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NUEVOS TRANSPORTADORES DE FARMACOS: NANOCÁPSULAS DE POLIASPARAGINA Y CASEÍNA

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INFORMAN:

Que la presente Memoria Experimental titulada: "Nuevos transportadores de fármacos: nanocápsulas de poliasparagina y caseína", elaborada por Gustavo Rodolfo Rivera Rodríguez, ha sido realizada bajo su dirección en el Departamento de Farmacia y Tecnología Farmacéutica y, hallándose concluida, autorizan su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

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Fdo. María José Alonso Fdo. Dolores Torres

A mi familia…

"Una vez descartado lo imposible, lo que queda, por improbable que parezca, debe de ser la verdad…"

Arthur Conan Doyle

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RESUMEN

Se han diseñado dos nuevas plataformas nanotecnológicas para la vehiculización selectiva de fármacos antitumorales. Estas nanoestructuras, constituidas por un núcleo oleoso y una cubierta polimérica, a base de secuencias de aminoácidos (poliasparagina o caseína), se prepararon mediante una modificación de la técnica de desplazamiento de disolvente. Las nanocápsulas de poliasparagina demostraron ser capaces de incrementar la actividad del docetaxel in vitro, sobre células de cáncer no microcítico de pulmón. Esta actividad se reflejó en el efecto logrado in vivo sobre un modelo de glioma implantado en ratones. Las nanocápsulas redujeron el crecimiento tumoral de un modo similar a la formulación comercial de docetaxel Taxotere ®, consiguiendo incrementar considerablemente los tiempos medios de supervivencia. En comparación a la nanoemulsión sin recubrir, las nanocápsulas de poliasparagina demostraron poseer características de larga permanencia en plasma, duplicando los valores del tiempo medio de residencia.

Por otro lado, como una alternativa a la recurrente vía i.v. en la administración de fármacos antitumorales, se han diseñado nuevas nanoestructuras de β- y κ-caseína utilizando la misma estrategia de formulación. Estas nanocápsulas mantuvieron una estabilidad adecuada tanto en fluído gástrico e intestinal, y se mostraron estables en el almacenamiento durante períodos prolongados, lo que las hace aparecer como una interesante plataforma para la administración oral de fármacos hidrofóbicos.

ABSTRACT

Two new platforms have been designed as nanovehicles for antitumor drug targeting. These nanostructures consist of an oily core and a polymeric coating based on amino acid sequences (polyasparagine or casein), and were prepared by a modification of the solvent displacement technique. Particularly, polyasparagine nanocapsules showed to be able of enhancing the in vitro activity of docetaxel in non-small lung cancer cells. This activity was reflected on the in vivo effect shown on a xenograft glioma mouse model. The tumoral growth was reduced by the nanocapsules in a similar manner than the marketed docetaxel formulation Taxotere ®, being the survival times considerably increased. In addition, polyasparagine nanocapsules behaved as long circulating carriers, showing mean residence time values 2 folds higher than the uncoated nanoemulsion.

On the other hand, as an alternative to the recurrent i.v. administration of anticancer drugs, new nanostructures based on beta and kappa casein have been designed, using the same formulation strategy. These nanocapsules showed adequate stability in gastric and intestinal fluids and were stable in the storage for prolonged periods of time, thus emerging as an interesting platform for the oral administration of hydrophobic drugs.

LISTADO DE ABREVIACIONES

AUC	Area under the curve	Area bajo la curva
ANOVA	Analysis of variance	Analisis de varianza
BKC	Benzalkonium chloride	Cloruro de Benzalconio
BSA	Bovine serum albumine	Albumina sérica bovina
B-CN	Beta-casein	Caseína tipo β
C max	Peak concentration	Concentración máxima
CN	Casein	Caseína
CTAB	Cetrimide	Cetrimida
CLp	Plasmatic cleareance	Aclaramiento plasmático
DiD	"Did" as Fluorescent probe	Compuesto fluorescente
DMEM	Dulbecco's Modified EagleMedia	
DLS	Dynamic Ligth Scattering	
DCX	Docetaxel	Docetaxel
HA	Hyaluronic acid	Ácido hialurónico
HPLC	High prefomarce liquid cromatography	Cromatografía líquida de alta eficacia
IVIS	in vivo imaging system	Sistema de Análisis de Imagen
κ-CN	Kappa casein	Caseína tipo kappa
LDA	Laser doppler anemometry	
LNC	Lipidic nanocapsules	Nanocapsulas lipídicas
MDR	Multi Drug Resistance	Resistencia multipla a fármacos
MPS	Mononuclear phagocytic system	Sistema fagocítico mononuclear
MRT	Mean retention time	Tiempo de retención media
MTD	Maximun tolerated dose	Dosis máxima tolerada
NCs	Nanocapsules	Nanocápsulas
NEs	Nanemulsions	Nanoelmulsiones
PACA	Polyalquil cyanoacrylate	poli(alquilcianoacrilato)
PASN	Poly-l-asparagine	Poli-l-asparagina
PBCA:	Poly(isobutylcyanoacrylate)	Poli(isobutilcianoacrilato)

PCL	Poly-epsylon-caprolactone	Poli-epsiolon-caprolactona
PCS	Photon correlation spectroscopy	Espectroscopia de correlacion fotonica
PEG	Poly ethileglicol	Poli etilenglicol
PLA	Poly lactic acid	Ácido Polilactico
PLGA	Poly lactic-co- glycolic acid	Ácido Polilactico Glicolico
RES	Reticular endothelial system	Sistema Retículo Endotelial
t1/2	Half life	Semivida de absorción
t1/2α	Distribution half live	Semivida de distribucion
t1/2β	Elimination half live	Semivida de eliminación
TEM	Transmission Electronic Microscopy	Microscopía de transmisión electrónica
Vdss	Distribution volume (steady state)	Volumen de distribución (estado de equilibrio)
W/O	Water in oil simple emulsion	Emulsion simple agua en aceite
O/W	Oil in Water simple emulsion	Emulsion simple aceite en agua

INTRODUCCIÓN. CAPÍTULO 1

CÁNCER Y NANOMEDICINA

La nanomedicina es una rama de la nanotecnología que engloba diversas aplicaciones de la biotecnología, las ingenierías, la medicina y la tecnología farmacéutica en el tratamiento, prevención y diagnóstico de múltiples enfermedades utilizando materiales y dispositivos a escala nanométrica, es decir de entre 1 y 1000 nm [1].

Durante los últimos años, una de las aplicaciones con más auge, y quizás la más representativa, se ha dirigido al tratamiento del cáncer en sus distintas formas mediante la vehiculización, de fármacos antitumorales incorporados en nanosistemas[2]. El término cáncer se utiliza para identificar a un conjunto de más de cien tipos diferentes de enfermedades, caracterizadas todas ellas por un crecimiento celular indiscriminado y no controlado. Dicho crecimiento con el paso del tiempo provoca la invasión y el daño a tejidos circundantes mediante la diseminación de las células enfermas a través del sistema linfático o sanguíneo [3]. Los recientes avances en oncología han permitido que actualmente se tenga un amplio y profundo conocimiento sobre esta enfermedad, llegando a conocer características muy específicas sobre la señalización y el desarrollo de los tumores y de las células que los componen.

