Universidad de Santiago de Compostela

Facultad de Farmacia

Departamento de Farmacia y Tecnología Farmacéutica

Doctoral Thesis

Hyaluronic acid nanocapsules for the intracellular delivery of anticancer drugs

Reduced version

Ana Cadete Pires

Santiago de Compostela, 2016

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Tesis Doctoral

Nanocápsulas de ácido hialurónico para la liberación intracelular de fármacos antitumorales

Ejemplar reducido

Ana Cadete Pires

Santiago de Compostela, 2016

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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 Benoit

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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 Benoit

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Aos meus pais.

À minha família.

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"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

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 solventes 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 NCs sin la adición de un surfactante catiónico, reduciendo así la posible toxicidad del sistema. Las NCs-HA se mantuvieran estables en condiciones de almacenamiento y después de 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 fármacos modelo: docetaxel, un fármaco citostático incorporado en el núcleo oleoso y una proteína terapéutica asociada a la cubierta polimérica. El docetaxel fue eficientemente encapsulado, manteniendo su citotoxicidad en la línea celular de cáncer de pulmón A549, y mostrando una liberación del sistema de un modo controlado. Finalmente, la proteína terapéutica fue eficazmente asociada 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 proteína terapéutica abandonó el compartimento endosomal y bloqueó de manera efectiva una oncoproteína, promoviendo así una importante reducción de la migración e invasión de las células 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 proteínas terapéuticas, constituyendo una estrategia ambiciosa en la lucha contra el cáncer.

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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 sensible 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 by electrostatic forces, and thus, it reduced the toxicity associated to the addition of a cationic surfactant as counterion. Once formulated, HA-NCs had a size around 130 nm and a negative zeta potential about -20 mV. Moreover, these NCs 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 drug models: docetaxel, a cytostatic drug, was incorporated into the oil core, whereas a therapeutic protein 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, the therapeutic protein was successfully associated to the polymeric shell of HA-NCs and its intracellular delivery confirmed by confocal microscopy. Once inside the cell, the therapeutic protein was able to escape the endosomal compartment and to target an oncoprotein, promoting an important decrease in the migratory and invasive behavior of breast cancer cells. All these results highlight the potential of self-emulsifying HA-NCs as multifunctional systems to carry diverse anticancer drugs, with special emphasis in the intracellular delivery of therapeutic proteins, an ambitious challenge that could open new avenues to fight cancer.

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Résumé

Cette thèse de doctorat avait pour principal objectif le développement d'une méthode viable pour la formulation de nanocapsules d'acide hyaluronique (HA-NCs) 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 HA-NCs 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 HA-NCs par des interactions hydrophobes, réduisant ainsi la toxicité due à l'addition d'un surfactant cationique. Une fois formulées, les HA-NCs 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 drogues modèles : le docétaxel, un agent cytostatique, a été incorporé au sein du cœur huileux, tandis qu'une protéine thérapeutique 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, la protéine thérapeutique a été associée avec succès à l'enveloppe polymérique de HA-NCs et, une fois à l'intérieur de la cellule, la protéine thérapeutique était capable d'échapper au compartiment endosomal et d'effectivement cibler une oncoprotéine, entraînant une importante diminution du comportement migratoire et invasif des cellules de cancer du sein. Tous ces résultats mettent en évidence le potenciel de HA-NCs auto-émulsifiées en tant que systèmes multifonctionnels pour transporter divers agents anticancéreux, en particulier pour la libération intracellulaire de protéines thérapeutiques, une approche ambitieuse qui pourrait passer au premier plan parmi les stratégies innovantes dans la lutte contre le cancer.

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Resumen *in extenso*

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Resumen *in extenso*

Introducción

El cáncer es una de las mayores causas de morbilidad y mortalidad en todo el mundo, resultando en más de 9 millones de muertes al año. Pese a los avances en investigación y el descubrimiento de nuevas dianas y moléculas terapéuticas, la cura del cáncer aún no es una realidad. Por tanto, es necesario seguir invirtiendo en el desarrollo 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 no específica, donde apenas una pequeña fracción del fármaco llega al tumor. Esto hace que dichos tratamientos sean ineficaces en muchos de los casos y 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 puedan 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 un área multidisciplinar que pretende utilizar plataformas nanométricas como transportadores de fármacos quimioterapéuticos, permitiendo así su liberación 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. Sin embargo, en los últimos años la atención se ha centrado considerablemente en el estudio de las nanocápsulas poliméricas con potencial aplicación en oncología. Las nanocápsulas (NCs) 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

promover una mejor protección del fármaco, controlar su liberación y orientar 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 NCs poliméricas se encuentra formando parte de la cubierta.

El HA es un polisacárido de origen natural compuesto 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 no tóxico, biocompatible y biodegradable. Además, su carácter aniónico (pKa = 3 – 4) le permite interaccionar con otros polímeros catiónicos, lípidos o tensoactivos, hecho mediante el cual tiene lugar 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 importantes características 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 interaccionar 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 orientar moléculas terapéuticas a células cancerosas, un efecto conocido como "vectorización activa".

Adicionalmente, cabe destacar que en la selección de un proceso de formulación, 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 formulación como, por ejemplo, el desplazamiento del solvente. Utilizando este método, las nanoemulsiones se producen en ausencia de solventes orgánicos, calor o energía, proporcionando la posibilidad de incorporar moléculas sensibles como

proteínas, péptidos o anticuerpos sin que sean degradados durante el proceso de formulación. El mecanismo 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, un alto rendimiento de formulación, un potencial escalado industrial y un bajo impacto ambiental, hecho por lo cual es considerado como "tecnología sostenible o tecnología verde". Por otro lado, las formulaciones basadas en este método pueden ver limitado su uso debido a una importante cantidad de tensoactivo, así a una baja solubilidad del fármaco en la fase oleosa.

El progreso en investigación ha llevado al descubrimiento de nuevas dianas terapéuticas como, por ejemplo, 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 tratamiento adecuado al "targeting" de las proteínas intracelulares.

El objetivo general de este trabajo se ha orientado al desarrollo de NCs-HA, diseñadas como una plataforma multifuncional para la incorporación de distintos fármacos antitumorales y su liberación dentro de las células cancerosas. Los objetivos específicos se han organizado de la siguiente manera:

1. Desarrollo de un método de auto-emulsificación adaptado a la formulación de NCs-HA, utilizando dos tipos de HA: un HA nativo y un HA-modificado con una molécula lipídica.
2. Incorporación en el núcleo oleoso de un fármaco hidrofóbico modelo, el docetaxel (DCX).
3. Asociación de una proteína terapéutica en la cubierta polimérica, destinada a ser liberada en el interior de las células tumorales y a bloquear una onco-proteína, responsable de la migración e invasión de las células tumorales.

1. Desarrollo de un método de auto-emulsificación para la formulación de NCs-HA

1.1 Métodos

Las NCs se han preparado por el método de auto-emulsificación utilizando un HA nativo (nat-HA) y un HA modificado con una cadena lipídica (mod-HA). El método ha sido inicialmente optimizado para la formulación de nanoemulsiones (NEs) y, posteriormente, adaptado para la preparación de las NCs. En primer lugar, se seleccionaron los compuestos más adecuados para la preparación de las NEs sin solventes orgánicos, eligiendo el núcleo oleoso y los tensoactivos más apropiados. A continuación, se estudiaron distintos parámetros que influyen en la formación del sistema: la cantidad de tensoactivo en la fase acuosa, el ratio óleo/tensoactivo en la fase oleosa y, por último, el ratio fase oleosa/fase acuosa. Una vez elegida la composición y ratios adecuados para la formulación de las NEs, las NCs fueron preparadas de la misma manera, pero incorporando el polímero en la fase acuosa. Los parámetros analizados en la preparación de las NCs fueron: la cantidad de tensoactivo catiónico (CTAB) en la fase oleosa y la concentración de HA en la fase acuosa, que fueron ajustados para la obtención de formulaciones con un tamaño nanométrico, menor a 150 nm, un índice de polidispersión inferior a 0.2 y una carga superficial negativa. Una vez preparadas, las NCs fueron aisladas por cromatografía de exclusión de tamaño, caracterizadas por espectroscopía de correlación fotónica (Zetasizer Nano ZS, Malvern) y su morfología evaluada mediante microscopia electrónica de transmisión (TEM, CM12, Phillips). La toxicidad de las NCs y su capacidad de internalización en las células tumorales fue evaluada *in vitro* utilizando como modelo las células de cáncer de pulmón A549 y evaluada utilizando el método de viabilidad celular Alamar®Blue. Para los ensayos de internalización, las NCs fueran preparadas con un fluoróforo en su núcleo oleoso, el Nile Red (NR), y su capacidad de internalización observada por microscopía confocal.

1.2 Resultados

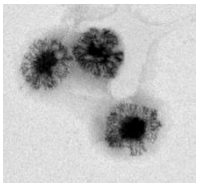
En primer lugar, la técnica de auto-emulsificación fue optimizada para la formulación de las NEs y la selección de los componentes se basó en unas propiedades físico-químicas adecuadas para la formulación de un sistema sin solventes orgánicos. Así, la fase oleosa

fue compuesta por Miglyol®812 y Tween®80 y, la fase acuosa, por agua y 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 DCX, permitiendo así su incorporación en el núcleo oleoso de las NCs. Respecto al tensoactivo, el Tween®80 fue seleccionado porque es uno de los tensoactivos más utilizados en procesos de auto-emulsificación. Tiene un balance hidrofilia-lipofilia (HLB) de 15 que le confiere una gran hidrofilia, favoreciendo la formación inmediata de nanoemulsiones aceite/agua. Comparando con otros tensoactivos similares, el Tween®80 presenta la ventaja de estar ya aprobado para administración por vía parenteral. La selección del Solutol®HS15 para formar parte de la fase acuosa está relacionada con sus propiedades físico-químicas y biológicas. Tiene un HLB 14-16, lo 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 fue optimizado y las NEs preparadas de acuerdo con el siguiente proceso: la fase oleosa, compuesta por Miglyol®812/Tween®80 (ratio 1:1 p/p) se añadió a una fase acuosa, constituida por una solución de Solutol®HS15 a 2.5 mg/mL. La fase oleosa fue añadida a la fase acuosa en un ratio 1:8 (v/v), bajo agitación magnética.

Las NCs-HA fueron preparadas utilizando el proceso de auto-emulsificación previamente descrito y su formulación fue inicialmente optimizada para el HA nativo. La incorporación del nat-HA a la superficie de las NCs fue conseguido mediante interacciones electrostáticas entre el polímero, cargado negativamente, y la superficie de las partículas modificadas con un tensoactivo catiónico, CTAB. La cubierta con el nat-HA (0.25 mg/mL) resultó en una inversión del potencial zeta de +10 mV, en las NEs catiónicas, a -18 mV después de la formulación de las NCs. Para evitar el uso del tensoactivo catiónico, muchas veces asociado a toxicidad celular, el nat-HA fue sustituido 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 mod-HA NCs presentaron características muy semejantes a las formuladas con el nat-HA. Sin embargo, fue necesario añadir 0.5 mg/mL

de mod-HA para conseguir un zeta potencial en torno a -20 mV. En la **Tabla 1** se representan las características físico-químicas de los sistemas formulados por auto-emulsificación y una imagen de las NCs de mod-HA. La imagen muestra una estructura núcleo-cubierta característica de las NCs.

Tabla 1. Caracterización de las distintas formulaciones preparadas por auto-emulsificación e imagen de las NCs de mod-HA por 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	
Nat-HA NCs	137 ± 11	0.2	-19 ± 1	
Mod-HA NCs	126 ± 5	0.2	-20 ± 2	

Nota: Los resultados se expresan en media ± desviación estándar (n=3)

Abreviaturas: PDI, índice de polidispersión; NE, nanoemulsicón; NCs, nanocáspulas; Nat-HA, ácido hialurónico nativo; Mod-HA, ácido hialurónico modificado

Los ensayos de toxicidad mostraron que, independientemente de la composición de los sistemas, las NCs formuladas con nat-HA o mod-HA no afectan la viabilidad celular de las células A549 hasta una concentración de 350 µg/mL. Sin embargo, a concentraciones mayores de hasta 1000 µg/mL, solo las NCs preparadas con mod-HA resultaron no tóxicas. Estos resultados podrían estar relacionados con la presencia del tensoactivo CTAB en las NCs de nat-HA, el cual se caracteriza por su toxicidad celular. Por otro lado, la mezcla de tensoactivos compuesta por Tween®80, Solutol®HS15 y CTAB a 350 µg/mL, resultó en una acentuada toxicidad celular, con 85% de muerte celular. Esto indica que los tensoactivos libres en solución presentan una toxicidad muy elevada que se ve disminuida una vez que estos son incluidos en la estructura de las NCs. Las NCs resultaron ser más seguras y menos tóxicas. La **Figura 1** muestra el perfil de toxicidad de las distintas formulaciones, en células A549, después de 72h de exposición.

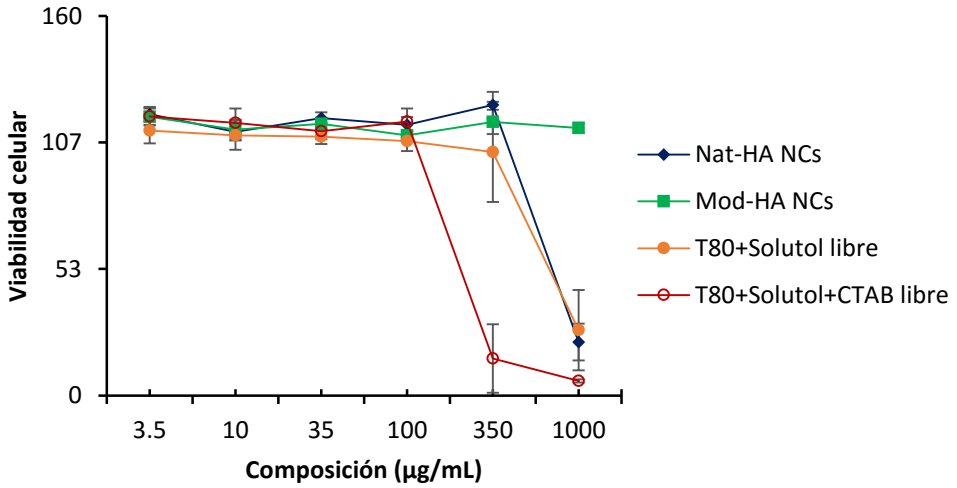


Figura 1. Toxicidad celular de las nanocápsulas y de las mezclas de tensoactivos en células de cáncer de pulmón, A549, trás 72h de incubación.

