic lipids or polymers to take advantage of the “proton sponge effect”, thus promoting the endosomal escape of proteins [79]. It has also been reported the adsorption or entrapment of proteins and antibodies to nanoparticles through non-ionic interactions. For example, β -galactosidase was entrapped within chitosan-conjugated Pluronic[®]-based nanoparticles [80] and the model protein, carbonic anhydrase, entrapped within self-assembled HA-nanoparticles [81]. Moreover, the adsorption of antibodies to the surface of polymeric nanoparticles has been also described for the non-covalent binding of mAbs to cyanoacrylate nanoparticles [73] as well as to PLGA nanoparticles [82–83].

With the purpose of promoting an electrostatic interaction between the mAb and the surface of HA nanocapsules, anti-GSDMB was protonated at pH 4.5 and ionically associated to negatively charged HA NCs. With an isoelectric point of 6.5-8.0 we had the option of working with negatively charged anti-GSDMB and positive nanocapsules, or with positively charged anti-GSDMB and anionic nanocapsules. We decided to protonate anti-GSDMB as it is described as a frequent intermediate step for antibody conjugation [84–85]. For example, SM5-1 single-chain antibody was protonated under acidic conditions and successfully adsorbed onto the surface of negatively charged PLGA nanoparticles [86]. On the other hand, anti-GSDMB adsorption to HA nanocapsules was carried by simple mixing a solution of nanocapsules with the antibody at neutral pH, thus promoting an interaction mainly based on hydrophobic forces rather than an ionic interaction.

Regardless of the strategy, anti-GSDMB was effectively incorporated within the polymeric shell of both types of nanocapsules. **Table 2** shows the physicochemical characterization of nanocapsules, which resulted in particles around 120 nm, monodisperses and with a high association efficiency. Moreover, the decrease in the zeta potential indicated the prevalence of HA on the surface of the nanostructure despite the high mAb association efficiency. The use of 0.75 mg/mL of HA and 0.5 mg/mL

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of C12-HA allowed us to obtain a drug loading of 25 μg mAb/mg nanocapsules, while maintaining a similar negative charge.

Table 2. Physicochemical characterization and association efficiency of anti-GSDMB and protonated anti-GSDMB+ associated to HA NCs and C12-HA NCs. (Blank nanocapsules are included for comparison)

Formulation	Anti-GSDMB (25 μg / mg NCs)	Size (nm)	PDI	ZP (mV)	Association efficiency (%)
HA NCs	-	126 \pm 5	0.2	-22 \pm 1	-
C12-HA NCs	-	123 \pm 2	0.2	-20 \pm 1	-
HA NCs	Anti-GSDMB+	117 \pm 6	0.2	-11 \pm 1	75 \pm 10
	Anti-GSDMB	126 \pm 7	0.2	-7 \pm 1	82 \pm 6
C12-HA NCs	Anti-GSDMB+	119 \pm 3	0.2	-6 \pm 1	84 \pm 8
	Anti-GSDMB	116 \pm 6	0.2	-10 \pm 1	93 \pm 3

Notes: HA NCs and C12-HA NCs were prepared with 0.75 mg/mL and 0.5 mg/mL of HA and C12-HA, respectively. Results are presented as mean value \pm standard deviation (n=3)

Abbreviations: PDI, polydispersion index; ZP, zeta potential

Electrostatic interactions are thought to be a major factor in protein binding to polymeric surfaces. As such, we obtained a high association efficiency when protonated anti-GSDMB+ was incubated with negatively charged HA NCs at a final pH of 4.5. Interestingly, the same association efficiency was observed when non-protonated anti-GSDMB was incubated with HA NCs (pH 7.4). These results could be justified by the interaction of the mAb with the polymeric shell by hydrophobic forces. At pH 7.4 the anti-GSDMB is within its isoelectric point, which could favor the entrapment of its hydrophobic domain within the interface of the nanocapsules [87]. Moreover, it could also be presumed that the hydroxyl-carboxyl groups in the polymeric structure might help the interaction of the protein by simple hydrogen bonds [88].