[1] **Hervella, P., et al.**, *Nanomedicine: New Challenges and Opportunities in Cancer Therapy*. J. Biomed. Nanotechnol., 2008. **4**(3): p. 276-292.

[2] **Wang, A.Z., et al.**, *Nanoparticle Delivery of Cancer Drugs.* Annual review of medicine, 2011. **63**(1).

[3] *Diccionario del Cáncer. National Cancer Institute*. 2011 15-06-2011 [cited 2011 24-10-2011]; Available from: http://www.cancer.gov/diccionario/.

No obstante, el cáncer sigue siendo una de las enfermedades más devastadoras a nivel mundial, con miles de casos nuevos y millones de muertes en el mundo a su alrededor [4]. Estas cifras se deben principalmente de la falta de un tratamiento eficaz y lo suficientemente accesible. Actualmente, el tratamiento del cáncer se encuentra limitado al uso de técnicas altamente invasivas como lo son la cirugía, radiación y quimioterapia, causantes de la aparición de múltiples efectos adversos y por ende de la alteración de la salud integral del paciente [5].

Los mayores obstáculos a los que la ciencia se enfrenta para la obtención de una terapia ideal son principalmente los asociados a las características fisicoquímicas y farmacocinéticas de los fármacos antitumorales y a los efectos adversos éstas generan. En este sentido, es bien sabido que la mayoría de los fármacos anticancerígenos presentan tras administración I.V. una biodistribución indiscriminada, además de un corto período de residencia en el organismo. A ello se suma, la escasa especificidad presentada por los medicamentos actuales, provocando que tanto los tejidos dañados como los sanos sufran los mismos efectos tóxicos [6]. Esta toxicidad se añade a la toxicidad intrínseca asociada a los excipientes utilizados para solubilizar estas moléculas, mismas que en la mayoría de los casos presentan una elevada lipofilia.

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[4] **Parkin, D.M.**, *Global cancer statistics in the year 2000*. The Lancet Oncology, 2001. **2**(9): p. 533-543.

[5] **Miranda, V., et al.**, *Adverse Drug Reactions and Drug Interactions as Causes of Hospital Admission in Oncology*. Journal of Pain and Symptom Management, 2011. **42**(3): p. 342-353.

[6] **Duncan, R.**, *Polymer conjugates as anticancer nanomedicines.* Nat. Rev. Cancer, 2006. **6**(9): p. 688-701.

VEHICULIZACIÓN SELECTIVA DE NANOESTRUCTURAS

La nanomedicina en los últimos años ha aportado múltiples herramientas a la hora de solucionar los problemas descritos previamente. Así, distintas nanoestructuras han demostrado su capacidad para alargar la permanencia del fármaco en circulación, vehiculizarlo hacia lugares específicos mediante diferentes técnicas de *targeting* [7], o bien mejorar la solubilización de los antitumorales hidrofóbicos, eliminando el uso de solventes y tensoactivos tóxicos.

Se conoce como *targeting*, o vehiculización selectiva, a la propiedad que presentan diferentes nanosistemas de conducir las moléculas que llevan asociadas, a través del organismo hasta un tejido específico denominado tejido diana. La nanomedicina contempla para conseguir este fin dos estrategias, conocidas como *targeting pasivo* y *targeting activo* [8].

TARGETING PASIVO

El *targeting* pasivo es probablemente en la actualidad la estrategia de vehiculización más común en el desarrollo de nuevos nanomedicamentos. Dicha estrategia se basa en dos aspectos fundamentales y complementarios como lo son el tamaño del sistema al que el fármaco se encuentra asociado y la capacidad de dichos sistemas de circular durante tiempos prolongados tras su administración, normalmente endovenosa. Además, se intenta aprovechar de una manera sencilla la fisiología aberrante de ciertos tejidos tumorales, especialmente de tumores sólidos.

[7] **Wang, M. and Thanou, M.**, *Targeting nanoparticles to cancer*. Pharmacol. Res., 2010. **62**(2): p. 90-99.

[8] **Danhier, F., et al.**, *To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery*. J Control Release, 2010. **148**(2): p. 135-146.

A diferencia de los tejidos sanos, en los que el endotelio capilar presenta espacios intercelulares lo suficientemente estrechos para evitar la extravasación de macromoléculas, la vascularización en tumores sólidos es generalmente anormal, con ramificaciones aberrantes y espacios intercelulares mucho más grandes, llegando a 400 nm de separación, lo que conduce a una escasa función barrera del endotelio. Esta extraña organización se debe fundamentalmente a la rápida proliferación de las células endoteliales[9].

La constitución tan característica de este tipo de tumores ocasiona la aparición tanto de una permeabilidad vascular alta como de una conductividad hidráulica elevada, permitiendo a macromoléculas y nanoestructuras, acceder al tumor de una forma en la que en los tejidos sanos sería imposible.

Los tumores sólidos presentan además una escasa irrigación linfática que, si tejidos sanos es la responsable del aclaramiento de macromoléculas, en el tejido enfermo causa una acumulación gradual de este tipo de moléculas, así como de nanoestructuras. Estos dos fenómenos: la penetración facilitada al tumor y su posterior acumulación debido al deficiente drenaje linfático, se le conoce como el Efecto de Permeabilidad y Retención Incrementada, generalmente abreviado como EPR [10].

Para conseguir la acumulación selectiva de nanoestructuras en tumor es necesario que estos sistemas presenten características de larga permanencia en plasma. Sin embargo, muchas nanopartículas son

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[9] **Jain, R.K. and Stylianopoulos, T.**, *Delivering nanomedicine to solid tumors.* Nat Rev Clin Oncol, 2010. **7**(11): p. 653-664.

[10] **Maeda, H.**, *The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting.* Adv. Enzyme Regul., 2001. **41**(1): p. 189-207.

rápidamente eliminadas de la circulación por acción del sistema fagocítico mononuclear (MPS), comúnmente llamado sistema retículo endotelial. El MPS comprende un conjunto considerable de células encargadas de la eliminación de cuerpos extraños de la circulación, sin embargo, son las células de Kupffer y los macrófagos del hígado y del bazo los encargados de la eliminación de macromoléculas [11].

TARGETING ACTIVO

En el caso del *targeting activo*, la estrategia utilizada consiste en aprovechar la expresión de receptores específicos sobre la superficie de células tumorales o endoteliales. La fijación a los nanosistemas de moléculas que se unen de manera específica a dichos receptores, se lleva a cabo con la finalidad de lograr una orientación específica al tumor [12]. En esta estrategia, la distribución a tumor no está basada únicamente en la acumulación indiferenciada, por lo que su efectividad no es dependiente de la fisiología vascular del tumor. Se distinguen dos estrategias bien diferenciadas, el *targeting* a células cancerosas y el *targeting* al endotelio tumoral.

En la orientación específica hacia las células cancerosas, son las características bioquímicas y fisiológicas particulares del tumor, la sobreexpresión de receptores y las condiciones del ambiente tumoral, los que determinan la posible especificidad tumoral. Así, la gran mayoría fundamenta el desarrollo de sistemas en la modificación de su superficie, bien enlazando moléculas con respuesta a diferentes estímulos o bien

[11] **Hume, D.A.**, *The mononuclear phagocyte system.* Current Opinion in Immunology, 2006. **18**(1): p. 49-53.