Los resultados se expresan en media ± desviación estándar (n=6)

Abreviaturas: NCs, nanocápsulas; Nat-HA, ácido hialurónico nativo; Mod-HA, ácido hialurónico modificado; T80, Tween®80

La capacidad de internalización de las NCs fue estudiada por microscopía confocal utilizando NCs cargadas con NR. Como control, las células fueron expuestas a una solución de NR libre, la cual no fue internalizada (**Figura 2B**). Una vez encapsulado, las NCs consiguieron penetrar la membrana celular y liberar dentro de las células una gran cantidad de NR, lo cual se confirmó mediante la elevada fluorescencia observada en el citoplasma celular (**Figura 2C**). Esta internalización está probablemente mediada por un proceso de endocitosis asociado a los receptores CD44 expresados en la superficie de las células A549.

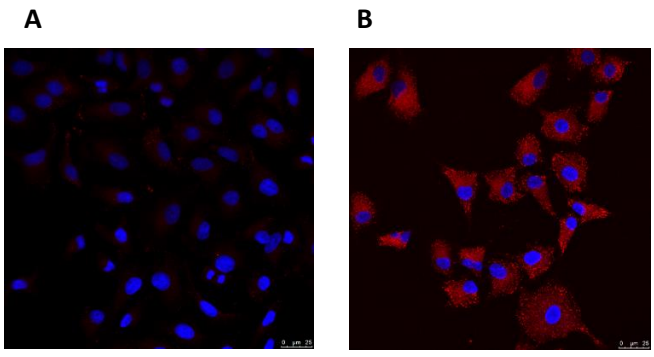


Figura 2. Estudios de internalización del fluoróforo *nile red* solo (a la izquierda) y de las HA-NCs cargadas con él (a la derecha).

2. Desarrollo de las NCs-HA para la incorporación del DCX en el núcleo oleoso

2.1 Métodos

En primer lugar, se hicieron estudios de solubilidad del DCX en Miglyol®812. Para ello, un exceso de DCX fue mezclado con Miglyol®812 mediante agitación magnética. Tras 24h, la suspensión de DCX en Miglyol®812 fue centrifugada y el DCX solubilizado cuantificado mediante una técnica de HPLC. La formulación de las NCs-HA cargadas con DCX se hizo de acuerdo con el procedimiento anterior, utilizando como núcleo oleoso el Miglyol®812 con DCX. Las características físico-químicas de las NCs se han analizado en términos de tamaño, indíce de polidispersión y potencial zeta. El fármaco libre se ha separado del encapsulado mediante cromatografía de exclusión por tamaño y la eficacia de encapsulación (EE%) calculada directamente de acuerdo con la siguiente ecuación: [fármaco encapsulado]/ [fármaco total] x 100. La liberación del DCX de las NCs fue evaluada después de diluir las NCs en PBS a 37ºC y de acuerdo con un método de transferencia del fármaco de una suspensión de NCs para una fase oleosa externa, capaz de solubilizar el fármaco libre. La actividad del fármaco encapsulado fue confirmada mediante ensayos de toxicidad en células A549.

2.2 Resultados

La solubilidad del DCX en Miglyol®812 fue de 2.03 ± 0.2 mg/mL. De acuerdo con estos resultados, se preparó una solución madre de DCX en Miglyol®812 a 1.8 mg/mL, garantizando la solubilidad total del fármaco y evitando su precipitación. Una vez preparadas, las NCs cargadas con DCX mantuvieron sus características físico-químicas y una elevada EE, alrededor del 90%, que se corresponde con una dosis de DCX en las NCs de 100 µg/mL **(Tabla 2**).

Tabla 2. Caracterización físico-química de las NCs-HA cargadas con DCX.

Nanocápsulas	Tamaño (nm)	PDI	Potencial Zeta (mV)	EE (%)
Nat-HA	140 ± 5	0.2	-18 ± 2	88 ± 9
Mod-HA	145 ± 6	0.2	-20 ± 1	86 ± 3

Nota: Los resultados se expresan en media ± desviación estándar (n=3)

Abreviaturas: PDI, índice de polidispersión; Nat-HA, ácido hialurónico nativo; Mod-HA, ácido hialurónico modificado; EE, eficiencia de encapsulación del DCX

En ambas formulaciones de NCs, preparadas con nat-HA o mod-HA, se produce una liberación rápida inicial de 45% y 55% de DCX, respectivamente. Sin embargo, los dos prototipos fueron capaces de mantener la liberación del DCX hasta 24h, con una liberación del 70% de ambos sistemas. Este perfil de liberación se puede justificar por la propia estructura de las NCs, la cual favorece una partición del fármaco entre el núcleo oleoso y el medio externo. Además, la capa polimérica evita su liberación inmediata, favoreciendo la retención del fármaco en el núcleo oleoso.

Las NCs-HA cargadas con el DCX demostraron una mejor inhibición de la viabilidad celular (IC50) en comparación con el fármaco libre. El IC50 para el fármaco encapsulado en las NCs-HA se correspondió con una concentración de 10µM trás 48h de incubación. Por otro lado, el fármaco libre no llegó a alcanzar el IC50 para a la misma concentración y durante el mismo tiempo de estudio (**Figura 3**). Las NCs-HA aparecen como nanosistemas prometedores para la incorporación y liberación del DCX dentro de las células tumorales, promoviendo una mayor toxicidad que el fármaco libre.

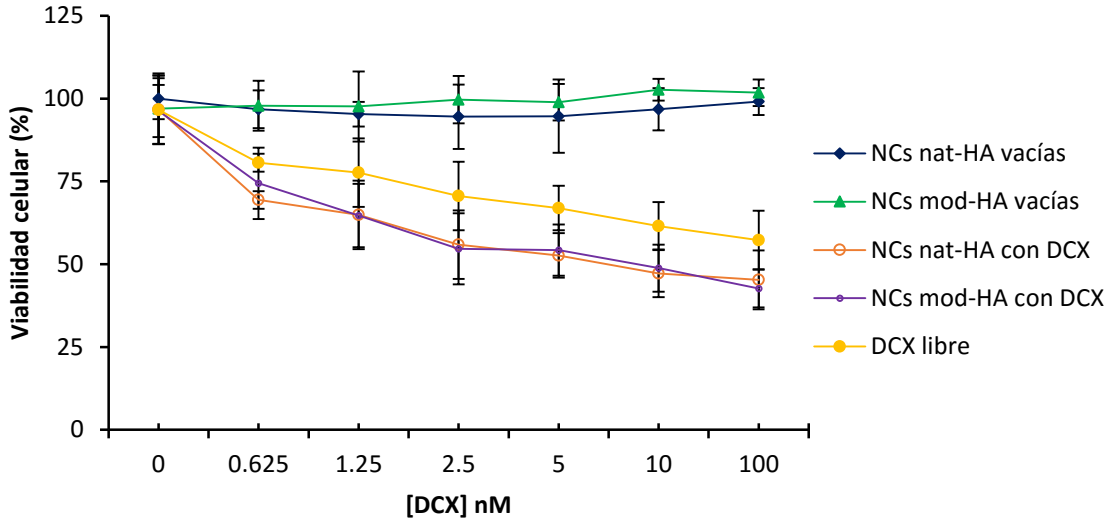


Figura 3. Toxicidad celular de las nanocápsulas cargadas con DCX en las células de cáncer de pulmón, A549, después de 48h de incubación.

Los resultados se expresan en media ± desviación estándar (n=6)

Abreviaturas: NCs, nanocápsulas; Nat-HA, ácido hialurónico nativo; Mod-HA, ácido hialurónico modificado; DCX

3. Asociación de una proteína terapéutica a la cubierta de las NCs-HA

Los contenidos de esta sección se han eliminado por motivos de confidencialidad.

Conclusiones

En este trabajo hemos demostrado el potencial de las NCs-HA como sistemas multifuncionales para la incorporación de diversos fármacos antitumorales y su liberación dentro de las células cancerosas. Las NCs-HA se desarrollaron mediante un nuevo método de auto-emulsificación que emerge como una tecnología sostenible y con bajo impacto medioambiental, ya que evita el uso de solventes orgánicos. Las NCs se han preparado utilizando dos tipos de HA, un HA nativo y un HA modificado químicamente con una molécula lipídica. El mod-HA permitió obtener formulaciones de nanocápsulas más seguras y menos tóxicas debido a la ausencia del tensoactivo catiónico. Estas NCs representan un sistema versátil para la incorporación de distintos fármacos, como el DCX o proteínas terapéuticas.

Introduction

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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. As a consequence, industries and researchers took hands and are working together with one purpose: fight 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 there has been an increase attention in “green technology” and the development of environmentally friendly techniques. Furthermore, nanotechnology have powered research 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 discuss how hard is to overtake conventional cytostatic drugs with nanotechnology and how reasonable is 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 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]. GSK is in a mission: 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 solvent 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 formulation 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 droplet 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 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]. As such, 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].

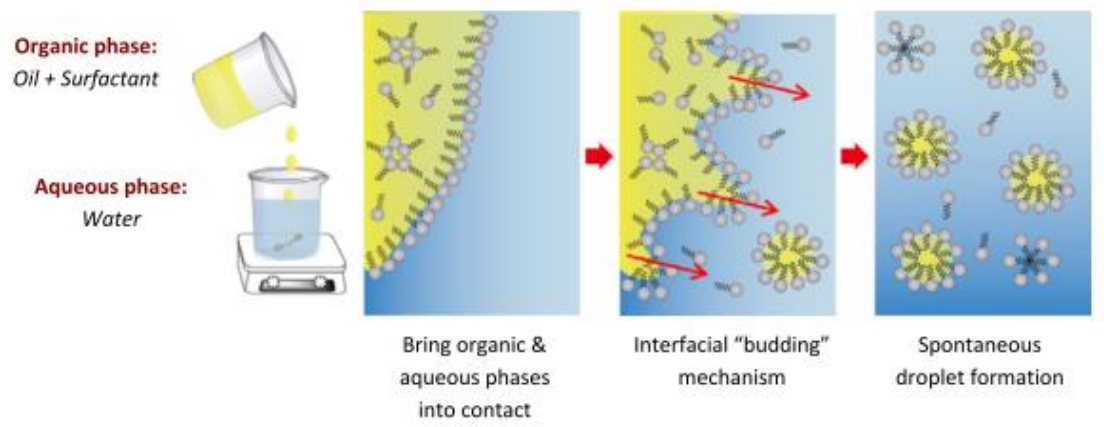

Figure 1. Schematic representation of a proposed mechanism for spontaneous emulsification: fine oil droplets are spontaneously formed when an organic phase containing a surfactant is mixed with an aqueous phase. The surfactant moves from the organic phase to the water phase (red arrows), leading to interfacial 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 formulation 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 high hydrophilic-lipophilic balance (HLB) values are usually applied for the formulation of self-emulsifying systems. The commonly used emulsifiers are various ethoxylated polyglycolyzed 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 [27]. 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 and limit its choice for the intended route of administration [28]. 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 surfactant monolayer providing additional fluidity to the interfacial film and disrupting the liquid crystalline phases [29]. 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 [30]. 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]. By resulting in 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 [31]. 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 [32]. 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 [33].

Several studies have reviewed the formulation of SEDDS for enhanced oral bioavailability of chemotherapeutic drugs, mainly paclitaxel [33–35], docetaxel (DCX) [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 fivefold compared to the marked formulation and the excipients presented a significantly reduced cytotoxicity [34]. In another study, 9-Nitrocamptotothecin (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 [43]. More 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 [44][45]. Recently, 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 [46].

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, isotropic and

low viscous. 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 the 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 component choice and the technical possibilities for formulating these systems [29].

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 [47]. For hydrophobic compounds, its incorporation into the oil phase can provide high encapsulation efficiency, great stability and avoid drug precipitation [48]. Additionally, the preparation process without solvents or heat can greatly decrease the production cost [49].

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 [50]. 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 [51].

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*

DCX 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 DCX is Taxotere®, which is composed of DCX and high quantities of surfactant and ethanol. As a consequence of its formulation composition, its efficacy is counterbalanced with serious side effects, including acute hypersensitivity reactions, cumulative fluid retention, neurotoxicity, among others [52]. To overcome secondary effects and improve DCX 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 [53]. 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 DCX, 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 DCX into the tumors through passive and active targeting strategies [54].

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

Table 1. Nanoformulations for DCX 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	[59]
Docetaxel-PNP	Polymeric NPs	Samyang	Phase I/II	[59–60]
CRLX-301	NP-drug-conjugates	Cerulean	Phase I/IIa	[62]
DEP-Docetaxel	Dendrimers	Starpharma	Phase I	[63]
AT-1123	Liposomes	Azaya Therapeutics	Phase II (soon)	[64]
Docecal	Micelles	Oasmia	Phase I (soon)	[65]

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 DCX, 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 DCX, leading consequently to a higher tumor accumulation and improved anti-tumor efficacy [66]. Preliminary Phase II studies in 40 patients with advanced metastatic non-small cell lung cancer (NSCLC) treated with 60mg/m^2 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 DCX [67]. 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 DCX 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 DCX 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 [68]. Cerulean has developed CRLX301, a self-assembled DCX 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 [69].

DEP-docetaxel comprises DCX 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® [70]. ATI-1123 is a liposomal formulation of DCX 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 [71]. Finally, Docecal from Oasmia will start a Phase I clinical stage this year [65].

4. Therapeutic proteins 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*

Part of this section was eliminated due to the strict connection with the confidential information.

4.1 Intracellular cancer-causing proteins (oncoproteins)

Oncogenes are a family of genes responsible for the mutation and dysfunctional 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 [72]. 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 [73]. 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 [72]. RAS (GTPases) [74], non-receptor tyrosine kinases (like Bcr-Abl) [75], BRAF [76] or heat shock proteins (like HSP90 that interacts and stabilize mutant p53) [77] are some examples of these proteins.

4.2 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 [78]. 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 [79]. In addition, protein kinases are attractive cancer targets as they are closely involved with tumor cell proliferation and survival [80]. It is important to note that the discovery of receptor tyrosine kinases as cancer targets has turned the way to the development of therapeutic antibodies that inhibit receptor activation however, for non-receptor tyrosine kinases, the majority of kinase inhibitors discovered to date are ATP competitive and almost exclusively directed against the ATP binding site of the kinase, which results in drug resistance and lack of inhibitory selectivity and efficacy [81]. 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 [84]. The small size of scFv and 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 beneficiating from a specific inactivation of the protein [85]. 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 [86]. 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 [87]. So far, these difficulties have limited the clinical application of intrabody therapies.