In order to study the ability of HA-based nanocapsules to transport the anti-GSDMB into the intracellular compartment, the first step was to perform *in vitro* cytotoxicity assays

for the blank prototypes in the HCC1954 breast cancer cell line. Likewise our previous results, HA NCs showed a marked cytotoxicity, with a decrease of 50% in cell viability (IC50) for 0.3 mg/mL. On the contrary, C12-HA NCs did not show considerable toxicity at concentrations up to 0.8 mg/mL and this formulation was chosen for the following assays.

The hypothetical internalization of the mAb was followed by confocal microscopy. For that, HCC1954 cells were incubated with anti-GSDMB-loaded C12-HA NCs at a concentration of 0.8 mg/mL, which corresponds to 20 μ g of mAb (125 nM). **Figure 12** shows the internalization of anti-GSDMB alone or after association to C12-HA NCs. Without a cell surface receptor and a nanocarrier, anti-GSDMB is described to be too large and hydrophilic to cross the cell membrane on its own [15]. On the other hand, anti-GSDMB associated to C12-HA NCs was effectively delivered into the cytosol of HCC1954 cells, as seen by the green dots in the XZ image. Similar results were obtained when protonated anti-GSDMB⁺ was associated to C12-HA NCs. Thus, in order to avoid the acidification of the medium and the protonation of the antibody, non-protonated anti-GSDMB associated to C12-HA NCs was selected to continue the study.

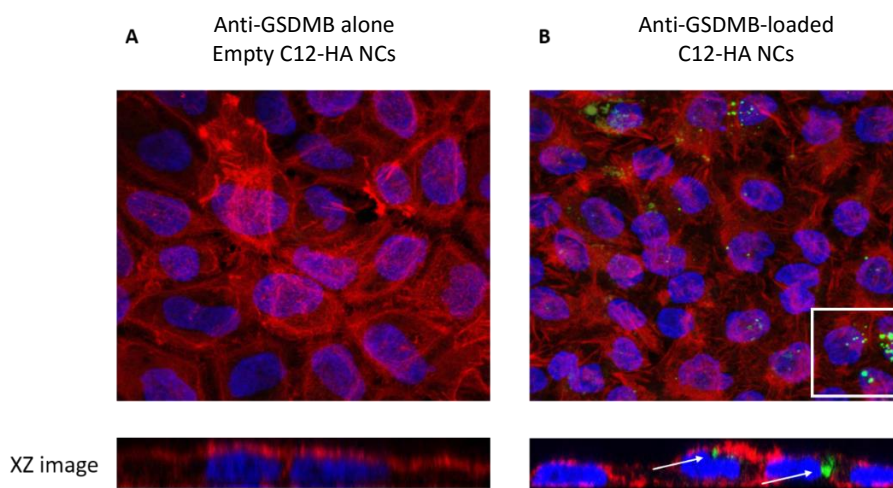


Figure 12. Internalization of anti-GSDMB mediated by its association to C12-HA NCs. HCC1954 cells were incubated with 0.8 mg/mL of C12-HA NCs, which corresponds to 20 μ g of anti-GSDMB, for 72h. **A)** Anti-GSDMB alone and blank C12-HA NCs were used as controls. Without a cell surface receptor, the anti-GSDMB on its own was not able to cross the cell membrane; **B)** C12-HA NCs promoted the internalization of anti-GSDMB after its association to the polymeric shell.

Notes: Images were taken by confocal microscopy. Cell nuclei were stained with DAPI (blue) and cell membrane with phalloidin (red).