[12] **Danhier, F., et al.**, *To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery.* J Control Release, 2010. **148**(2): p. 135-146.

enlazando ligandos específicos de la biología tumoral [13], utilizando desde sencillas moléculas hasta complejas macromoléculas diseñadas específicamente.

Actualmente, el uso de anticuerpos monoclonales emerge como una de las técnicas con más futuro en el desarrollo de nanotransportadores con orientación activa [14]. Otras moléculas de bajo peso molecular como el ácido fólico [15], o polisacáridos, como el ácido hialurónico, han sido utilizados con el mismo fin. Así, el ácido fólico ha sido ampliamente utilizado en investigación explotando la sobreexpresión de receptores folato en determinados tumores como el de ovario, mientras que el ácido hialurónico, orientado al receptor CD-44, se ha investigado diferentes tipos de tumores sólidos y leucemias [16]. En este sentido, los receptores más estudiados y explotados en la búsqueda de una orientación activa a células tumorales han sido el receptor de transferrina [17], el receptor de folato [18], algunas glicoproteínas expresadas en la superficie celular como las lectinas [19], y el receptor del factor de crecimiento epidermal [20].

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[13] **Wang, A.Z., et al.**, *Nanoparticle Delivery of Cancer Drugs.* Annual review of medicine, 2011. **63**(1).

[14] **Schliemann, C. and Neri, D.**, *Antibody-based targeting of the tumor vasculature.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2007. **1776**(2): p. 175-192.

[15] **Kalli, K.R., et al.**, *Folate receptor alpha as a tumor target in epithelial ovarian cancer.* Gynecologic Oncology, 2008. **108**(3): p. 619-626.

[16] **Mizrahy, S., et al.**, *Hyaluronan-coated nanoparticles: The influence of the molecular weight on CD44-hyaluronan interactions and on the immune response.* Journal of controlled release : official journal of the Controlled Release Society, 2011(0).

[17] **Cho, K., et al.**, *Therapeutic Nanoparticles for Drug Delivery in Cancer.* Clinical Cancer Research, 2008. **14**(5): p. 1310-1316.

[18] **Low, P.S. and Kularatne, S.A.**, *Folate-targeted therapeutic and imaging agents for cancer.* Current Opinion in Chemical Biology, 2009. **13**(3): p. 256-262.

[19] **Tamara, M.**, *Drug targeting to the colon with lectins and neoglycoconjugates.* Advanced Drug Delivery Reviews, 2004. **56**(4): p. 491-509.

[20] **Sebastian, S., et al.**, *The complexity of targeting EGFR signalling in cancer: From expression to turnover.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2006. **1766**(1): p. 120-139.

Más recientemente, el *targeting* hacia el endotelio tumoral es una estrategia que ha sido utilizada en el tratamiento de tumores sólidos. Mediante esta estrategia es posible vehiculizar moléculas activas para provocar la destrucción del endotelio en este tipo de tumores, esto puede representar la destrucción del tumor propio ya que se origina la falta de oxígeno y nutrientes evitando que el tumor tenga acceso a estos mediante nuevos vasos sanguíneos[21]. Algunas de las ventajas que aporta esta orientación selectiva serían 1) los nanotransportadores no necesitan extravasación para alcanzar su diana; 2) la unión a los receptores específicos puede darse directamente tras administración intravenosa; 3) el desarrollo de resistencia a este tipo de terapias es prácticamente imposible, debido principalmente a la alta estabilidad y homogeneidad genética de las células endoteliales, al contrario de lo que sucede en las células del tumor y 4) la mayoría de los marcadores biológicos utilizados en éste tipo de *targeting* son útiles para el tratamiento de cualquier tipo de tumor sólido, incrementando de manera considerable el espectro terapéutico de estos nanomedicamentos [22]. Los principales receptores explotados para el *targeting* a endotelio tumoral son los de los factores de crecimiento endotelial vascular (VEGF por sus siglas en inglés) [23], la integrina $\alpha_V\beta_3$ [24], la molécula-1 de adhesión celular vascular (VCAM-1) [25] y finalmente la matriz de metaloproteinasas [26].

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[21] **Lammers, T., et al.**, *Tumour-targeted nanomedicines: principles and practice.* Br J Cancer, 2008. **99**(3): p. 392-397.

[22] **Danhier, F., et al.**, *To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery.* J Control Release, 2010. **148**(2): p. 135-146.

[23] **Shadidi, M. and Sioud, M.**, *Selective targeting of cancer cells using synthetic peptides.* Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy, 2003. **6**(6): p. 363-371.

[24] **Desgrosellier, J.S. and Cheresh, D.A.**, *Integrins in cancer: biological implications and therapeutic opportunities.* Nat Rev Cancer, 2010. **10**(1): p. 9-22.

REQUISITOS FISICO-QUÍMICOS DE LOS NANOMEDICAMENTOS

Cabe aclarar que para que ambos tipos de orientación selectiva puedan tener lugar, los nanomedicamentos deben cumplir ciertas características físico-químicas que les permitan alcanzar el tejido diana una vez que se encuentren circulando por el organismo. A continuación se detallan algunas de las características necesarias y algunas estrategias utilizadas para conseguir que los nanosistemas alcancen el tejido diana.

TAMAÑO, FORMA Y CARGA ELÉCTRICA SUPERFICIAL

Las características físico-químicas de los nanotransportadores condicionan irremediablemente la forma en que éstos interaccionan con el medio biológico y el organismo. Dichas características determinan la forma en que se producirá su eliminación, el tiempo que permanecerán en circulación, los lugares en los que se acumularán y su capacidad para alcanzar el tejido diana. Es sabido, por ejemplo, que para que ocurra una eficiente extravasación a través de las fenestras del complejo tumoral los nanosistemas deberán poseer un tamaño inferior a los 400 nm, sin embargo, por otro lado, para evitar la filtración renal, las nanoestructuras deben de presentar un tamaño superior a los 10 nm, y más específicamente, para promover la captura hepática deberán de presentar un tamaño menor a los 100 nm [27].

[25] **Dienst, A., et al.**, *Specific Occlusion of Murine and Human Tumor Vasculature by VCAM-1–Targeted Recombinant Fusion Proteins.* J. Natl. Cancer Inst., 2005. **97**(10): p. 733-747.

[26] **Zhao, T., et al.**, *A novel strategy to tag matrix metalloproteinases-positive cells for in vivo imaging of invasive and metastatic activity of tumor cells.* J Control Release, 2010. **144**(1): p. 109-114.

[27] **Danhier, F., et al.**, *To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery.* J Control Release, 2010. **148**(2): p. 135-146.

Por otro lado, la carga eléctrica superficial de las nanopartículas juega también un rol fundamental a la hora de conseguir nanotransportadores de larga permanencia en circulación tras su administración intravenosa o intramuscular. Dicha carga deberá ser preferentemente neutra o ligeramente aniónica para evitar la interacción con las opsoninas y, en general, con las células sanguíneas [28].

Finalmente, la forma de los nanotransportadores ha sido un parámetro geométrico que se ha descuidado durante mucho tiempo en el diseño de los nanosistemas. En este sentido, se ha demostrado que algunas nanopartículas con formas no esféricas, y más bien alargadas u ovoides, han demostrado poseer mayor capacidad de carga de fármaco y lograr mejoras en la biodistribución, tiempo de circulación y acumulación en ciertos tejidos, al ser comparadas con las esféricas [29]. Sin embargo, es importante señalar que de momento las conclusiones que se puedan extraer con respecto a forma de las nanopartículas siguen estando poco definidas, y suponen un reto importante en el diseño de nanosistemas.