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Chapter 1

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

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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 for the design of multifunctional structures, which are 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 the targeted delivery of cytostatic drugs, polynucleotides, combination therapies, immunomodulation and theranostics. In the end, we will discuss the main advances in the technology that are enabling these carriers towards the clinical development.

Keywords: hyaluronic acid, cancer nanotechnology, immunotherapy, translational research

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1. Introduction

Progress in cancer research has led to the development of a wide array of anti-cancer agents, from the well-known cytostatic drugs to complex molecules, such as peptides, proteins and polynucleotides. Unfortunately, the pharmacological effect of these molecules has been highly compromised by a number of problems, including poor solubility, inadequate biodistribution and, ultimately, limited efficacy associated to a significant toxicity. To address these limitations, diverse drug delivery systems have been designed with the purpose to carry, protect and control the delivery of therapeutic drugs [1]. Of those, polymeric nanocarriers have been extensively studied, especially those made of biocompatible and biodegradable polymers. In particular, HA-based nanocarriers have gained a significant attention, as noted by the increased number of publications in the field, especially in the last five years (**Figure 1**). The versatility of HA has allowed the design of multifunctional nanocarriers specifically tailored for the incorporation of diverse molecules. Within this frame, although cytostatic drugs continue to be of great interest, other molecules, such as immunomodulators, and polynucleotides are raising significant expectation. In this review, we first outline the physicochemical and functional characteristics of HA, which make it a suitable biomaterial for the design of the anticancer targeted nanocarriers. Then, we critically analyzed the potential the most recent nanocarriers developed for the delivery of cytostatic drugs and polynucleotides, single or in combination, as well as immunostimulants and imaging agents. The review ends with the evaluation of the candidates undergoing clinical assessment and the prospective of the therapeutic impact of HA-based nanocarriers for successful anticancer therapy.

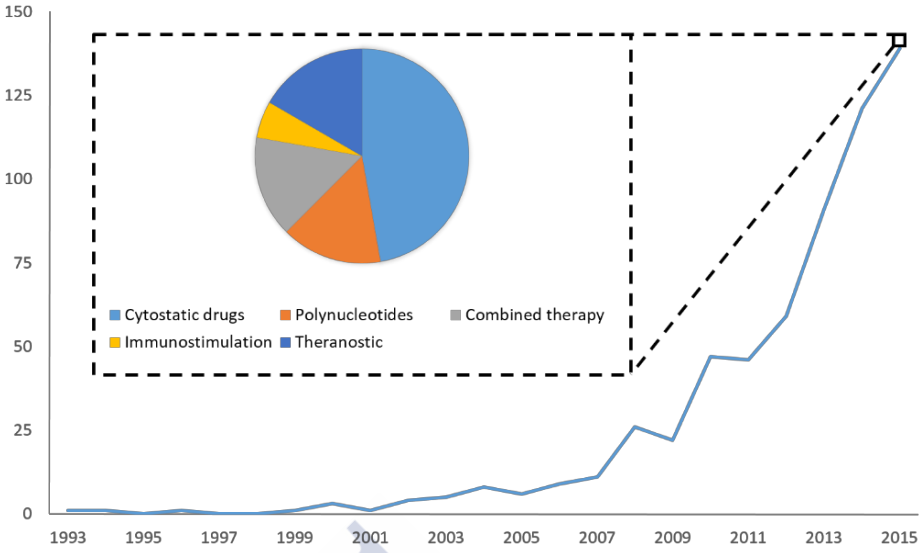

Figure 1. Evolution in the study of HA-based nanocarriers for anticancer drug delivery since the 90's. In 2015, almost 150 publications were released for the application of HA for the delivery of cytostatic drugs or polynucleotides, for combined therapy, as immunostimulating vehicles and for theranostic. Data from Scopus (1993-2015) with the words "hyaluronic" 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 >10^6Da is the main component of the extracellular matrix of mammalians and it is responsible, among others, for cell division, adhesion and matrix renovation [3]. These cellular events are mainly regulated by two major cell-surface receptors for HA, the 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]. Among these receptors, CD44 is the most studied one from the drug delivery perspective due to its abnormal overexpression in a large number of solid tumors [7].

From the physicochemical point of view, HA exhibit 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, a result of

which is the formation of a variety of nanostructures. Finally, HA holds reactive functional groups, which offer the possibility of obtaining a variety of HA-based derivatives with modulated properties and targeting capacities [10].

In general, HA is known as 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 to the consideration of HA as an immunostimulatory material.

The good biocompatibility and immunotolerance of HA is further illustrated by the fact that it is part of a number of marketed products since 2003, first as a dermal filler and, later as a biomaterial for surgery, 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, as it can be extracted from animal tissues, and it can also be produced by microbial fermentation. The latter one is nowadays the main source of HA for pharmaceutical purposes because it results in the production of reproducible batches of highly purified polymer [17], with a broad range of molecular weight grades, going from 4 Da and up to 5,000 KDa [18].

3. Design of HA-based nanocarriers for cancer therapy

HA have been engineered to deliver anticancer drugs using different strategies. HA can be directly conjugated to therapeutic molecules, self-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. Particularly, this technological approach has so far resulted in: (i) an enhancement of the drug solubility and stability in biological fluids, (ii) an improvement of the pharmacokinetics profile through the increase in the blood circulation time (passive targeting) and, (iii) an improvement of the biodistribution pattern, based on HA ability to target tumor cells (active targeting).

The concept of **passive targeting** is associated to the ability of the nanocarrier to circulate in the blood stream for extended periods of time, so that the nanocarrier has the chance to passively diffuse 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 providing the nanocarriers with a hydrophilic polyethyleneglycol (PEG) coating [19]. In this sense, some authors have argued that HA might also have this stealth role [20], however, this issue needs to be further explored as this behavior might be highly dependent on the molecular weight (MW) of HA. 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 higher MW (1,200 KDa) was known to be rapidly eliminated through the liver and kidneys. This mechanism of elimination has been associated to the high affinity of HA to the HA receptor for endocytosis, HARE-1, mostly located in the liver and spleen [21].

While the passive targeting mechanism needs to be further elucidated, the **active targeting** has been well justified by the binding affinity of HA to specific receptors overexpressed by cancer cells [22]. Indeed, it is well known that HA has the capacity to 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 has been less investigated [23]. The main strategy to target CD44 has been described by the use of HA as a drug carrier. As such, HA can bind CD44 receptors, be internalized and effectively transport anticancer drugs to the intracellular compartment [24]. It is important to highlight 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, in an *in vitro* CD44-mediated cell uptake study with HA-coated liposomes, it was concluded that the binding affinity was higher for high MW (HMW) HA (175-350 KDa) than for LMW HA (up to 150KDa) [25]. These results were in agreement with a previous report showing that

HMW HA-coating lipid nanoparticles improved ligand accessibility towards CD44 [13]. The authors justified these results by the fact that larger molecules may have a greater chance to interact with CD44 receptors than smaller molecules [26]. Nevertheless, *in vivo*, the tumor binding affinity might counter-balance the fast clearance of HMW HA when compared to 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 longer time in circulation. This fast clearance must be explained by the high affinity of HMW HA to HARE-1 receptors expressed in the liver, which results in a fast elimination when compared to LMW HA-coated nanoparticles [27].

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

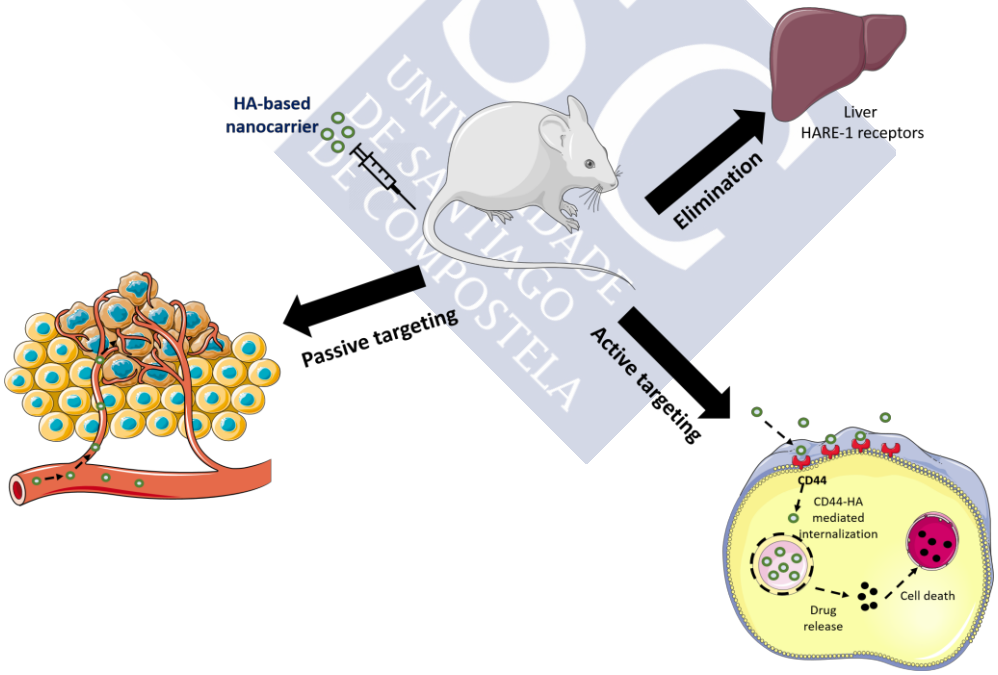

Figure 2. Design of HA-based nanocarriers to achieve the optimal antitumor efficacy. The optimal response must be achieved by a balance between the clearance and the target affinity of HA-based nanocarriers.

4. HA-based nanocarriers for the delivery of anti-cancer drugs

HA-based nanocarriers are being developed as suitable carriers for the delivery diverse therapeutic molecules, such as cytostatic drugs, polynucleotides, immunostimulating molecules and imaging agents. HA can be directly conjugated to therapeutic drugs, self-assembled into micelles, forming polymeric nanoparticles or decorating lipid and magnetic carriers. **Figure 3** represents the leading HA-based nanocarriers for anticancer drug delivery.

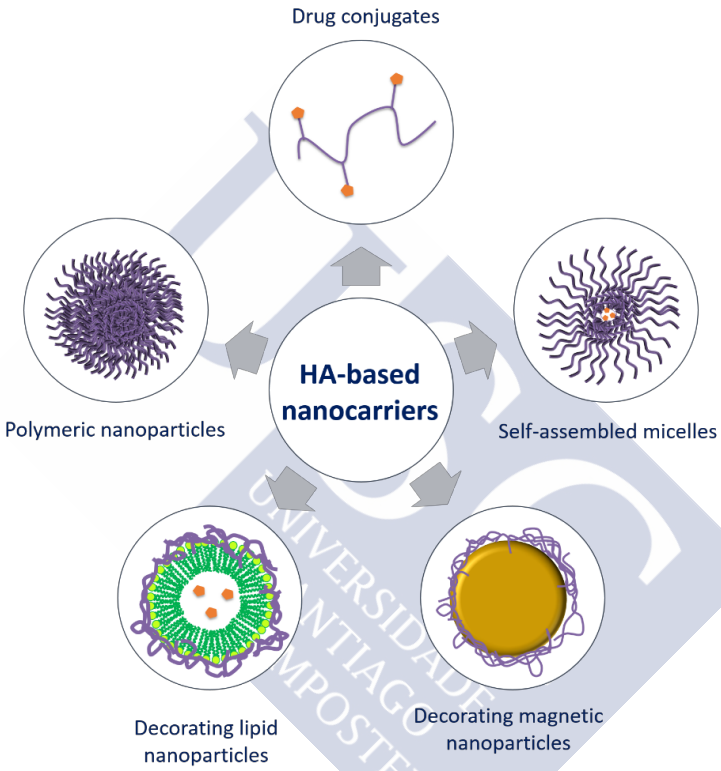

Figure 3. Leading HA-based nanocarriers for anticancer drug delivery. HA can be chemically conjugated with therapeutic drugs to form HA-based drug conjugates or with a hydrophobic molecule to self-assemble in micelles. HA can ionically interact with other polymers to formulated polymeric nanoparticles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

4.1 Delivery of cytostatic drugs

Cytostatic molecules are recognized as very efficient anticancer drugs; however, 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].

4.1.1 HA-drug conjugates and complexes

As indicated, HA holds reactive functional groups useful for the chemical modification with, among others, small cytostatic drugs [29]. As such, **HA-drug conjugates** need to be adequately designed in order to preserve the activity of the drug while maintaining the inherent HA properties and notable capacity to bind to CD44. For example, an extended degree of substitution can result in HA-CD44 low binding affinity [30]. Moreover, the polymer can lose its aqueous solubility which can influence the biodistribution of the system [31]. The most recent studies report the conjugation of HA with different cytostatic drugs, among them docetaxel [32], camptothecin [31, 33], doxorubicin [30] and paclitaxel [34–35] which have led to some promising data. 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 unloaded drug [36]. Nevertheless, the bioconjugate resulted inefficacious 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, following 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 longer than the controls (49 days for HA-paclitaxel comparing with 42 and 37 days for paclitaxel or non-treated mice, respectively) and significant reduction of the lesion burden in 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, the redox potential of a HA-drug conjugate was investigated for the crosslinking of HA with

paclitaxel using disulfide bonds, which resulted in the rapid release of the drug in the presence of glutathione and a significant tumor suppression *in vivo* [39].

Owing to the hydrophilic properties of HA and the hydrophobic character of most cytotoxic drugs, HA-drug conjugates are expected to self-assembled into micelles in aqueous solution. As such, HA was conjugated with quercetin [40] and doxorubicin [41] which formed self-assembled micelles when dissolved in water. HA-quercetin self-assembled micelles resulted in a 20-fold increase in the half-life and 5-fold increase in the area under the curve, when compared with the free drug [40]. Although HA-drug conjugates can self-assemble into micelles, the majority of the publications concerning HA-based micelles are described for the conjugation of HA with a hydrophobic molecule, as report in the next paragraph.

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], 5β-cholanic acid [45], copoly(styrene maleic acid) [46] and cholesteryl [47–48] for the delivery of diverse 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 inhibiting P-gp pump and overcoming multi-drug resistance [50–51]. As a result, a multifunctional nanoparticle composed of HA-α-TOS (HT) conjugate and D-α-tocopheryl polyethylene glycol succinate (TPGS) with docetaxel loaded in its hydrophobic core demonstrated, *in vivo*, a higher tumor tissue accumulation and a

pronounced anti-resistance tumor efficacy in resistance breast cancer xenograft tumor compared with the commercial formulation, Taxotere® [52].