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Furthermore, to be able to increase the concentration of C12-HA NCs without compromising cell viability, serial steps of incubation were performed using different concentrations and incubation times. As a result, it was demonstrated that by incubating HCC1954 cells with C12-HA NCs for short periods of time, up to 2h, followed by repeated washing and incubation steps, it was possible to increase the nanocapsules concentration to 3.2 mg/ml without additional cytotoxicity. Accordingly, the concentration of anti-GSDMB could be increased four-fold, to 80 µg of anti-GSDMB (which corresponds to 500 nM). We decided to study the trafficking pathways and therapeutic efficacy of anti-GSDMB into HCC1954 cells using the highest concentration tested for intracellular uptake, i.e. 500 nM. For example, it was reported that the cell-mediated cytotoxicity of trastuzumab in HCC1954 cells was assessed following treatment with 100 nM or 200 nM of the mAb [89–90]. Because our mAb was intended to act intracellularly and we were not sure about the therapeutic concentration required, we decided to perform these experiments using 500 nM of anti-GSDMB.

Once it was shown that the mAb was able to enter the cells, the next step was to study the trafficking pathway of anti-GSDMB and its co-localization with early endosomes, as well as the endosomal escape. It is well known that one of the main hurdles in the delivery of proteins or RNA into the cytosol is the *in vivo* degradation by lysosomes [91]. In fact, once inside the cell, anti-GSDMB might escape lysosomes and maintain its conformational structure in order to target GSDMB. The results showed that anti-GSDMB (green dots) was carried into the cell by endocytosis (red dots) and was able to escape lysosomes (yellow dots) (**Figure 13**).