CARACTERISTICAS DE SUPERFICIE

La composición química y la hidrofília de la superficie de los nanotransportadores son otros dos factores de gran importancia a la hora de evitar el proceso de eliminación por parte del MPS. Así se sabe que partículas con superficies hidrofílicas son generalmente invisibles para las células del MPS, presentando por lo tanto un mayor tiempo de permanencia en plasma. Se han utilizado diversas estrategias para conseguir nanovehículos de larga permanencia en sangre, con el objetivo

[28] **Owens Iii, D.E. and Peppas, N.A.**, *Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles.* International Journal of Pharmaceutics, 2006. **307**(1): p. 93-102.

[29] **Venkataraman, S., et al.**, *The effects of polymeric nanostructure shape on drug delivery.* Advanced Drug Delivery Reviews, 2011. **63**(14–15): p. 1228-1246.

de favorecer tanto su acumulación en el área tumoral como el reconocimiento entre ligandos y receptores. Las más sencillas y eficaces se basan en la modificación de su superficie con diferentes materiales, con la intención de dotarlos de una cubierta tipo "stealth" o "camuflaje" a los sistemas, evitando que sean capturados por el MPS.

La modificación más popular para conseguir este "camuflaje" es el recubrimiento en superficie con cadenas de moléculas hidrofílicas, en especial de polietilenglicol (PEG), estrategia denominada pegilación. Hasta la fecha se ha descrito la pegilación de una gran variedad de nanosistemas, consiguiendo en muchos casos aumentos significativos de los tiempos de vida media de fármacos y marcadores[30]. Además de aportar la hidrofilia y movilidad de sus cadenas, el PEG aporta también carácter eléctrico negativo a la superficie de los nanosistemas, lo que minimiza las posibles interacciones electrostáticas con superficies celulares [31].

Además del PEG, se han estudiado otros polímeros para dotar a los nanosistemas de larga permanencia plasmática [32]. Entre ellos cabe resaltar biomateriales como proteínas [33], poliaminoácidos [34] y polisacáridos [35].

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[30] **Molineux, G.**, *Pegylation: engineering improved pharmaceuticals for enhanced therapy.* Cancer Treatment Reviews, 2002. **28, Supplement 1**(0): p. 13-16.

[31] **Howard, M.D., et al.**, *PEGylation of nanocarrier drug delivery systems: State of the art.* J. Biomed. Nanotechnol., 2008. **4**(2): p. 133-148.

[32] **Torchilin, V.P. and Trubetskoy, V.S.**, *Which polymers can make nanoparticulate drug carriers long-circulating?* Advanced Drug Delivery Reviews, 1995. **16**(2–3): p. 141-155.

[33] **Elzoghby, A.O., et al.**, *Albumin-based nanoparticles as potential controlled release drug delivery systems.* J Control Release, (0).

[34] **Romberg, B., et al.**, *Poly(amino acid)s: Promising enzymatically degradable stealth coatings for liposomes.* International Journal of Pharmaceutics, 2007. **331**(2): p. 186-189.

[35] **Lemarchand, C., et al.**, *Polysaccharide-decorated nanoparticles.* European Journal of Pharmaceutics and Biopharmaceutics, 2004. **58**(2): p. 327-341.

NANOMEDICAMENTOS Y TERAPIA ANTITUMORAL

CLASIFICACIÓN Y AVANCES CLÍNICOS

En la actualidad, el desarrollo de la nanomedicina ha llevado a que una gran variedad de nanoestructuras se encuentren en fases avanzadas de desarrollo clínico para su aplicación en la terapia del cáncer. Entre ellas, nos encontramos liposomas, conjugados poliméricos, micelas poliméricas y nanopartículas. A continuación se describen las principales características de cada una y sus avances clínicos.

LIPOSOMAS

Los liposomas (Figura 1), son vesículas constituidas en su forma más sencilla por una bicapa lipídica que rodea a una cavidad acuosa central. No obstante, se pueden obtener desde sistemas bicapa hasta sistemas multicapa, dependiendo de la técnica de obtención y los materiales empleados, pudiendo presentar tamaños de decenas o de centenares de nanómetros.

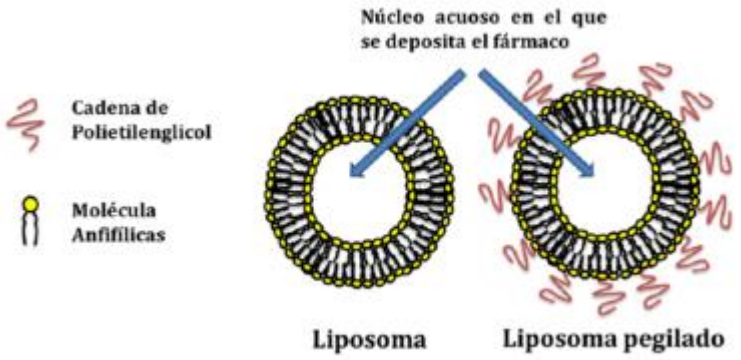

Figura 1. Esquema de dos estructuras de liposomas. La más sencilla construida a partir de moléculas anfifílicas a la izquierda, y a la derecha una estructura liposomal modificada con PEG

Estos sistemas, como la gran mayoría de las nanoestructuras estudiadas en clínica, presentan un perfil de toxicidad y seguridad lo suficientemente aceptable, además de que generalmente están elaborados a partir de materiales biocompatibles.

Tabla 1. Formulaciones liposomales actualmente comercializadas o en fase de evaluación clínica.

LIPOSOMAS			
Nombre comercial	**Fármaco**	**Indicación**	**Status (año)**
Doxil®	Doxorubicina	Cáncer de ovario, mama y sarcoma de Kaposi	Aprobado (1995)
Myocet®	Doxorubicina	Cáncer de mama metastático en mujeres adultas	Aprobado (1995)*
DaunoXome®	Doxorubicina	Sarcoma de Kaposi	Aprobado (1996)
Onco-TCS® (Marqibo®)	Vincristina	Varios tipos de linfoma, leucemia y melanoma	Aprobado (2004)
Thermodox®	Doxorubicina	Cáncer de mama y de pulmón.	Fase III

*Aprobado por la EMA

Una formulación liposomal, el Doxil®, fue el primer nanomedicamento aprobado para su uso en clínica para el tratamiento del cáncer. En la actualidad son cuatro las formulaciones que se encuentran comercializadas (Tabla 1). Tanto el Doxil® como el Myocet® se encapsulan la antraciclina doxorubicina. Mientras que el Myocet®, que a

diferencia del Doxil® no se encuentra pegilado, está aprobado únicamente en Canadá y Europa en una terapia asociada a ciclofosfamida [36].

Además de las formulaciones ya mencionadas, el Onco-TCS® (Marqibo®) es otra formulación liposomal no peguilada diseñada para la vehiculización de la vincristina. El Onco-TCS®, ha demostrado reducir la neurotoxicidad de la vincristina y está indicado en el tratamiento del linfoma no-Hodgkin en asociación con otros citostáticos [37]. Finalmente, en la actualidad se encuentran cerca de 512 estudios de fase clínica en Estados Unidos y 17 en Europa (http://www.clinicaltrials.gov, Febrero 2012) . Destacan entre estos el Thermodox®, un sistema que encapsula doxorubicina, y que actualmente se encuentra en estudios de Fase III con la indicación de cáncer de mama y de pulmón.