Finally, self-assembled conjugates can also be tailored in order 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 retarded under physiological conditions (pH 7.4), whereas 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. The system was very stable in circulation, leading to 30-fold higher plasma concentration than the free drug, and a consequent tumor accumulation which resulted in 60% tumor growth inhibition [56].

4.1.3 HA-based nanoparticles

Polymeric nanoparticles, consisting of a matrix of HA and additional counter ion polymers, have been mainly proposed 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 hepatocyte HepG2 cells [59]. Curcumin loading HA-chitosan nanoparticles resulted in a strong dose dependent cytotoxicity and high uptake efficiency in C6 cells [58]. The same kind of nanoparticles were also evaluated for the targeted delivery of 5-fluorouracilo, following oral administration. Here, the HA targeting affinity to colon cancer cells was combined with the mucoadhesive properties of chitosan [60].

A different nanoparticle composition is 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 85% of 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 be incorporated into the liposomes during its preparation [66], or after their formation by simple incubation [67]. Liposomes have also been decorated with HA conjugated to 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 sense, it was found that 5% PEG coating was 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 have claimed that the coating of this carriers with HA contributed to their steric stabilization and a substantial amount of doxorubicin was still detected in plasma of mice 72h post-administration. After 24h post-i.v. injection, around 25% of the dose injected via GAGs was accumulated into the tumor, when compared with less than 0.5% when free doxorubicin was administered. As a consequence, the encapsulated drug significantly attenuated the growth of the tumors relative to the free drug, without any clinical toxicity. The authors justify these results by three main reasons: the hydrophilicity of the HA shell conferred 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 was also 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-fluorouracilo [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, to MUC-1 binding DNA aptamer [74]. Unfortunately, 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 to 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 superior efficacy was attributed to the combined target affinity of HA to CD44 receptors and the tumor tissue penetration conferred by the peptide [75].

4.2 Delivery of polynucleotides

The growing interest of small and micro interfering RNAs (siRNA and miRNA) in cancer therapy has encouraged significant activity in the drug delivery field intended to overcome critical biological barriers, that these nucleic acid-based molecules need to confront before reaching their target. These barriers include their clearance by the mononuclear phagocytic system (MPS), followed by their limited access to the tumor cells, and their degradation all along this pathway as well as 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 have demonstrated great potential to promote intracellular delivery of siRNA/miRNA. By condensing anionic nucleic acids into the cationic chain, these positive charged polyplexes protect genetic material from enzymatic degradation and enhance cellular penetrance. On the other hand, the high positive charge density contributes to 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 the entrapment of the material into a polymeric/lipidic matrix or by decorating the surface of polynucleotide loaded nanocarriers. As described in the next sections, specific 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 system in order to mask their positive charge and to provide a hydrophilic PEG corona. The *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. *In vivo*, the mice treated with the siRNA loaded nanoparticles together with paclitaxel resulted in a 3-fold smaller tumor volume than the mice treated with paclitaxel alone. These results reflected an increase in the chemosensitivity to paclitaxel in mice treated with HA-PEI/HA-PEG/MDR1 siRNA and the ability of the system to deliver siRNA *in vivo* [79]. A simpler composition is the one 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 was quantitatively examined in tumor tissues and the results showed that the miR-34a expression level increased 200-fold for the mice treated with encapsulated miRNA [80].

4.2.2 HA-based nanocomplexes

The conjugation of HA with lipophilic molecules has not only be confined for the delivery of hydrophobic drugs. Recently, **self-assembled** HA-cholesterol [81] and HA-5β-cholanic acid [82] nanocomplexes were described as suitable reservoirs for the delivery of siRNA. For this, 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-5β-cholanic acid micelles with a RNA receptor, the DPA/Zn, which promoted the incorporation of siRNA into the self-assembled carrier [82]. Based on the observed 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 evaluated for *in vitro* cytotoxicity and gene silencing efficacy in HeLa-cells. Comparing to commercialized transfection reagents, HA-siRNA/cationic nanoparticles demonstrated a remarkable safety as a delivery vehicle for siRNA, as well as a 10-fold higher therapeutic index (LC50/IC50), confirming its feasibility 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 greater *in vivo* stability and tumor targeting ability when compared with the complex in which HA was physically adsorbed [89]. In a different study, it was reported that the graft of HA to cationic lipoplexes resulted in a multilayer system, with the siRNA entrapped within the multilamellar structures, surrounded by the polymer. The binding affinity towards CD44 receptors of

non-coated and 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 an drastic reduction in the PLK1 mRNA levels and an increased survival 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 cancer chemoresistance, which is responsible for the most failed causes 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** represents examples of multifunctional HA-based nanocarriers for the delivery of cytostatic drugs and cytostatic drugs together with siRNA therapies.

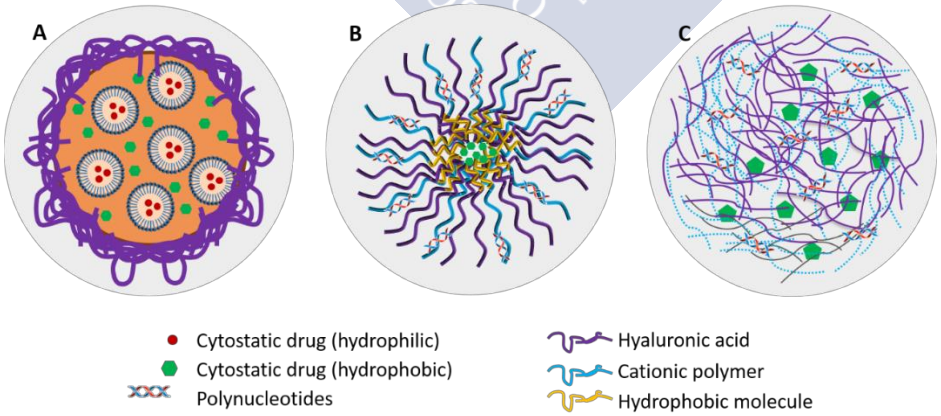

Figure 4. Multifunctional HA-based nanocarriers for the co-encapsulation of different drugs. A) HA coating w/o/w nanoparticles for the co-delivery of hydrophilic and hydrophobic drugs. B) Self-assembled HA-micelles for the delivery of a hydrophobic drug (inner core) and siRNA (ionically attach to a cationic polymer). C) Polymeric nanoparticles prepared from electrostatic interactions between negatively charged HA and siRNA and 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 as they are able to avoid drug incompatibility, achieve adequate pharmacokinetics profiles and overcome multidrug resistance [92–93]. The combinatorial effect can be achieved by mixing different HA-drug conjugates [94–95], or by associating different drugs to a HA-based nanocarriers [93,96–98]. Among the various combination strategies explored so far, it is worthwhile to highlight HA-ss-PLGA nanoparticles loaded with doxorrubicine and cyclopamide. The dual-drug loaded particles were prepared by double emulsion, allowing the incorporation of doxorubicin (hydrophilic) and cyclopamide (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 has been achieved by the design of (i) self-assembled micelles with HA-octandioic acid and PEI, which resulted in a system with the paclitaxel entrapped into the oil core and the siRNA ionically attach 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 achieved a synergistic effect against triple negative breast cancer and overcome drug resistance, which was successfully achieved. For example, the *in vivo* antitumor efficacy of doxorubicin/miR-34a loaded HA-chitosan nanoparticles resulted in a tumor 2-fold and 4-fold smaller than the drug and 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 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. 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 did not showed significant therapeutic effect after administration to immunized mice, the intravenous administration of HA-OVA in the same murine model enhanced the production of cytotoxic T cells against the tumor, leading to the inhibition of the tumor growth [104]. In the other work, 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 a major increase of cytokine IL-6 levels in blood, 77-times higher than after 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 the generation of a tumor specific memory response, demonstrated by the significant inhibition of a secondary tumor growth in mice vaccinated with PLL/HA-CpG complexes [105]. A different study explored the idea of inhibiting 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 the reduction of TGF-β around 50% in the late stage tumor

microenvironment. This nanotherapy aided to boost the vaccine efficacy and to inhibit the tumor growth by 52% when compared with the vaccine treatment alone [106].

It is important to note that several recent studies have been focused on the evaluation of the immunomodulatory properties of HA "per se". For example, LMW (MW 50-200KDa) HA has been shown to be able 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 for a detailed mechanism of action, interestingly, these studies show signs of an inherent capacity of LMW HA 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 manuscript, HA-coated (MW 40 KDa) mannan-conjugated manganese dioxide nanoparticles (HA-Man-MnO_2 NPs) have been used as a multifunctional platform to enhance the chemotherapeutic response of doxorubicin in a 4T1 murine breast cancer model. Although we suggest that the mechanism of action should be elucidated with more detail, the authors demonstrated that HA-Man-MnO_2 NPs were efficiently taken by macrophages, and they suggest that HA is responsible for reprograming anti-inflammatory M2-like into pro-inflammatory antitumor M1-like macrophages via a TLR2-MyD88-IRAK1-TRAF6-PKCζ-NK-κB-dependent pathway. The promotion of M1 macrophages results in a higher secretion of cytokines and H_2O_2 generation, which reacts with MnO_2 NPs for the production of O2 and Mn2+, resulting in decreased tumor hypoxia. These *in vitro* data were in line with the *in vivo* response observed after administration of the HA-Man-MnO_2 NPs in combination with doxorubicin into 4T1 tumor-bearing mice. The reduction of tumor hypoxia by HA-Man-MnO_2 NPs could contribute to the enhancement of chemotherapy response, resulting in the improved efficacy of doxorubicin and consequent tumor inhibition [109]. **Figure 5** drafts a schematic representation of the possibilities of using HA with immunomodulatory purposes, either by the stimulation of the adaptive immune system (dendritic cells and consequent activation of a T cell response) or by the modulation of the macrophage

polarization favoring the M1-like anti-tumoral phenotype with ability to kill tumor cells, inhibit angiogenesis and promote adaptive immune responses. Overall, these are some of the most relevant studies in the last years related to the use of HA with immunotherapeutic purposes. We strongly believe that important research related to the study of the mechanisms of action and, the possibilities of applying this idea into the clinic, will be presented in the coming years.

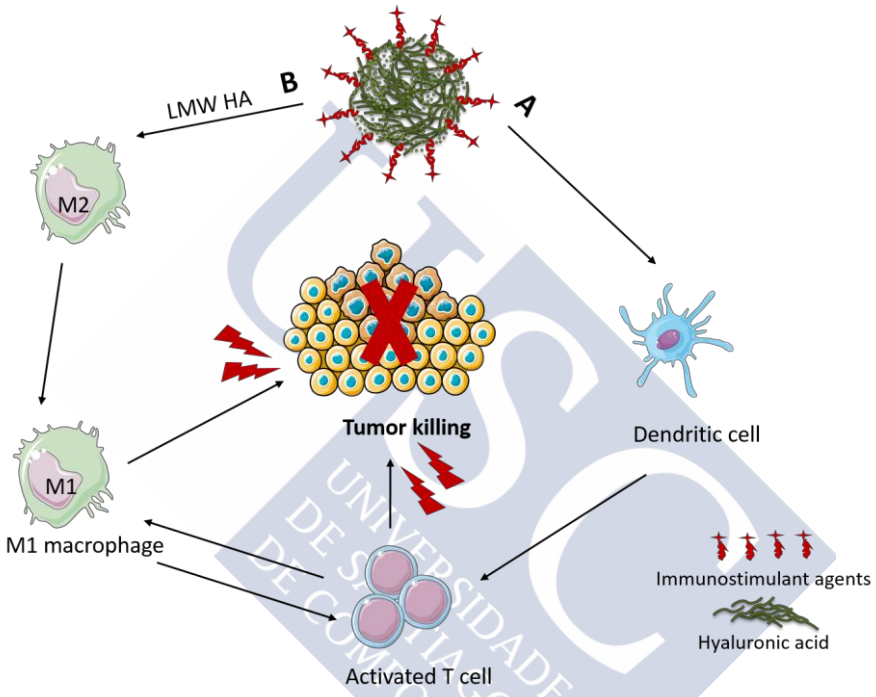

Figure 5. Immunotherapeutic possibilities for the use of HA-based nanocarriers in cancer. In addition to the immunomodulatory properties of HA "per se", HA-based nanocarriers can be designed through the association of antigens or immunostimulatory molecules to A) promote an adaptive immune response through 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 involves 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]. For example, computed tomography (CT) imaging and antitumor effect 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 high tumor accumulation compared with non-coated nanoparticles, demonstrating a bright CT signal 24h after administration. Moreover, these nanoparticles resulted in an 88% of tumor growth inhibition, when compared with the free drug [113]. Photothermal therapy (PTT) is a modality that 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 into tumors. Moreover, mice treated with cooper sulfide loaded Cy5.5-conjugated HA nanoparticles and laser irradiation led to a remarkable tumor growth inhibition, resulting in a 10-fold smaller tumor than any other control [117].

5. Conclusion and future perspectives

HA-based nanocarriers have received an exponentially increasing interest in the field of oncology. A few years ago the majority of publications on HA-based nanocarriers referred to their use for the delivery of cytostatic drugs, however nowadays the interest application has been extended to emerging therapies, including immunotherapies, polynucleotide-based therapies, combined therapies and theranostics. This increased attention is associated to an improved knowledge of HA biological properties, including its well-known CD44 targeting ability and, importantly, to the deep understanding of its chemical versatility. As a simple polymeric chain or in the form of 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 to revert 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 proprieties with image guided diagnosis and treatment.

The development of HA-based nanocarriers for cancer therapy is cited in hundreds of publications and patents in the last years. This interest is also notorious in the industry area, where diverse companies are taking forward the application of HA into possible clinical products. Currently, there are two clinical trials ongoing: (i) the ONCOFID™-P, a HA-paclitaxel conjugate for the treatment of refractory bladder cancer is in phase II (EudraCT number 2009-012274-13) [118–119], and (ii) FOLF(HA)iri, a phase III clinical study that uses the HA Chemotransport Technology (HyATC®), consisting of 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 envisaged that the 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 cure 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 which are useful for chemical modifications and functionalization. It has two carboxyl groups ionized at physiologic pH, it is highly hydrophilic and predisposed to be associated to counter ions.
- Mainly produced by microbial fermentation which results in 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 like cytostatic drugs, proteins, polynucleotides, immunomodulators and imaging agents.