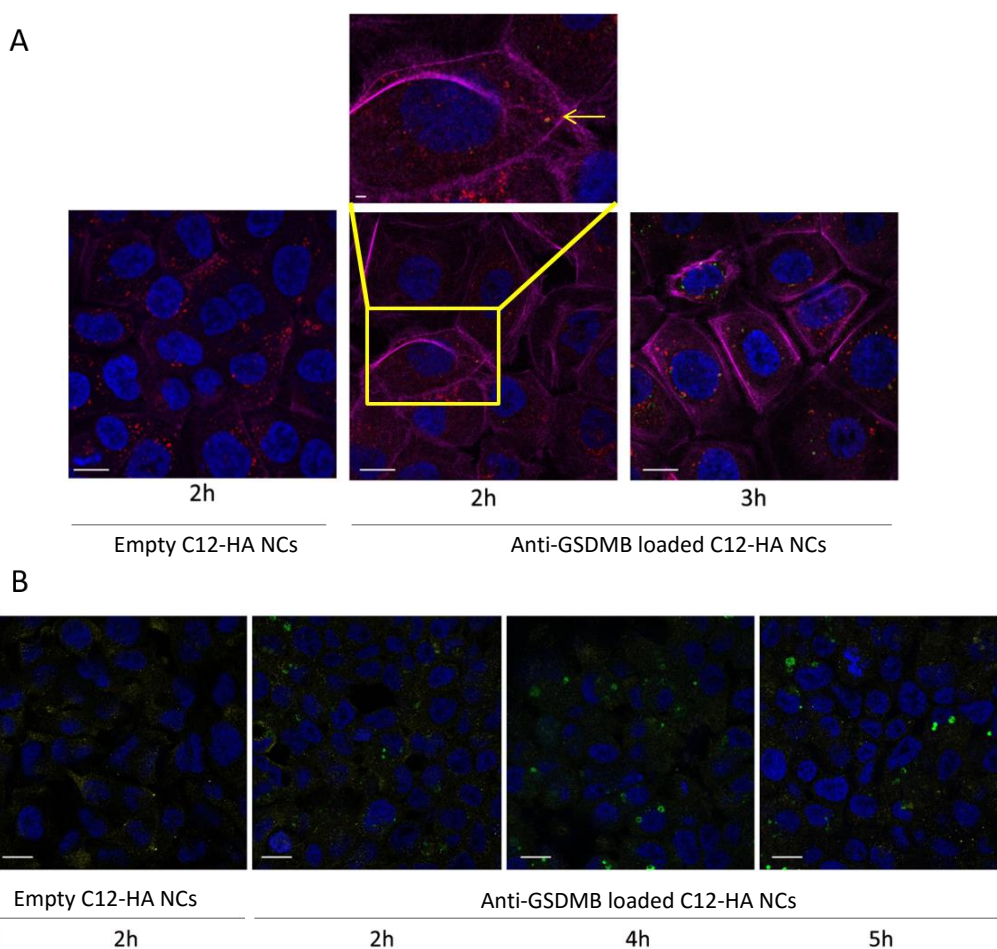


Figure 13. Trafficking of anti-GSDMB (green) through endosomes. HCC1954 cells were incubated with 3.2 mg/mL of C12-HA NCs containing 80 μ g of anti-GSDMB for 2h, 3h, 4h and 5h. **A)** Early endosomes were detected by early endosome antigen 1 (EEA1) (red). **B)** Late endosomes were detected by lysosomal-associated membrane protein (LAMP1) (yellow).

Notes: Images were taken by confocal microscopy. Nuclei were stained with DAPI (blue) and the cytoskeleton with phalloidin (purple).

The internalization through endocytosis might probably be mediated by CD44 receptors, overexpressed on the surface of HCC1954 cells [92]. CD44 is the principal receptor for HA and its binding affinity and enhanced uptake through this mechanism is well known [93]. Moreover, HA-based nanocapsules could be internalized by other passive mechanisms, for example after particle adsorption onto the cell surface [94–95] as well as by the penetration enhancing nature of some of the compounds of the nanocarrier. For example, it has been described that Tween®80 has de capacity to enhance cell permeability. To better understand the internalization pathway, three different assays could be taken into consideration: (i) blocking of CD44 receptors with free HA to confirm

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the intracellular uptake by CD44-receptor mediated transport [96]; (ii) inhibition of the cell uptake mechanism using three endocytosis inhibitors, namely, chlorpromazine (clathrin inhibitor), nystatin (caveolin inhibitor), and cytochalasin D (actin inhibitor) [78] and; (iii) expose the cell to ice-cold medium (0-4°C) for nonspecific inhibition of endocytosis [97].

The endosomal escape was visualized by confocal microscopy and qualitatively confirmed a negative co-localization with LAMP1 labeled lysosomes (yellow) (**Figure 13 B**). Considering the effective endosomal escape, this mechanism must be explained by two main reasons: (i) a pH-dependent mechanism, similar to the “proton sponge effect” caused by cationic lipids [98] and, (ii) the influence of the anionic surfactant Tween®80 in destabilizing the endosomal membrane [99]. It has been described that cationic nanoparticles with a buffering capacity at pH from 5 to 7 have displayed the ability to escape the endosomes (pH 5 – 6). Under acidic conditions, cationic lipids/polymers are capable of buffering the endosomal vesicle, leading to endosomal swelling and lysis, and thus releasing the payload into the cytoplasm [100]. Following the same explanation, we can adapt this mechanism to explain the endosomal escape of anti-GSDMB. Thus, at pH 7.4 anti-GSDMB is within its isoelectric point, which favored the association to C12-HA NCs and promoted its internalization into the cells. Nevertheless, when inside the endosomes the pH drops to acidic values, the anti-GSDMB becomes positively charged and the interaction with the nanocapsules structure becomes weak [101]. Once protonated, cationic anti-GSDMB⁺ might form ion pairs with the anionic lipids within the endosome and destabilize the endosomal membrane [100]. These results would be in accordance with those described for doxorubicin-loaded HA-Poly-L-Histidine/ D- α -Tocopherol PEG 1000 succinate polyhistidine with the highest release occurring at endosomal pH [102]. Moreover, the endosomal escape of protonated anti-GSDMB can be correlated with the trafficking pathway of positively supercharged proteins. For example, positively charged green fluorescent protein was found in high amounts inside early endosomes nevertheless, two hours later, only a small fraction (< 20%) was eventually co-localized with late endosomes. Furthermore, 80% of the original protein signal remained within cells after 16h, indicating that the proteolysis of the endocytosed