NANOPARTÍCULAS

Las nanopartículas son sistemas matriciales obtenidos a partir de una gran variedad de materiales, en su mayoría polímeros, tanto naturales como sintéticos. Dentro de los polímeros naturales, o biopolímeros, a partir de los cuales se han elaborado nanopartículas, es interesante resaltar algunas proteínas como la albumina, polisacáridos como el quitosano o el ácido hialurónico, polipéptidos y poliaminoácidos. Dentro de la infinidad de materiales de origen sintético, se puede hacer particular mención de los poliésteres y los poliacrilatos [38].

[36] **Martin, F.** *Comparison of Liposomal Doxorubicin Products: Myocet Vs. DOXIL. Apples to Apples?* 2011 01/25/2011 [cited 2012 13-01-2012]; Available from: http://www.fda.gov/ohrms/dockets/ac/01/slides/3763s2_08_martin/sld001.htm.

[37] **Yang, F., et al.**, *Liposome based delivery systems in pancreatic cancer treatment: From bench to bedside.* Cancer Treatment Reviews, 2011. **37**(8): p. 633-642.

[38] **Hervella, P., et al.**, *Nanomedicine: New Challenges and Opportunities in Cancer Therapy.* J. Biomed. Nanotechnol., 2008. **4**(3): p. 276-292.

Tabla 2. Formulaciones de nanopartículas actualmente comercializadas o en fase de evaluación clínica.

NANOPARTÍCULAS				
Nombre comercial	**Fármaco**	**Ingredientes**	**Indicación**	**Status (año)**
Abraxane ®	PTX	Albumina	Cáncer de mama	A (2005)
Livatag® (Transdrug ®)	DOX	Polialquil-cianoacrilatos	Hepatocarcino-ma	FC I/II
NBTXR3	-	Cristales de óxido de hafnio	Sarcoma de tejido blando	FC I
Panzem®	Metoxi-estradiol	Dispersión nanocristalina de 2-metoxiestradiol	Cáncer de ovario y glioblastoma multiforme	FC III

PTX – Paclitaxel; DOX – Doxorubicina; A – Aprobado; FC – Fase clínica

Cabe destacar que aunque existe solamente una formulación de nanopartículas aprobada para su uso en humanos, otras se encuentran en fases avanzadas de estudios clínicos tanto en Estados Unidos, Europa y Asía (Tabla 2). La formulación aprobada es el Abraxane®, un sistema constituido por albumina, diseñado para la vehiculización del paclitaxel. Actualmente, tanto a FDA como la EMA la consideran para el tratamiento del cáncer de mama metastático [39]. Otro de los grandes avances en la clínica de las nanopartículas lo representa el Livatag® (tecnología Transdrug®), un sistema nanoparticulado a base de poli-isocianoacrilatos diseñado para la vehiculización de doxorubicina. Este sistema, actualmente en ensayos clínicos Fase II, ha demostrado la capacidad de

[39] **Elsadek, B. and Kratz, F.**, *Impact of albumin on drug delivery - New applications on the horizon.* Journal of controlled release : official journal of the Controlled Release Society, 2011(0).

aumentar significativamente la supervivencia en pacientes con carcinoma hepatocelular, en comparación a la conseguida con el tratamiento clásico de quimioembolización [40].

Cabe aclarar que dentro del arsenal de los sistemas conocidos como nanopartículas existe otro tipo de sistemas no poliméricos, como pueden ser las nanopartículas metálicas, magnéticas o cristalinas. Así por ejemplo, actualmente, existe una formulación, el Panzem®, 2-methoxyestradiol en forma de una dispersión nanocristalina en ensayos clínicos Fase III para el tratamiento de cáncer ovárico y en glioblastoma multiforme. Cabe señalar que esta formulación en forma de nanocristales de fármaco se administra por vía oral. Otro ejemplo lo constituyen las nanopartículas metálicas de óxido de hafnio, propuestas como potenciadoras del efecto de la radioterapia, que se encuentran en ensayos clínicos Fase I.

CONJUGADOS POLIMÉRICOS

El término conjugado se refiere a nanoestructuras híbridas consistentes en polímeros enlazados covalentemente a un agente terapéutico [41]. Dentro de los conjugados poliméricos se distinguen dos grupos principales, los conjugados polímero-proteína y los conjugados polímero-fármaco (Figura 2).

[40] **Merle, P.** *Presentation of Livatag® (BioAlliance Pharma) survival results*. in *International liver cancer congress*. 2011. Hong Kong.

[41] **Duncan, R.**, *Polymer conjugates as anticancer nanomedicines.* Nat. Rev. Cancer, 2006. **6**(9): p. 688-701.

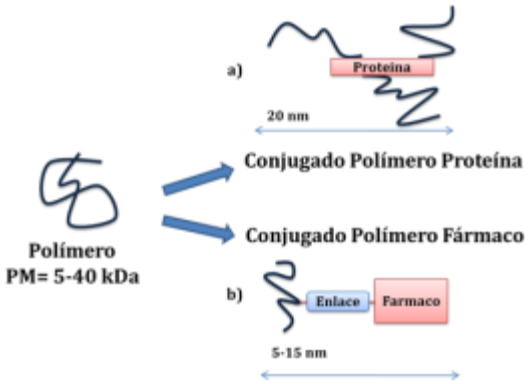

Figura 2. Esquema de conjugados poliméricos. Se muestran los dos tipos de conjugados poliméricos que se encuentran en estudio clínico: a) Conjugado polímero-proteína, en éste caso el ingrediente activo terapéutico es una proteína, pudiendo ser un enzima o un anticuerpo. b) Conjugado polímero-fármaco, en cuyo caso el ingrediente activo es una molécula terapéutica.

Dentro de los conjugados polímero-proteína, en 1990, se comercializó el primero de ellos bajo el nombre de Zinostatin Stimalamer®, también conocido por las siglas SMANCS. Este sistema es un conjugado de estireno-anhídrido maléico (SAM) y una proteína con actividad antitumoral, la neocarzinostatina (NCS). Se encuentra indicado para el tratamiento de carcinoma hepatocelular. La asociación al agente de contraste Lipiodol® ha permitido una correcta visualización del tumor [42].

[42] **Matsumura, Y. and Maeda, H.**, *A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs.* Cancer Research, 1986. **46**(12 Part 1): p. 6387-6392.

Tabla 3. Formulaciones a base de conjugados poliméricos actualmente comercializadas o en fase de evaluación clínica.