Cancer selectivity

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

HA nanocarriers and its application in oncology

- HA nanocarriers can be applied 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 or 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 promote an immune response against tumor cells antigens.
- Imaging and theranostic: to simultaneously deliver an imaging agent for diagnostic or/and an anticancer drug for therapy, HA nanocarriers are becoming important theranostic tools.

Challenges

- The binding affinity of HA to specific cells receptors confers to the polymer advantages and disadvantages. Although it is known that HA has the ability to target the receptors that are overexpressed in cancer cells, it is also recognized that HA has the same capacity to interact with receptors expressed in healthy cells. As such, the main challenge is how to enhance the binding affinity of HA to cancer cells receptors, for example CD44, without enhancing its affinity to the other ones, for example HARE-1, associated to the high elimination of HA from blood circulation.

- Even though HA is commonly considered non-toxic and biocompatible, recent studies are reporting the immunogenicity of LMW HA and its role in macrophage polarization. Although in a preliminary stage, these results must be deeply evaluated in order to better understand the potential of HA in cancer immunotherapy.

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Background, hypothesis and objectives

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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 diverse 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 formulation of nanoemulsions without the need of organic solvents and heat [4]. This method has important advantages, as such: (i) ease of preparation, (ii) allows the incorporation of different therapeutic molecules, such as cytostatic drugs or sensible molecules 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 gives to the polymer 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 in 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 chain variable fragments (ScFv). Unfortunately, these approaches are ineffective and intracellular oncoproteins still lack from valid treatment options [11–13].

5. This section was eliminated for confidentiality reasons.

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 formulation of nanocapsules without the need of a cationic surfactant as 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 therapeutic proteins.

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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 formulation of HA nanocapsules intended for the encapsulation of docetaxel, as a hydrophobic drug model, and the association of a monoclonal antibody aimed for intracellular delivery. This goal was achieved by following the next steps:

Preparation of HA nanocapsules using a spontaneous emulsification method.

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

These results are presented in Chapter 2.

Evaluation of the capacity of HA nanocapsules to encapsulate the hydrophobic drug model, docetaxel

3. Formulation and characterization of unmodified and modified-HA nanocapsules loaded with docetaxel.
4. Docetaxel release was determined using an original drug transfer method. In vitro cytotoxicity was evaluated in A549 lung cancer cell line.

These results are presented in Chapter 2.

Study the ability of HA nanocapsules to associate a therapeutic protein and to promote its intracellular delivery

This section was eliminated for confidentiality reasons.

These results should be 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

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

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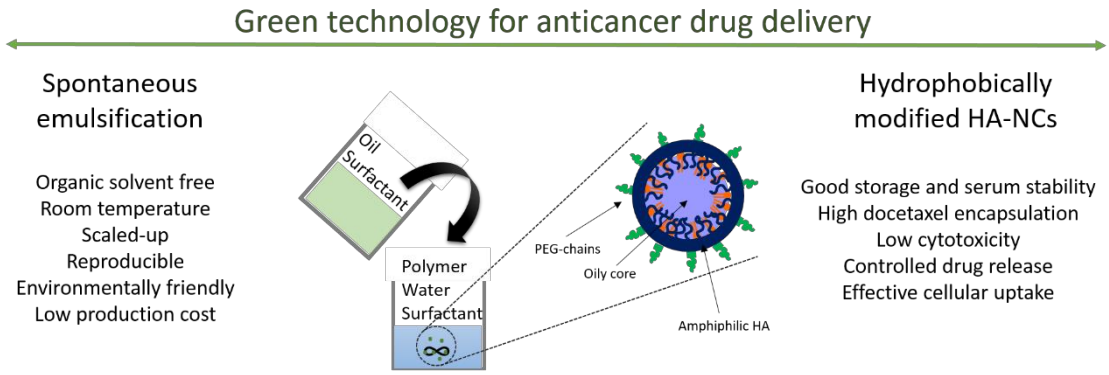
Graphical abstract



Abstract

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

Keywords*:* Nanocapsules, self-emulsifying, hyaluronic acid, anticancer, drug delivery

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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 (NCs) have been 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 in creating effective nanocarriers has been engineering them with the optimal physicochemical characteristics to guide them to the tumor [5]. As such, the development and optimization of NCs can be achieved by tailoring the carrier with adequate properties, such as size, shape and surface characteristics [2]. Besides, it is desirable that the nanocarrier can be formulated through industry-friendly techniques, without organic solvents and with simple scaling-up [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 process 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 for 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 NCs with a polymeric shell made of hyaluronic acid (HA) is an attractive approach to achieve active targeting. Hyaluronic acid (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]. Most published work reports 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-NCs by a one-step solvent-free emulsification process by utilizing amphiphilic HA precursors. By using docetaxel, we evaluate the capacity of HA-NCs to encapsulate hydrophobic drugs and further improve its therapeutic efficacy. This formulation process may constitute a green nanotechnology for drug delivery application.

2. Materials and methods

2.1 Materials

Native sodium hyaluronate of 200 KDa molecular weight was provided by Sanofi Genzyme, USA. Miglyol®812 was a kind gift from Cremer, Germany. Tween®80, Solutol®HS15, Haxadecyltrimethylammonium bromide (CTAB), Nile Red and DAPI were obtained from Sigma-Aldrich, Spain. Centripure P10 columns were purchased from EmpBiotech, Germany, and Dulbecco's Modified Eagles Medium (DMEM) from Themo Fisher Scientific, Spain. All other chemicals used were of reagent grade.

2.2 Synthesis of dodecylamide functionalized sodium hyaluronate

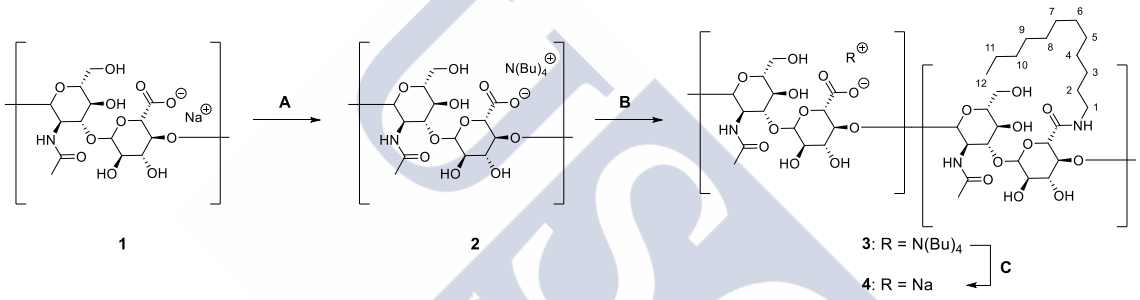

Figure 1. Synthetic scheme of dodecylamide functionalized sodium hyaluronate. A) i) DOWEX 50WX8-400 ii) $N(Bu)_4OH$; B) i) 2-Bromo-1-ethyl pyridinium tetrafluoroborate ii) 1-aminododecane; C) DOWEX 50WX8-400 ii) NaOH

200mg of native sodium hyaluronate was dissolved in water (concentration below 10mg/mL) and treated with 5mL Dowex 50WX8-400 (1.7 miliequivalents/mL, H^+ form; freshly washed with water/methanol/water). The pH of the solution was <4. The resin was filtered off and washed with water. The resulting polymer solution was treated with tetrabutylammonium hydroxide (40wt solution in water) until the pH was 12.0. The whole procedure was repeated twice and the final pH was subsequently adjusted to 7.5–8.0 by bubbling CO_2 followed by bubbling with N_2. The solution was concentrated by tangential flow using a 30 KDa cut-off Pellicon XL Biomax filter cassette (EMD Millipore). The concentrate was lyophilized.

To the above prepared tetrabutylammonium hyaluronate (400mg, 0.64 miliequivalents) was added DMF (45mL) and monomethyl formamide (4mL). To this solution was added

2-bromo-1-ethyl pyridinium tetrafluoroborate (8.8mg, 0.032mmol, 0.05 equiv.) dissolved in 1mL DMF. After aging the reaction for 1 hour, a solution of 1-aminododecane (12mg, 0.064mmol, 0.1 equiv) and triethyl amine (150mL, 1.08mmol, 1.7 equiv.) in 1mL DMF was added to the reaction, and the mixture was left at ambient temperature for 48 hours. The reaction mixture was added drop-by-drop to 150mL of a solution consisting of 1:1 acetone/tetrahydro-2-methylfuran. The precipitate was collected and redissolved in water and collected as an amorphous 50mL of deionized water.

The above solution was treated with 5mL 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 CO_2 followed by bubbling with N_2. 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 was analyzed by 1HNMR spectroscopy to confirm its structure and degree of substitution.

From now on, native HA would be defined as unmodified HA (unmod-HA) and hydrophobically modified HA as dodecylamide functionalized HA (C12-amide HA).

2.3 Development of the self-emulsification method – primary emulsions

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

Oil in water (o/w) NEs 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. NE 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®80ratios (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 2.5mg/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-NCs

Using the optimized NE as a template, HA-NCs were prepared by dissolving HA into the aqueous phase. Unmod- and C12-amide HA-NCs were prepared using the same procedure. Nevertheless, to prepare unmod HA-NCs the cationic surfactant CTAB was dissolved into the oil phase at different concentrations: 0.05, 0.1 and 0.15 mg/mL. For both prototypes, increased concentrations of HA 0.25, 0.5 and 1 mg/mL were dissolved in water.

2.5 Characterization

The amphiphilic HA was characterized by ^{1}H NMR spectroscopy using Varian Mercury Plus 400 MHZ spectrometer.

HA-NCs were characterized by 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 was performed under storage conditions and in the presence of human plasma. For long term stability, samples were kept undiluted at 4ºC 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 37ºC. 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 0.5mL 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 DCX loaded HA-NCs

DCX was solubilized in Miglyol®812 at 1.8 mg/mL and DCX loaded HA-NCs were prepared as described before at a concentration of 112 µg/mL. Briefly, for DCX loaded unmod 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 unmod-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-amide HA-NCs were prepared using the same procedure but without the cationic surfactant CTAB and by dissolving 0.5 mg/mL of C12-amide HA in water.

2.9 Nanocapsules isolation and DCX encapsulation efficiency

All formulations (empty and DCX loaded HA-NCs) 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 NCs was transferred to the column and eluted with water. The opalescent fraction, corresponding to 1.2mL of the formulation, was collected and characterized. Encapsulation efficiency (EE%) was calculated by quantifying drug concentration in the collected elute and in the initial formulation.

2.10 DCX quantification

Docetaxel 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-NCs under sink conditions. Using 50mL falcon tubes, DCX loaded HA-NCs were diluted in phosphate buffer saline (PBS) at 37ºC and placed inside a water bath incubator. At specific time points, 15min, 3h, 6h and 24h, 500µL 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 30min, 4000rpm at 20ºC. After centrifugation, the oil and aqueous phase were separated, the NCs 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 was evaluated by using the cell viability AlamarBlue® assay in A549 human lung adenocarcinoma cell line for both blank and DCX loaded HA-NCs, 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 $5x10^4$ cells/mL. Cells were incubated at 37ºC and 5% CO_2. On the first day, medium was replaced by fresh medium containing different formulations and each concentration was tested in six wells per plate. Cells were incubated for 24h, 48h and 72h and after each time of exposition, medium was replaced by fresh medium containing 5mM AlamarBlue® and incubated for 3h at 37ºC. Fluorescence was measured at 530 and 590nm (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 DCX loaded HA-NCs

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

2.13 Fluorescent labeled HA-NCs

Nile red (NR) loaded 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

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-amide HA was carried out by following a designed procedure. The reaction scheme is illustrated in **Figure 1.** The reaction yield for each synthetic step was between 50-70%. The final product was characterized by ^{1}H NMR spectroscopy and the spectrum of the compound is shown in **Figure 2**. The degree of substitution (DS) of 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%.

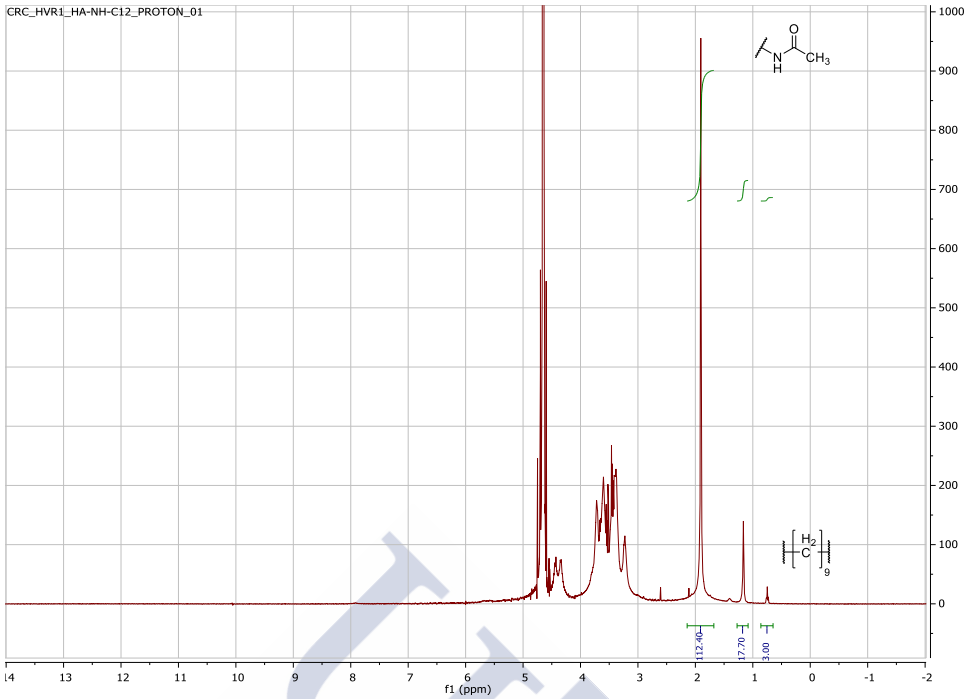

Figure 2. ^{1}H NMR of dodecylamide functionalized sodium hyaluronate in D_2O

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

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 NEs.

Table 1. Optimized parameters and correspondent physicochemical characterization of 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	**1.5 :1**	1:2	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 presented as mean value ± standard error (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 PDI.

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), NEs 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 NEs was as small as in the case of

formulations based on high amounts of oil phase, resulting in NEs 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 physicochemical properties of NCs (Results not shown).

Based on the above findings, the following conditions were employed for the formulation of NEs: the oil phase was composed of Miglyol®812/ Tween®80 in a ratio 1:1 (w/w) and the aqueous phase with 2.5mg/mL of Solutol®HS15 in water. The oil phase was poured into the aqueous phase (ratio 1:8 v/v). NCs were prepared using the optimized self-emulsifying process as a template in addition to dissolving the HA in the aqueous phase. NCs based on unmodified HA were prepared in the same way but adding CTAB to the oil phase.