cargo occurred in a little extension [103]. On the other hand, another hypothesis for the endosomal escape could be related to the components choice to formulate HA nanocapsules, namely the Tween[®]80. It was described that the Tween[®]80 could interact with the endosomal membrane [99] or form lamellar-to-inverted hexagonal phases [104], which can disturb the endosome membrane and promote endosomal escape.

In order to confirm these results, a possible experiment to take into consideration would be the cellular uptake performed by confocal microscopy with live cells using a real-time imaging experiment. In this case, anti-GSDMB would be conjugated with a fluorophore, for example Alexa[®]Fluor, and the trafficking pathway observed without the need of cell fixation. It has been reported that cargos delivered by cell penetration peptides can artificially “escape” from endocytic vesicles, thus resulting in misleading conclusions [105].

It is known that the overexpression of GSDMB is responsible for cell migration and invasion whereas its knockdown with shRNA results in a marked decrease of the migratory ability and the invasive capacity of HCC1954 cells. Therefore, we performed a last experiment in order to assess the ability of anti-GSDMB to effectively block the intracellular cancer-protein GSDMB and inhibit cell migration and invasion. The binding affinity and targeting ability of anti-GSDMB against intracellular GSDMB was demonstrated by wound healing migration assays. We observed that HCC1954 cells treated with anti-GSDMB-loaded C12-HA NCs inhibited cell migration as compared to the negative control (**Figure 14**). As a result, the scratch healing of HCC1954 cells treated with anti-GSDMB-loaded C12-HA NCs was bigger than the cell treated with only NCs, denoting the slow capacity of cells to migrate and close the wound.

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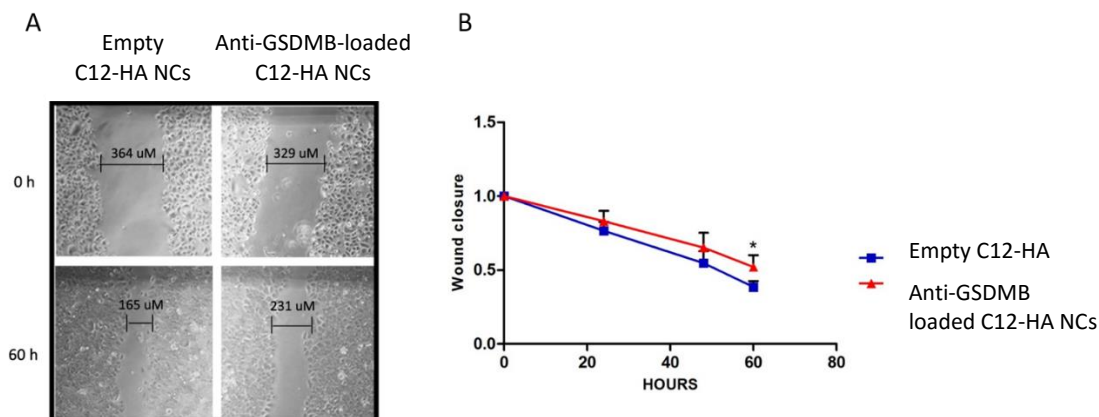


Figure 14. Effect of anti-GSDMB loaded C12-HA NCs on the migration of HCC1954 cells. **A)** Scratch wound healing assay was performed to examine the effect of C12-HA NCs entrapped anti-GSDMB on cell migration. **B)** Mobility rate histograms of each group across the selected times (hours). * $p < 0.05$