CONJUGADOS				
Conjugados polímero-proteína				
Nombre comercial	**Proteína**	**Polímero**	**Indicación**	**Status (año)**
Zinostatin Stimalmer®	SMANCS	Estireno-anhídrido maléico	Carcinoma hepatocelular	A(1990)
Oncaspar®	L-asparaginasa	PEG	Leucemias	A(1994)
PEG-Asys®	Interferon α -2[a]	PEG	Melanoma y carcinoma renal	Fase I-II
PEG-Intron™	Interferon α -2b	PEG	Melanoma y carcinoma renal	Fase I-II
Conjugados polímero-fármaco				
Nombre comercial	**Fármaco**	**Polímero**	**Indicación**	**Status**
Opaxio® (Xyotax®)	Paclitaxel	Poliglutamato	Cáncer de mama y de ovario	Fase II-III
Prolindac (AP5346)	Platino-DACH	HPMA	Cáncer de ovario	Fase II
PK1	DOX	HPMA	Cáncer de mama, de pulmón y de colon.	Fase II
PK2	DOX	HPMA – Galacto-samina	Carcinoma hepatocelular	Fase II
NKTR-102	Irinotecan	PEG	Cáncer de mama Cáncer colorectal, de pulmón y de ovario	Fase III Fase II
Prothecan®	Campto-tecina	PEG	Cáncer gástrico y de esófago	Fase II
CRLX101	Campto-tecina	Ciclo-dextrinas	Cáncer de pulmón no microcítico	Fase II

HPMA – Hidroxipropilmetracrilamida; DOX – Doxorubicina; A - Aprobado

Como se mencionó anteriormente, una de las estrategias de conjugación que merece ser destacada por su importancia es la pegilación. Precisamente, el Oncaspar®, comercializado en 1994, consiste en la unión covalente del enzima L-asparaginasa a una cadena de PEG. Está indicado como fármaco de primera línea en pacientes con leucemia linfoblástica. Mediante la conjugación se consiguió aumentar el tiempo de vida media del enzima, disminuyendo así la frecuencia de la administración [43].

Existen, además de las comercializadas, dos formulaciones actualmente en estudios clínicos Fase II. El PEG-Asys® y el PEG-Intron™, ambos constituidos por interferones alfa pegilados, están indicados en el tratamiento de melanoma y carcinoma renal.

En lo que respecta a los conjugados polímero-fármaco, hasta el momento, no se ha obtenido la aprobación en clínica para ninguno de ellos, sin embargo, existen numerosas formulaciones en fases avanzadas de estudios clínicos. Entre ellos, cabe destacar la importancia del Opaxio®, formulación también conocida con el nombre de Xyotax®. Esta formulación fue el primero de los conjugados de este tipo en alcanzar una Fase clínica III. Está constituido por la unión covalente entre el ácido poliglutámico y el paclitaxel y sus indicaciones son el tratamiento de cáncer de esófago, recto, mama, ovario y pulmón [44]. Mediante la combinación con el ácido poliglutámico se ha conseguido aumentar la actividad antitumoral del paclitaxel, además de eliminar todos aquellos excipientes que ocasionan reacciones adversas en la formulación comercial del paclitaxel, como el Cremophor®.

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[43] **Graham, M.L.**, *Pegaspargase: a review of clinical studies.* Adv Drug Deliv Rev, 2003. **55**(10): p. 1293-302.

[44] **Canal, F., et al.**, *Polymer–drug conjugates as nano-sized medicines.* Current Opinion in Biotechnology, 2011. **22**(6): p. 894-900.

Además del Xyotax®, hay otros conjugados polímero-fármaco que se encuentran actualmente en fase clínica, entre ellos los constituidos por PEG o por el polímero hidroxipropilmetacrilamida (HPMA). En el caso de los constituidos por PEG, se puede destacar al sistema NKTR-102 [45], un conjugado de irinotecan en Fase II con la indicación de cáncer de colon, mama y ovario, que ha conseguido un aumento de su eficacia, al incrementar la concentración en el área tumoral. Otra formulación de éste tipo es el Prothecan®, conjugado PEG-camptotecina, que a pesar de estar actualmente en suspensión, alcanzó en su momento la Fase II, demostrando un aumento considerable en la eficacia antitumoral gracias a la conjugación. El Prolindac® es un conjugado HPMA-oxiplatino, que se encuentra actualmente en Fase clínica II y que ha conseguido aumentar la eficacia del fármaco en el tratamiento del cáncer de ovario [46-47]. Además, existen dos formulaciones de conjugados de doxorubicina a partir de HPMA en Fase clínica II, el PK1 y el PK2.

Finalmente, el CRLX101, es una formulación de nanopartículas a base de un polímero de cadena lineal de ciclodextrinas que conjugan al fármaco camptotecina. Esta formulación está siendo ensayada en estudios clínicos Fase II para el tratamiento del cáncer de pulmón no microcítico, observándose un aumento considerable de la residencia del fármaco en tejido tumoral [48].

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[45] **Merle, P.** *Presentation of Livatag® (BioAlliance Pharma) survival results*. in *International liver cancer congress*. 2011. Hong Kong.

[46] **Campone, M., et al.**, *Phase I and pharmacokinetic trial of AP5346, a DACH–platinum–polymer conjugate, administered weekly for three out of every 4 weeks to advanced solid tumor patients.* Cancer Chemotherapy and Pharmacology, 2007. **60**(4): p. 523-533.

[47] **Nowotnik, D.P. and Cvitkovic, E.**, *ProLindac™ (AP5346): A review of the development of an HPMA DACH platinum Polymer Therapeutic.* Advanced Drug Delivery Reviews, 2009. **61**(13): p. 1214-1219.

[48] **Schluep, T., et al.**, *Preclinical Efficacy of the Camptothecin-Polymer Conjugate IT-101 in Multiple Cancer Models.* Clinical Cancer Research, 2006. **12**(5): p. 1606-1614.

MICELAS POLIMÉRICAS

Otra de las nanoestructuras en desarrollo clínico la constituyen las micelas. Son nanoestructuras originadas a partir del auto-ensamblaje de moléculas anfifílicas, generalmente tensoactivos, proteínas o polímeros sintéticos o naturales, de tamaño comprendido entre los 10 y los 100 nm. Estos sistemas presentan una estructura tipo reservorio con un núcleo generalmente hidrofóbico, que comúnmente incluye el fármaco, y una superficie hidrofílica [49] (Figura 3).

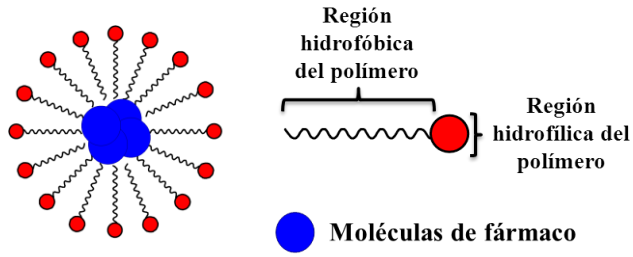

Figura 3. Esquema de una micela polimérica mostrando los componentes de la misma.

En la Tabla 4 se resumen los mayores avances clínicos obtenidos con micelas.

[49] **Blanco, E., et al.**, *Nanomedicine in cancer therapy: Innovative trends and prospects.* Cancer Sci., 2011. **102**(7): p. 1247-1252.

Tabla 4. Formulaciones de micelas poliméricas actualmente en fase de evaluación clínica.