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

Cationic NEs were initially prepared by varying the concentration of CTAB in the oil phase. The cationic surfactant promoted an inversion in the negatively charged NE to positive values. Also, increased amounts of CTAB resulted in a high zeta potential (ZP), without influencing the mean droplet size (**Table 2**). Since no further ZP increase was observed, 0.15 mg/mL of CTAB was used for NCs formation. HA coating resulted in a ZP shift from +10 mV to -19 mV after NCs formation regardless of polymer concentration. Further experiments were done with 0.25 mg/mL of unmod-HA.

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

Table 2 shows the characterization of NCs prepared using C12-amide HA. The modified prototype did not change the physicochemical properties of the system. However, the ZP was dependent on the concentration of the hydrophobically modified HA. At least 0.5 mg/mL of modified HA was needed to give appropriate negative ZP to the NCs. Subsequently, all NCs formulations were prepared using functionalized HA at a concentration of 0.5 mg/mL.

Table 2. Physicochemical characterization of anionic NE, cationic NEs, unmod and C12-amide HA-NCs for different component concentrations.

Formulation	Compound varied	Conc. (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
Unmod HA-NCs	**Unmod-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-amide HA-NCs	**C12-amide 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 presented as mean value ± standard error (n=3)

Abbreviations: PDI, polydispersity index; ZP, zeta potential

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

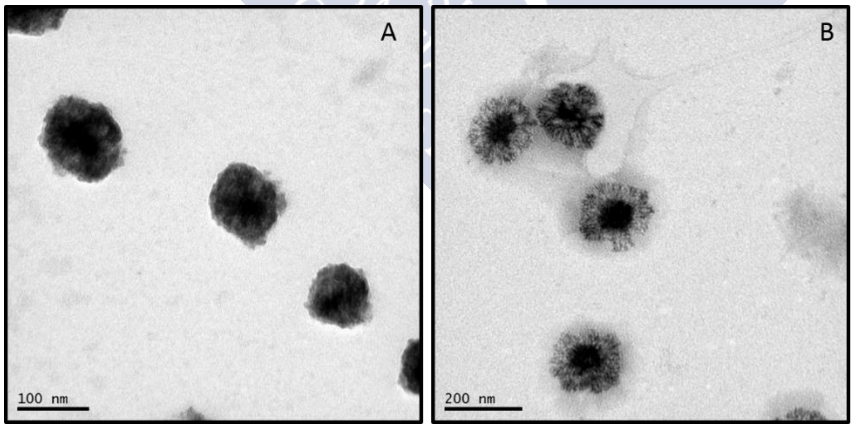

Figure 3. TEM images of HA-NCs. A: unmod HA-NCs; B: C12-amide HA-NCs

3.5 Stability of HA-NCs

Stabilities of both unmod HA (0.25 mg/mL) and C12-amide HA (0.5 mg/mL) NCs were tested under storage conditions at 4ºC for 6 months, and in human plasma at 37ºC for 24h. Under storage conditions, both formulations were very stable, without significant change in particle size, PDI or ZP for up to 6 months (**Table 3**).

Table 3. Physical stability of self-emulsifying HA-NCs in storage conditions.

Time period	Size (nm)		PDI		ZP (mV)	
	Unmod HA-NCs	C12-amide HA-NCs	Unmod HA-NCs	C12-amide HA-NCs	Unmod HA-NCs	C12-amide 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 presented as mean value ± standard error (n=3)

Abbreviations: PDI, polydispersity index; ZP, zeta potential

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

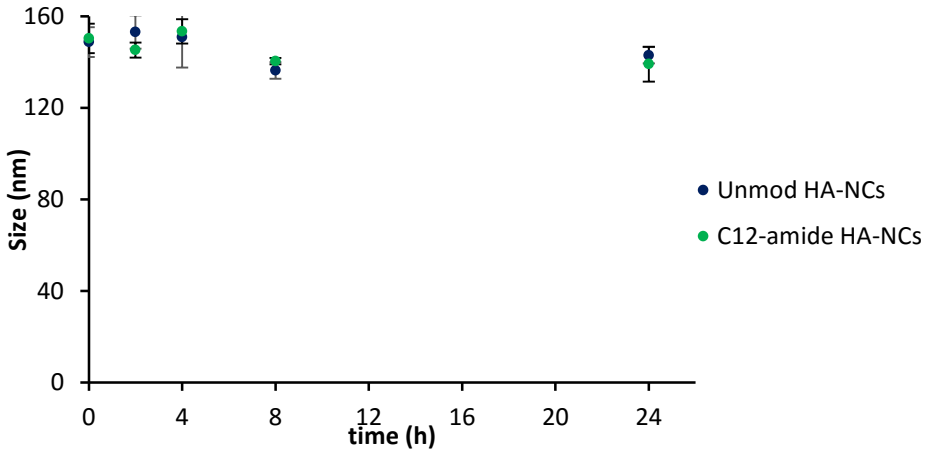

Figure 4. Size distribution of HA-NCs incubated with human plasma, at 37ºC.

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

3.6 Characterization of DCX loaded HA-NCs

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. **Figure 5** presents the elution profile of free and encapsulated DCX by SEC. DCX was successfully encapsulated into the oil core of both types of NCs, without affecting its physicochemical characteristics. The percentage encapsulation efficiencies were 88 ± 9 and 86 ± 3 (n=3) for the NCs based on unmod-HA and C12-amide functionalized HA, respectively.

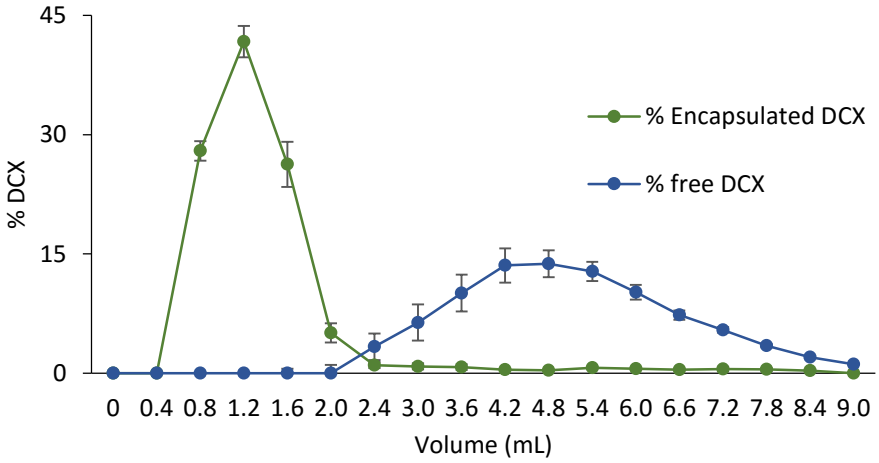

Figure 5. Elution profile of free and encapsulated DCX into HA-NCs.

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

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 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 nanocapsule suspension. After separation, NCs 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-NCs when compared with the free drug. As observed, 100% of free DCX was transferred to the oil compartment, evidencing the ability of Miglyol®812 to solubilize all the free DCX in solution. The release behavior of DCX from unmod and C12-amide HA-NCs exhibited an initial burst release of 55% and 40%, respectively, followed by a continuous release for 24h.

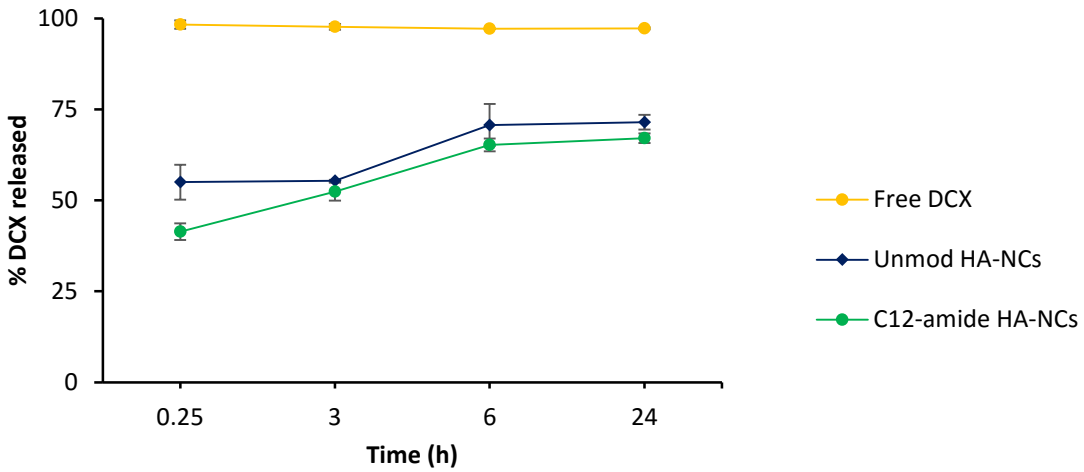

Figure 6. Release of docetaxel from unmod and C12-amide HA-NCs.

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

3.8 *In vitro* toxicity of empty HA-NCs

Cytotoxicity of unmod and C12-amide HA-NCs was assessed in A549 cells at different concentrations. Additionally, two surfactant solutions were prepared at the same concentration required for the formulation of NCs, and they were used as controls. As can be seen from **Figure 7**, either type of NCs affected cell viability when tested at concentrations up to 350 µg/mL. On the other hand, amphiphilic HA functionalized NCs showed no toxicity even when tested at the highest concentration (1000 µg/mL). The highest cytotoxicity was observed for the free surfactant mixture with CTAB, where only 20% of cells survived at 350 µg/mL after 72h. It appears that by eliminating the use of a cationic surfactant it offers the possibility to prepare nanocapsules with improved biocompatibility profiles.

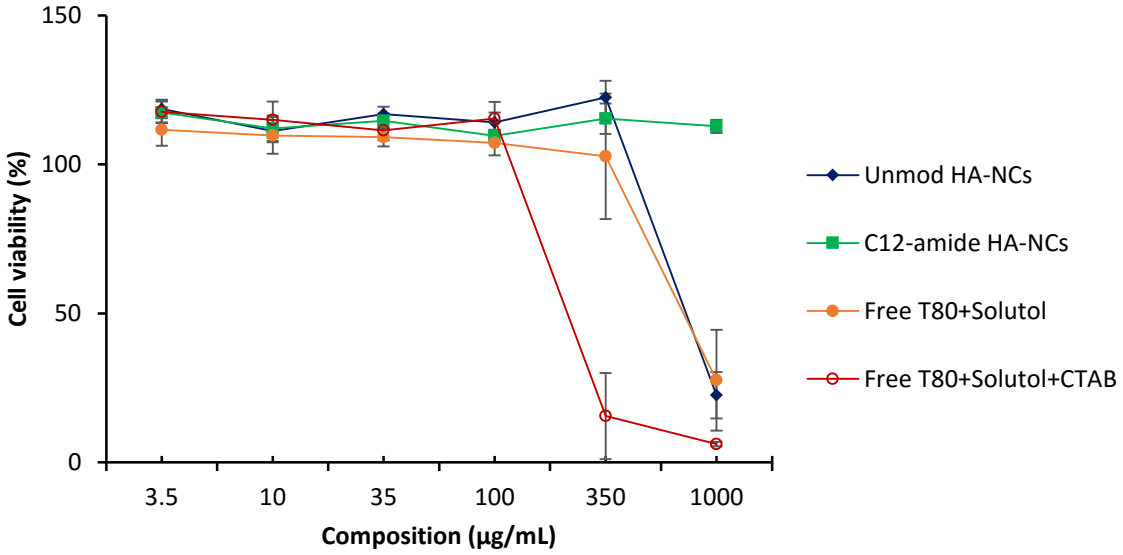

Figure 7. *In vitro* cell toxicity of A549 cells after exposition to different concentrations of unmod HA-NCs, C12-amide HA-NCs and free surfactant mixture for 72h.

Notes: Results presented as mean value ± standard error (n=6)

3.9 *In vitro* toxicity of DCX loaded HA-NCs

Both free and DCX loaded HA-NCs showed a dose dependent cytotoxicity against A549 cells in the concentration range from 0.625 to 100µM (**Figure 8**). The half minimal inhibitory concentration (IC50) was reached only by the drug-loaded HA-NCs at 10µM 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.

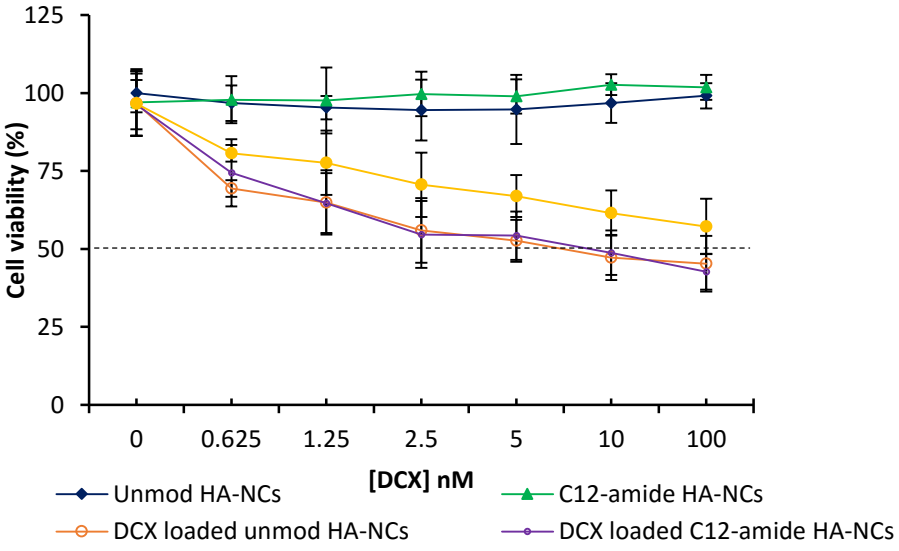

Figure 8. *In vitro* cytotoxicity of free DCX, DCX loaded HA-NCs and blank HA-NCs.

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

3.10 Intracellular uptake of HA-NCs

To evaluate the intracellular uptake of unmod and C12-amide HA-NCs, NR was loaded into both NCs 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 NR was delivered into unmod and C12-amide HA-NCs (**Figure 9B, C)**.