After 60h, HCC1954 cells treated with blank nanocapsules migrate and close the wound healing to 165 μm whereas, HCC1954 cells treated with anti-GSDMB loaded C12-HA NCs maintain an opening heal of 291 μm , which prove the slow migration of cells. We can speculate that, once inside the cell, anti-GSDMB is maintaining its original conformation and binding affinity to the GSDMB protein. Although, to our knowledge, this was the first time that a scratch migration assay was performed to analyze the effect of a mAb delivered intracellularly, this experiment has been largely used as a tool to analyze the binding effect of mAbs to extracellular cancer receptors in the migration and invasive behavior of tumor cells [107]

Finally, these results demonstrate the capacity of C12-HA NCs to incorporate and deliver anti-GSDMB into cancer cells. This approach must represent a promising strategy against the “undruggable targets” and broaden the application of mAbs, confined so far to the target of extracellular proteins.

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Overall discussion

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Conclusions and Future Perspectives

Conclusions

The experimental work enclosed in this manuscript was aimed at designing a new spontaneous emulsification method for the preparation of polymeric nanocapsules as a multifunctional platform for the delivery of conventional anticancer drugs and new biomolecules. The results allowed us to withdraw the following conclusions:

1. The assessment of a “green technology” process for the preparation of nanocarriers was successfully achieved by the development of a self-emulsification method, where nanoemulsions and polymeric nanocapsules were prepared without the need of organic solvents, heat or high energy input. Using these mild conditions, the formation of self-emulsifying systems with less than 150 nm and monodisperse was mainly influenced by components choice and the oil/surfactant ratio.
2. Self-emulsifying hyaluronic acid (HA) based nanocapsules were prepared with two types of polymer: HA and a dodecylamide-functionalized HA. Both systems had similar physicochemical characteristics, presenting a size around 130 nm, a polydispersity index less than 0.2 and a negative charge about -20 mV. The use of a hydrophobically modified HA derivate allowed the formulation of nanocapsules without a cationic surfactant, which resulted in systems with low toxicity and a safer profile.
3. Self-emulsifying HA-based nanocapsules exhibited a satisfactory capacity to encapsulate and release the hydrophobic drug docetaxel in a controlled manner. *In vitro* cytotoxicity assays in A549 cells demonstrated that HA-based nanocapsules showed an improvement in the inhibitory cell viability when compared with the free drug. Moreover, cell uptake assays showed that the internalization of the fluorophore Nile Red was only achieved after its incorporation into the nanocapsules.
4. The monoclonal antibody, anti-gasdermin B (anti-GSDMB), was associated to the polymeric shell of self-emulsifying HA-based nanocapsules and successfully delivered

into the cytosol of HCC1954 breast cancer cells. Once inside the cell, anti-GSDMB-loaded dodecylamide-HA nanocapsules was able to escape early endosomes and effectively block the oncoprotein, gasdermin B, resulting in the inhibition of cancer cell migration and its invasive behavior.

Future perspectives

Cancer is a complex disease and despite all the efforts that researchers and companies have been doing during the last years, it is still worth to continue developing new drug delivery systems with the hope that, maybe this time, we are getting closer to fight cancer.

The development of self-emulsifying HA-based nanocapsules resulted in attractive carriers, from an industrial perspective or a therapeutic application. The method, without organic solvents and heat, becomes advantageous for the pharmaceutical industry each time more concerned about cost-effective and environmentally sustainable technologies. As a drug carrier, these nanocapsules showed adequate capacity to be loaded with small hydrophobic drugs, such as docetaxel, and to promote the intracellular delivery of biomolecules, like antibodies, intended to be the therapeutic payload and not the surface active targeting ligand.

About the intracellular delivery of monoclonal antibodies, this work might represent a change in the way we look at the so called “undrugable proteins”. We face an era where new oncoproteins are continuously being discovered; however, finding an effective molecule against them continues to be the main hurdle. If we can use antibodies against these oncoproteins and specifically deliver them into the cell compartment, we might start a new way of fighting cancer.



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