MICELAS				
Nombre comercial	**Fármaco**	**Composición**	**Indicación**	**Status (año)**
Genexol-PM®	Paclitaxel	Ácido poliláctico -PEG	Cáncer de mama y de ovario	Fase II
			Cáncer de ovario	Fase I-II[1]
			Cáncer de pulmón no microcítico	Fase II[1]
			Cáncer pancreático	Fase III[2]
NK105	Paclitaxel	PEG-poliaspartato	Cáncer de estómago	Fase II
Paclical®	Paclitaxel	Derivado de Vitamina A	Cáncer de ovario	Fase III
NK012	SN 38	PEG-poliglutamato	Cáncer de mama	Fase II
Nanoxel-PM®	Docetaxel	PEG-ácido-poli-D-l-láctico	Cáncer de mama	Fase I
NC-6004/ Nanoplatin	Cisplatino	Poliglutámico-PEG	Cáncer pancreático	Fase I
SP1049C	Doxoru-bicina	Pluronics	Cáncer de esófago y de estómago	Fase III

[1]En terapia combinada con la administración de carboplatino. [2]En terapia combinada con la administración de gemcitabina

Existen actualmente cinco formulaciones de micelas en ensayos clínicos. El estado más avanzado es el del Genexol® PM, obtenido a partir del autoensamblaje de un polímero dibloque ácido poliláctico-PEG (PLA-PEG) encapsulando paclitaxel. Estas micelas se encuentran en

estudios clínicos de Fase III en su indicación para cáncer de mama, de pulmón no microcítico y páncreas [50].

El NK105 es otra formulación que encapsula paclitaxel, consiste en micelas de PEG-poliaspartato con un tamaño medio de 80 nm. Se encuentra en Fase II en su indicación para cáncer de estómago [51]. Y finalmente, el Paclical® está formado por micelas de un derivado de Vitamina A, que encapsula paclitaxel. Se encuentra actualmente en estudios Fase III y está indicado para el tratamiento del carcinoma ovárico Otra formulación actualmente en Fase II en el tratamiento de cáncer de mama es el NK012, micelas de PEG-poliglutamato que contienen un análogo de camptotecina, metabolito del irinotecan. En esta formulación el principio activo se encuentra unido covalentemente a los residuos hidrofóbicos del copolímero, lo que permite una lenta liberación del mismo a partir de la degradación del propio sistema [52].

Las micelas de ácido poliglutámico-PEG conteniendo cisplatino, denominadas NC-6004 (Nanoplatin™) [53], se encuentran en estudios clínicos Fase II evaluando el tratamiento del cáncer de páncreas en terapia combinada con gemcitabina. Los primeros resultados indican que se ha conseguido mediante esta estrategia una reducción significativa de los

[50] **Oerlemans, C., et al.**, *Polymeric Micelles in Anticancer Therapy: Targeting, Imaging and Triggered Release.* Pharm. Res., 2010. **27**(12): p. 2569-2589.

[51] **Kato, K., et al.**, *Phase II study of NK105, a paclitaxel-incorporating micellar nanoparticle, for previously treated advanced or recurrent gastric cancer.* Investigational New Drugs: p. 1-7.

[52] **Yasuhiro, M.**, *Preclinical and clinical studies of NK012, an SN-38-incorporating polymeric micelles, which is designed based on EPR effect.* Advanced Drug Delivery Reviews, 2011. **63**(3): p. 184-192.

[53] **Kataoka, K., et al.**, *Polymeric micelle containing cisplatin enclosed therein and use thereof*, 2006, Toudai TLO, Ltd.: USA.

efectos adversos relacionados a la neurotoxicidad y nefrotoxicidad del cisplatino [54].

El docetaxel formulado en micelas de PEG-ácido poli-D-l-láctico [55], se encuentra en estudios clínicos Fase I, para el tratamiento del cáncer de mama bajo el nombre de Nanoxel-PM®. Esta formulación ha conseguido una reducción significativa de los efectos adversos del Taxotere®.

Finalmente, el SP1049C, micelas formadas por los copolímeros, Pluronic L61 y Pluronic F127, para la vehiculización de doxorubicina se encuentra actualmente en Fase clínica III, indicado para el tratamiento de adenocarcinomas y de cáncer de estómago [56]. Esta formulación presenta un perfil de toxicidad mucho más favorable, y presenta actividad frente a tumores generalmente resistentes a la doxorubicina.

Además de las formulaciones nanométricas ya mencionadas, muchas otras se encuentran en investigación oncológica. Debido a su reciente desarrollo muchas de ellas no se encuentran aún en ensayos clínicos. Entre ellas están las nanocápsulas, que al ser objeto de estudio de esta tesis doctoral, se presentan en un apartado específico.

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[54] **Uchino, H., et al.**, *Cisplatin-incorporating polymeric micelles (NC-6004) can reduce nephrotoxicity and neurotoxicity of cisplatin in rats.* Br. J. Cancer, 2005. **93**(6): p. 678-687.

[55] **Lee, S.-W., et al.**, *Development of docetaxel-loaded intravenous formulation, Nanoxel-PM™ using polymer-based delivery system.* J Control Release, (0).

[56] **Hervella, P., et al.**, *Nanomedicine: New Challenges and Opportunities in Cancer Therapy.* J. Biomed. Nanotechnol., 2008. **4**(3): p. 276-292.

NANOCÁPSULAS

Las nanocápsulas fueron descritas por primera vez por Couvreur *et al* [57] a finales de los años 70s. Estos sistemas ofrecen una gran alternativa para solucionar diferentes retos farmacológicos, ya que sus ventajas van desde un aumento de la biodisponibilidad de fármacos hidrofóbicos, hasta mejoras en la captura celular en los sitios diana, o disminuciones en los mecanismos de resistencia celular [58]. Las nanocápsulas presentan una organización vesicular con un reservorio interno, que puede estar constituido de agua o de aceite, rodeado en general de un recubrimiento polimérico [59] (Figura 4). Un espacio físico como el que ofrece el reservorio de las nanocápsulas, incrementa la posibilidad de obtener una gran carga de fármaco, en comparación a otro tipo de nanoestructuras. Otra función que ofrece el núcleo, en este tipo de sistemas, es el de proteger al fármaco de las condiciones ambientales dentro y fuera del organismo. Por otro lado, la naturaleza liquida del núcleo, y por lo tanto su elasticidad, puede facilitar el contacto de la nanoestructura con el epitelio y de alguna así forma mejorar la internalización. Finalmente, la cubierta polimérica en estos sistemas ofrece la posibilidad de obtener una liberación controlada al fármaco encapsulado.

Se han empleado diversos métodos para la obtención de nanocápsulas. Todos ellos están basados en diferentes principios fisicoquímicos e

[57] **Couvreur, P., et al.**, *Nanocapsules: a new type of lysosomotropic carrier.* FEBS letters, 1977. **84**(2): p. 323-6.

[58] **Hervella P, Lollo G, Oyarzun-Ampuero F, Rivera-Rodriguez G, Torres D, Alonso MJ**, *Nanocapsules as Carriers for the Transport and Targeted Delivery of Bioactive Molecules*, in *Nanocomposite particles for bio-aplications: Materials and bio-interfaces*, Trindade T, Daniel A.L.,, Editor 2011, Pan Stanford Publishing: Singapore. p. 350.

[59] **Legrand, P., et al.**, *Polymeric nanocapsules as drug delivery systems: A review.* S.T.P. Pharma Sciences, 1999. **9**(5): p. 411-418.

incluyen i) la polimerización interfacial [60], ii) el desplazamiento del disolvente, también llamado método de precipitación instantánea[61], iii) inversión de fase por temperatura [62] y iv) la adsorción de un polímero a una nanoemulsion preformada [63]. Como en todas las nanoestructuras, la elección de los materiales y el método de preparación correcto son parámetros cruciales para el éxito del nanosistema. Las nanocápsulas se han aplicado también a la encapsulación de moléculas diferentes a las antitumorales, incluyendo macromoléculas hidrofílicas como el siRNA [64]. A continuación se realizará una descripción más detallada sobre las formulaciones de nanocápsulas enfocadas en el tratamiento de ciertos tipos de cáncer.