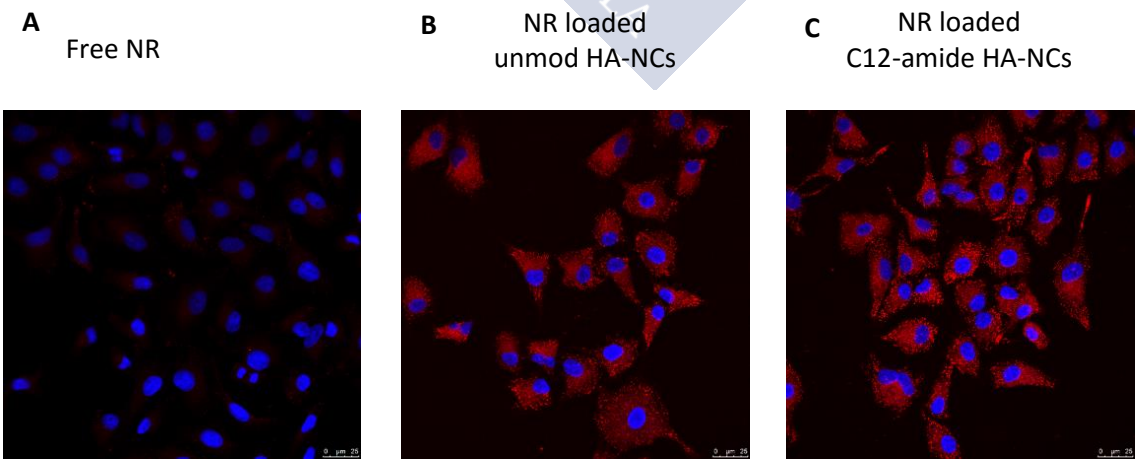

Figure 9. Intracellular uptake of NR loaded unmod and C12-amide HA-NCs in A549 cells.

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

4. Discussion

4.1 Synthesis and characterization of C12-amide functionalized HA

Hydrophobically modified HA was synthesized by chemical modification of native sodium hyaluronate (average molecular weight of 200 KDa) by 1-aminodecane via an amide bond. The degree of substitution was kept in the range 2.5 to 3.0 mole%. The modified polymer was characterized by ^{1}H 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 Preparation 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-NCs. 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, show 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 relative high hydrophilic/lipophilic balance (HLB) 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 important side effects [40] it showed remarkably less toxicity and apoptosis then Labrasol® and Cremophor® RL [41]. For example, Ma *et al* [42] had formulated DCX loaded poly-Ɛ-caprolactone (PLC)-Tween®80 nanoparticles and demonstrated that the carrier showed better *in vitro* toxicity than commercial Taxotere® at the same surfactant concentration.

As such, we think that its localization at the interface of the particle surrounded by the polymeric shell must decrease its free circulating exposure. We decided to include Solutol®HS15 in the formulation for two main reasons. First, Solutol®HS15 includes a PEG chain in its structure, which provide stability and prolonged circulation times [43]. Additionally, it possesses the required high HLB, 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 NE 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 NE 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 reducing the oil/aqueous phase ratio to 1:8 (v/v) we were able to produce NEs 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 went even further up to 1:30 (v/v) oil/aqueous phase ratio, however, those formulations were limited by a very low amount of oil which constrain consequently the amount of drug incorporated into the system.

The effective attachment of the unmod-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

NE and the possible toxicity associated with high amounts of CTAB [22], we chose a concentration of 0.15 mg/mL of CTAB for the NCs preparation. The chosen concentration was previously demonstrated to be enough for polymer attachment and nanocapsule stabilization [21]. 0.25 mg/mL of unmod-HA was enough to promote the attachment of the polymer to the particle surface, resulting in a negative zeta potential (ZP). By shielding the NCs 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, unmod-HA was replaced by C12-amide functionalized HA. The hydrophobic dodecyl chains of HA facilitated the self-assembly of the polymer within the oil/surfactant NE 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 ZP as the unmodified prototype.

4.3 Stability assays

The stability of NCs was assessed thorough storage conditions and in human plasma. No significant difference in size, PDI and ZP was observed on either unmod and C12-amide HA-NCs after storage for 6 months, at 4ºC. The stability could be attributed to the high negative charge that prevents particle aggregation due to charge-charge repulsion. Moreover, the presence of Tween®80 should also add steric stability to the system [50]. The stability of the NCs in plasma was determined by their physical integrity, mainly the particle size [51]. The observed increase in particle size after 24h at 37^{o}C might be due to protein deposit. Nevertheless, this increase was less than 20% compared to the initial particle size, which means that these NCs are suitable for IV administration [52].

4.4 *In vitro* release assays

HA-NCs either prepared with unmod or C12-amide functionalized 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-NCs, which presented an initial burst release between 45-65%. The initial burst release has been justified by the own structure of the NCs, favoring the partition of the drug between the oil core and the aqueous external medium [53]. Furthermore, the release was not affected by the ionic or hydrophobic forces that drove the formation of NCs with unmod and C12-amide HA, respectively. With a Pka1=2.82 and Pka2=3.42, unmod-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 provide us information about mechanistic details, it is important to highlight tow important points: (i) the limitation of the method, where an external oil phase may force the release of DCX from the oil core of the NCs and, (ii) the *in vitro* release behavior it 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 cytotoxic behavior of C12-amide HA-NCs was expected since the formulation of these NCs with 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 NCs prepared with C12-amide modified HA did not cause any toxicity when tested at the highest concentration (1000µg/mL). The higher toxicity for te NCs prepared with unmod-HA 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 the unmodified 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 free surfactants [57].

DCX-loaded unmod and C12-amide HA-NCs showed an improvement in the inhibitory cell viability when compared with the free drug. The IC50 was reached only by the drug-loaded HA-NCs at 10µM concentration 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-NCs, which in turn did not express any inherent cytotoxicity themselves, it is fair to assume that these NCs 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-NCs, both prototypes were incubated with A549 cells overexpressing CD44 receptors. As seen by confocal microscopy, strong fluorescent signal was detected in the cytoplasm for both HA-NCs 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 unmod and C12-amide HA-NCs, which suggests that the functionalization of HA with C12-amide did not affect its binding affinity through CD44 receptors. As such, this must confirm 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 for the first time the application of a green methodology for the preparation of HA-NCs. A self-emulsification method was developed for the preparation of HA-NCs without the aid of organic solvents and heat, which offers a promising and sustainable approach to prepare nanoformulations for therapeutic molecules. The formulation of HA-NCs based on an amphiphilic functionalized HA derivative led to the development of nanoparticles with low toxicity and the potential to 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 therapeutic proteins

This section was eliminated for confidentiality reasons.

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Overall discussion

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

As a multifunctional drug carrier, HA-NCs were designed to encapsulate docetaxel (DCX) as a cytostatic drug model and to entrap within the HA shell a therapeutic protein, intended for intracellular delivery. Small hydrophobic drugs, such as docetaxel, continue to be a challenge in nanomedicine. For example, besides its toxic side effects, Taxotere® continues to be the only commercialized formulation for DCX. As such, new delivery systems must be developed and the encapsulation of DCX within HA-NCs must improve its therapeutic efficacy, while decreasing the cytotoxicity associated to the drug solvents used in the commercial formulation [13].

In this study, self-emulsifying HA-NCs were designed as multifunctional nanocarriers for the delivery of different anticancer molecules, such as DCX and a therapeutic protein. **Figure 1** illustrates a summary of this work.

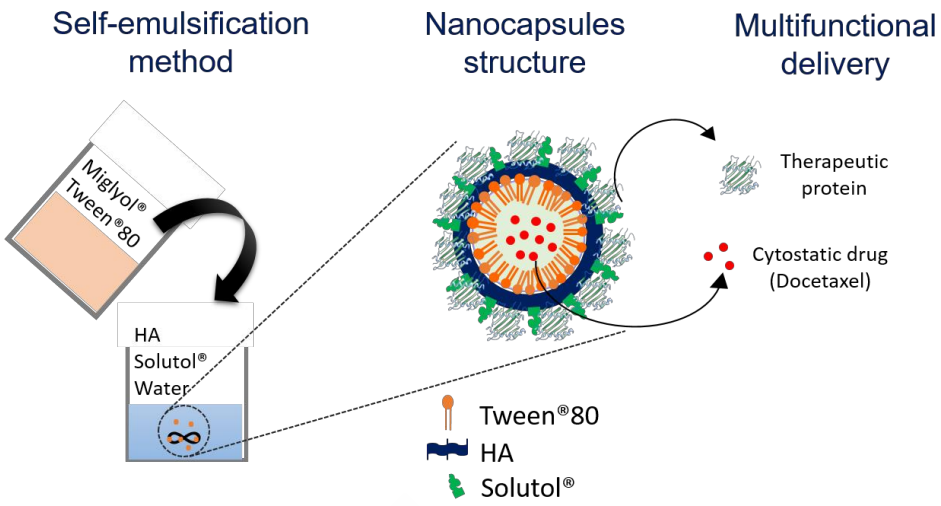

Figure 1. Schematic representation of the preparation of HA-NCs by self-emulsification and their structure as a multifunctional carrier for the delivery of cytostatic drugs and therapeutic proteins.

1. Green nanotechnology – method choice

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 [14]. 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 [15]. In nanotechnology, the application of "green methodologies" can be seen 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 [16] and (ii) the formulation of biodegradable nanoparticles using organic solvent-free and mild methods, for example, ionotropic gelation [17], phase inversion temperature (PIT) [18] and spontaneous emulsification [8]. In this work, we have decided to develop a green formulation process based on the spontaneous emulsification technique. Comparing

with the ionotropic gelation, which has been reported for the formulation of polymeric nanoparticles intended for the delivery of hydrophilic drugs [19], 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 [20]. The main disadvantage of the PIT method is the use of heating-cooling cycles, which might compromise thermolabile drugs [21].

The self or spontaneous emulsification technique has been widely described for the formulation 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 [26]. The formulation 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.

2. Spontaneous emulsification design

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, the medium chain triglycerides reduce the interfacial tension and have better partitioning ability to emulsify [29–30]. In addition, DCX can be effectively incorporated within Miglyol®812 with enhanced drug loading capacity [29]. 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 [30]. 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 [31]. 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 an inhibitor 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 template for HA-NCs preparation.

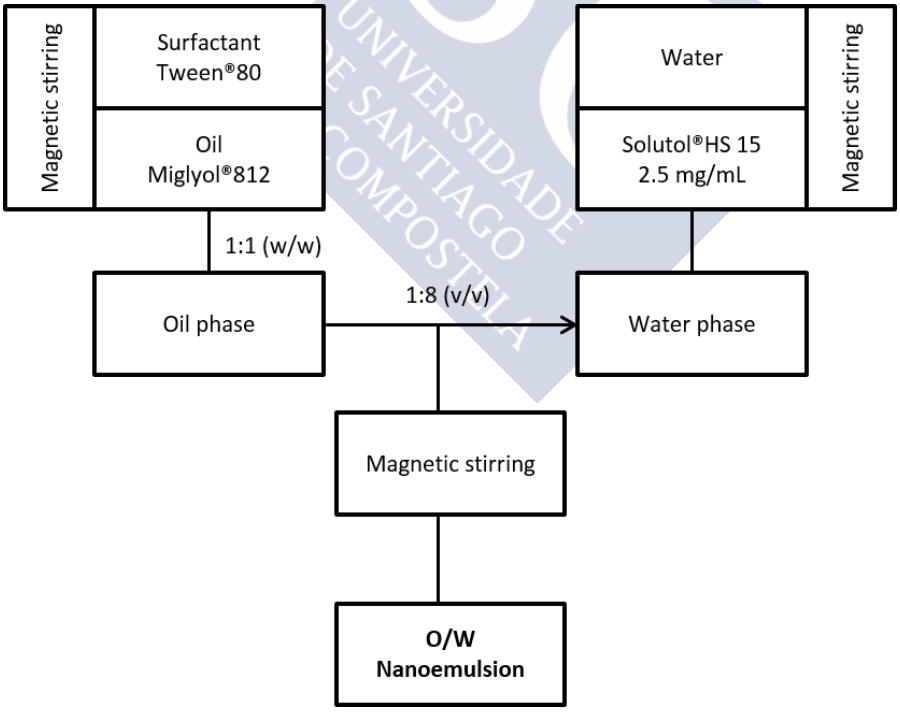

Figure 2. Flow chart of the preparation of self-emulsifying nanoemulsions.

3. Preparation of HA-NCs using unmodified and dodecylamide-functionalized HA

HA-NCs were prepared using the optimized spontaneous emulsification technique by dissolving the polymer into the aqueous phase. HA-NCs were prepared using two types of HA: an unmodified structure (unmod-HA) and a hydrophobically functionalized HA with a dodecylamide chain (C12-amide HA) (**Figure 3**). C12-amide HA has a 2.0-3.0% 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 [34]. Additionally, it is not expected that this degree of substitution interferes with CD44 recognition, an important feature for active targeted delivery [35].

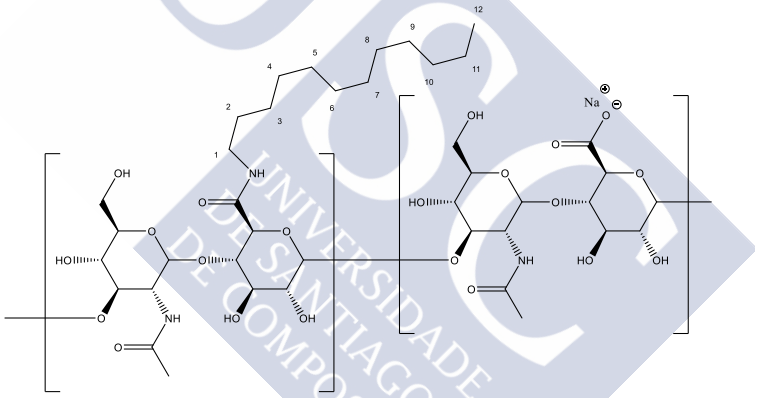

Figure 3. Chemical structure of dodecylamide-functionalized HA

The formulation of unmod HA-NCs has been reported by our group and consists on the interfacial deposition of negatively charged HA onto a positive charged surface [36]. Accordingly, self-emulsifying unmod 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 unmod-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 to -19 mV (**Figure 4**). Different CTAB and unmod HA concentrations were studied and the characterization of the optimized

formulation (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**.

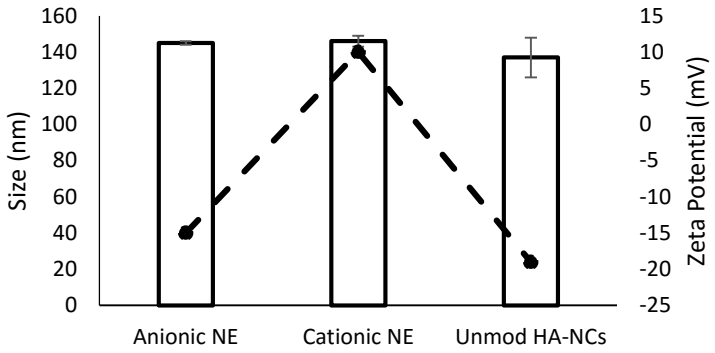

Figure 4. Physicochemical characterization of the optimized anionic nanoemulsion (NE), cationic nanoemulsion and unmod HA-NCs prepared by the spontaneous emulsification method. There was an inversion of the zeta potential after CTAB addition and again after HA deposition.

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

To simplify the process and avoid the use of a cationic surfactant, which is commonly associated to cytotoxic effects, unmod-HA was replaced by the C12-amide 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 formulation 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 [40]. Moreover, HA can be grafted with phospholipids, like L-α-Dioleylphosphatidylethanolamine (DOPE), and incorporated onto the surface of liposomes [43–45].

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

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

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

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

Abbreviations: PDI, polydispersity index; ZP, zeta potential

TEM images presented the effective formation of nanocapsules, where a core/shell structure is visualized for both HA-systems (**Figure 5**). These results highlighted the formation of C12-amide HA-NCs by hydrophobic interactions, with the dodecyl chains of 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 liposomes, where the DOPE plays the role of anchor in the lipid membrane [44].

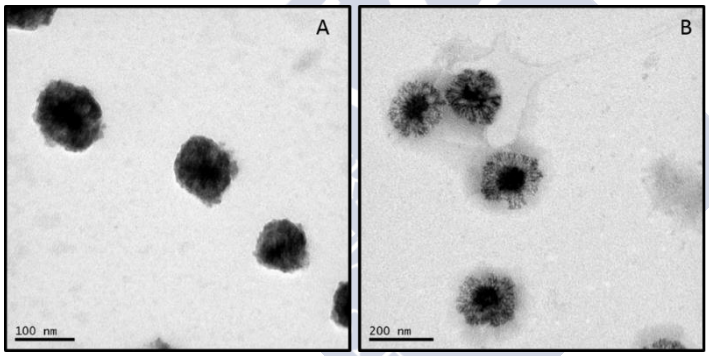

Figure 5. TEM images of unmodified HA-NCs (A) and C12-amide functionalized HA-NCs (B).

Self-emulsifying HA-NCs were evaluated for stability under storage conditions at 4ºC and after dilution in human plasma at 37ºC. Under storage conditions, both prototypes were very stable, without significant changes in particle size, polydispersity index, and zeta potential up to 6 months. This high stability must result from different factors: (i) the negative charge conferred by the HA shell can avoid particle aggregation [45], (ii) the PEGylated chains from Solutol®HS15 are described as a stabilizer [46] and, (iii) the presence of Tween®80 can also provide steric stability [47]. When incubated in human plasma at 37ºC 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 NCs nevertheless.

The cytotoxicity of unmod HA-NCs, C12-amide 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 HA-NCs did not affect cell viability when tested at concentrations up to 350 µg/mL. Nonetheless, only C12-amide HA-NCs did not cause toxicity at the maximum concentration tested (1000 µg/mL). These results relate the presence of CTAB in the unmod system with its greater cytotoxicity, as reveled in other studies [48]. 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. These results indicate that free surfactants in solution were responsible for a significant toxicity profile. However, when encapsulated within the nanocapsules structure, this toxic profile changed and the HA-NCs themselves were less toxic [49]. C12-amide HA-NCs were also prepared by the solvent displacement technique and the anionic surfactant Tween®80 was replaced by lecithin [36] (results not shown). Regardless its composition, both nanocapsules presented the same cell viability profile, which demonstrated that the amount of Tween®80 needed to formulated HA-NCs by the self-emulsification method was not responsible for additional toxicity. This result must be related to the correct isolation of NCs and/or to the capacity of the polymeric shell to mask surfactant toxicity.

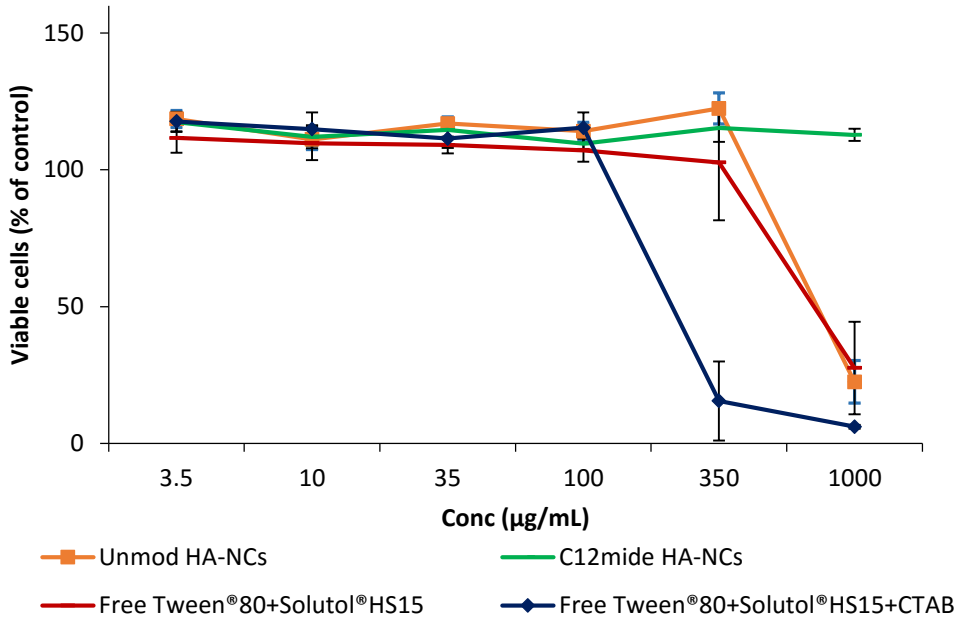

Figure 6. *In vitro* cytotoxicity of unmod HA-NCs, C12-amide HA-NCs and free surfactant mixture in A549 cells after 72h of incubation.

Notes: Results presented as mean value ± standard error (n=6)

4. DCX-loaded HA nanocapsules – hydrophobic drug model

DCX was used as a hydrophobic drug model to be encapsulated within the oil core of HA-NCs. The drug was first solubilized within Miglyol®812 and then incorporated into unmod and C12-amide HA-NCs following the initial protocol. DCX was efficiently encapsulated in both prototypes, without changing their physicochemical characteristics. The solubility of DCX 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, DCX was solubilized in a small amount of ethanol (<10%), followed by evaporation. DCX-loaded HA-NCs were formulated with a loading up to 2.75% (corresponding to 2.5 mg/mL of DCX) 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 [50]. The encapsulation efficiency was between 86-89% for both types of nanocapsules. The cytotoxicity of DCX-loaded HA-NCs was studied by the AlamarBlue® assay. Both free and encapsulated DCX showed

a dose dependent toxicity in A549 cells. Nevertheless, the IC50 was only achieved for DCX delivered from nanocapsules, demonstrating the potential of HA-NCs for cytostatic drug delivery.

Commonly, the majority of *in vitro* release assays for hydrophobic drugs are performed by ultracentrifugation and dialysis and in less extension by size exclusion chromatography or continuous flow filtration. Nevertheless, it is often the case that the drug and the carrier cannot be separated using those methods; for example, ultracentrifugation cannot be applied to samples that aggregate under this separation conditions [51]. Regarding dialysis, the addition of surfactants to achieve the total solubility of DCX in the medium under sink conditions can interfere with the structure of colloidal particles and change the drug release [52]. Considering the limitations of this isolation methods, *in vitro* release assays of DCX from both nanocapsules prototypes were assessed using a drug transfer method adapted from Bastiat *et al* [53]. For that, DCX-loaded HA-NCs were diluted under sink conditions in PBS at 37ºC, and at fixed time points (15minutes, 3h, 6h and 24h) a sample volume was taken, mixed 1:1 (v/v) with Miglyol®812 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 encapsulated DCX would be kept into the nanocapsules suspension (**Figure 7**).

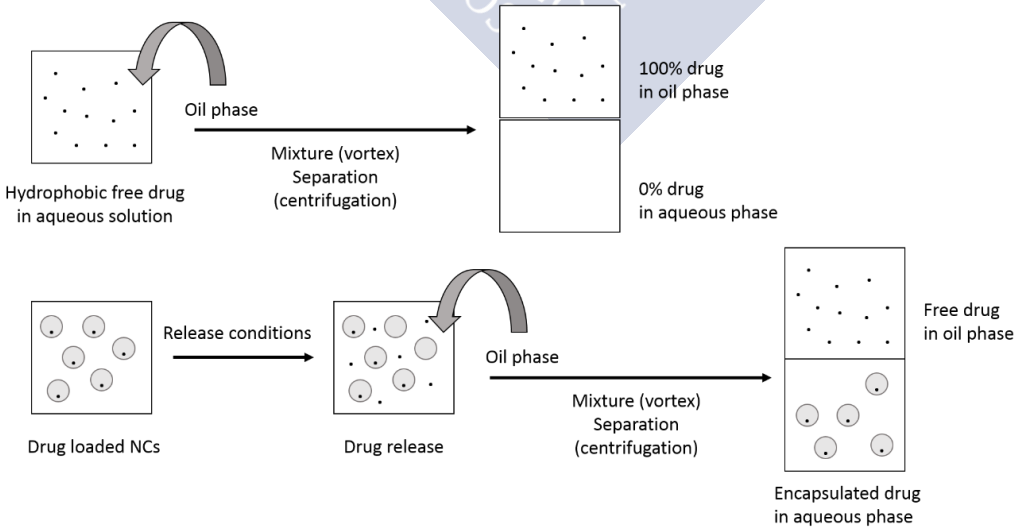

Figure 7. Schematic representation of the drug transfer method for the *in vitro* release of DCX from HA-NCs.

To validate the method, an initial experiment was performed only with free DCX dissolved in PBS at sink conditions. As observed in **Figure 8**, all the free DCX 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 DCX from HA-NCs, the results demonstrated an improved profile. DCX was released from unmod and C12-amide 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**).

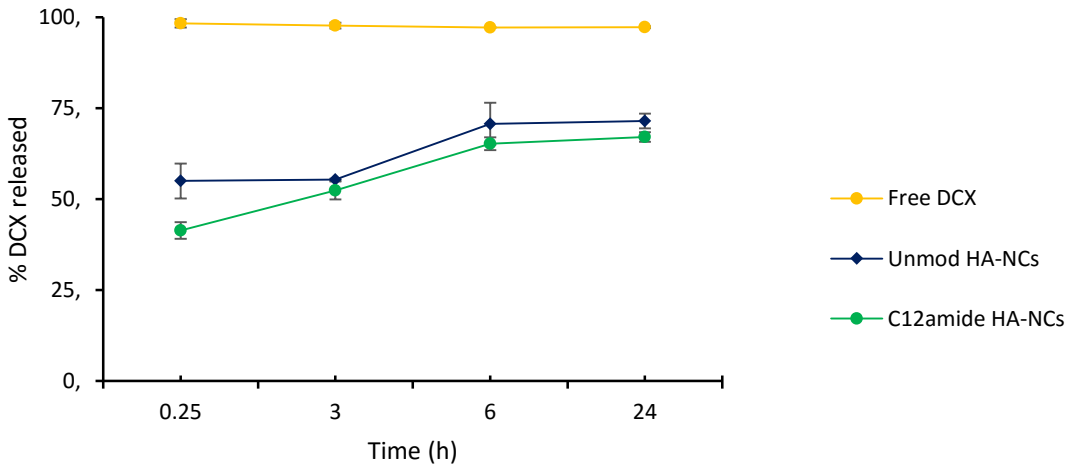

Figure 8. *In vitro* release profiles of DCX from unmod and C12amide HA-NCs in PBS.
Notes: Results presented as mean value ± standard error (n=3)

This release behavior can be justified by the structure of the NCs and the partition coefficient of the drug between the oil core and the aqueous external medium [54]. 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 DCX from HA-NCs.

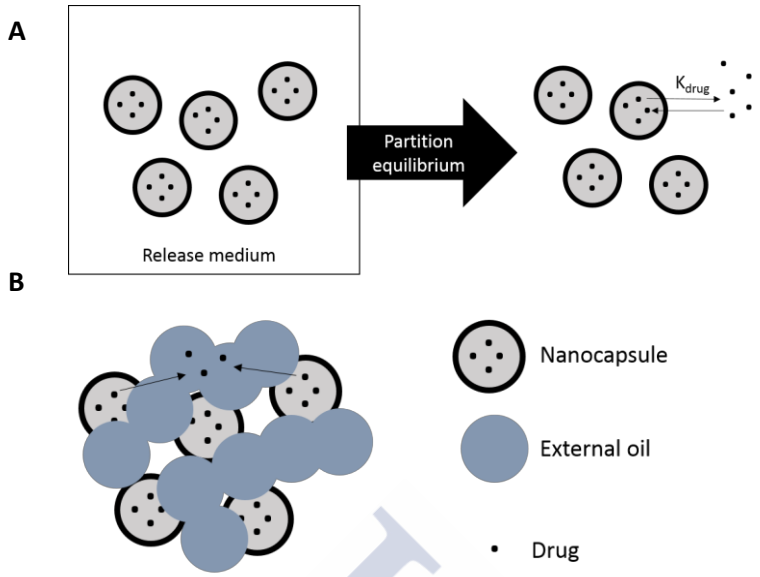

Figure 9. Schematic illustration of the mechanisms responsible for the release behavior of DCX from HA-NCs. A) Influence of the partition equilibrium in the drug release of nanocapsules; B) Oil transfer from a NCs nucleus to an external oil phase during the mixing process.

5. Intracellular delivery of a therapeutic protein associated to HA-NCs

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Conclusions and Future Perspectives

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Conclusions

The experimental work enclosed in this manuscript was aimed at designing a new spontaneous emulsification method for the formulation 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 the component choice and the oil/surfactant ratio.

2. Self-emulsifying hyaluronic acid (HA) nanocapsules were prepared with two HA structures, a native HA and a dodecylamido-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 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 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. This section was eliminated for confidentiality reasons.

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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 close to fight cancer.

The development of self-emulsifying HA 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 every 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 proteins.

This platform demonstrated promising characteristics for the intracellular delivery of diverse anticancer drugs which can represent a new strategy against cancer progression.

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