NANOCÁPSULAS POLIMÉRICAS

La primera generación de nanocápsulas fue desarrollada por Couvreur y su grupo a finales de los años 70 utilizando poli(alquilcianoacrilatos) (PACA) como materiales de cubierta [65]. Desde entonces, las nanocápsulas de PACA han sido investigadas ampliamente en distintas aplicaciones como la mejora de la biodisponibilidad oral de macromoléculas como la insulina. En el desarrollo de estas nanocápsulas, se han utilizado

[60] **Couvreur, P., et al.**, *Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties.* Journal of Pharmacy and Pharmacology, 1979. **31**(1): p. 331-332.

[61] **Fessi, H., et al.**, *Nanocapsule formation by interfacial polymer deposition following solvent displacement.* International Journal of Pharmaceutics, 1989. **55**(1): p. R1-R4.

[62] **Heurtault, B., et al.**, *A Novel Phase Inversion-Based Process for the Preparation of Lipid Nanocarriers.* Pharm. Res., 2002. **19**(6): p. 875-880.

[63] **Prego, C., et al.**, *Efficacy and Mechanism of Action of Chitosan Nanocapsules for Oral Peptide Delivery.* Pharm. Res., 2006. **23**(3): p. 549-556.

[64] **Lambert, G., et al.**, *Polyisobutylcyanoacrylate nanocapsules containing an aqueous core as a novel colloidal carrier for the delivery of oligonucleotides.* Pharm. Res., 2000. **17**(6): p. 707-714.

[65] **Couvreur, P., et al.**, *Nanocapsules: a new type of lysosomotropic carrier.* FEBS letters, 1977. **84**(2): p. 323-6.

fundamentalmente técnicas de polimerización interfacial y de desplazamiento del solvente [66].

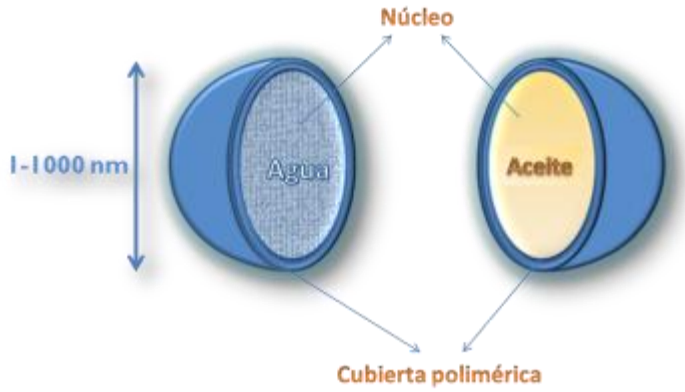

Figura 4. Esquema de dos tipos diferentes de nanocápsulas poliméricas. Con núcleo acuoso (izquierda) y con núcleo oleoso (derecha).

Otros materiales utilizados en la formación de nanocápsulas son los poliésteres, como la poli-ε-caprolactona (PCL), el ácido poliláctico (PLA) y su copolímero el ácido poli(láctico-glicólico) (PLGA)[67;68;69].

En este caso, la superficie y características de los nanosistemas se han modificado en diferentes ocasiones con polímeros hidrofílicos para

[66] **Vauthier, C. and Bouchemal, K.**, *Methods for the Preparation and Manufacture of Polymeric Nanoparticles.* Pharm. Res., 2009. **26**(5): p. 1025-1058.

[67] **Fessi, H., et al.**, *Nanocapsule formation by interfacial polymer deposition following solvent displacement.* International Journal of Pharmaceutics, 1989. **55**(1): p. R1-R4.

[68] **Quintanar-Guerrero, D., et al.**, *Preparation Techniques and Mechanisms of Formation of Biodegradable Nanoparticles from Preformed Polymers.* Drug Development and Industrial Pharmacy, 1998. **24**(12): p. 1113-1128.

[69] **Moinard-Chécot, D., et al.**, *Mechanism of nanocapsules formation by the emulsion–diffusion process.* Journal of Colloid and Interface Science, 2008. **317**(2): p. 458-468.

cumplir distintos objetivos. Por ejemplo, el recubrimiento con un biopolímero mucoadhesivo como el quitosano, por medio de incubación, ha permitido obtener mejoras en la biodisponibilidad oral de moléculas peptídicas [70]. También, la técnica de pegilación ha sido ampliamente utilizada en la modificación superficial de nanocápsulas de poliésteres, principalmente mediante la obtención de copolímeros como el PEG-PCL [71], PEG-PLA [72] o PEG-PLGA [73].

Los conocidos como biopolímeros, polímeros presentes comúnmente en la naturaleza, como polisacáridos y proteínas, han sido utilizados también en la preparación de nanocápsulas. Nuestro grupo ha descrito por primera vez la preparación de nanocápsulas de quitosano a partir de la técnica de desplazamiento del disolvente [74]. También se ha propuesto una alternativa a este método, mediante la incubación de una nanoemulsión pre-formada en una solución de quitosano, o de quitosano-PEG [75] [76]. Este procedimiento ha permitido también la obtención de nanocápsulas con

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[70] **Garcia-Fuentes, M., et al.**, *A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery.* Eur J Pharm Sci, 2005. **25**(1): p. 133-143.

[71] **De Campos, A.M., et al.**, *The effect of a PEG versus a chitosan coating on the interaction of drug colloidal carriers with the ocular mucosa.* Eur J Pharm Sci, 2003. **20**(1): p. 73-81.

[72] **Mosqueira, V.C.F., et al.**, *Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules.* Biomaterials, 2001. **22**(22): p. 2967-2979.

[73] **Mosqueira, V.C.F., et al.**, *Biodistribution of Long-Circulating PEG-Grafted Nanocapsules in Mice: Effects of PEG Chain Length and Density.* Pharm. Res., 2001. **18**(10): p. 1411-1419.

[74] **Calvo, P., et al.**, *Development of positively charged colloidal drug carriers: Chitosan-coated polyester nanocapsules and submicron-emulsions.* Colloid & Polymer Science, 1997. **275**(1): p. 46-53-53.

[75] **Prego, C., et al.**, *Chitosan Nanocapsules as Carriers for Oral Peptide Delivery: Effect of Chitosan Molecular Weight and Type of Salt on the In Vitro Behaviour and In Vivo Effectiveness.* Journal of Nanoscience and Nanotechnology, 2006. **6**(9-10): p. 2921-2928.

[76] **Prego, C., et al.**, *Chitosan-PEG nanocapsules as new carriers for oral peptide delivery: Effect of chitosan pegylation degree.* J Controlled Release, 2006. **111**(3): p. 299-308.

más de un recubrimiento, con el caso de las nanocápsulas de quitosano recubiertas con lambda carragenina [77].

NANOCÁPSULAS LIPÍDICAS

Benoit y colaboradores [78] han desarrollado recientemente una nueva generación de nanocápsulas, denominadas nanocápsulas lipídicas (LNC). Estos sistemas consisten en un núcleo oleoso rodeado por un ligero recubrimiento polimérico, a base de PEG-hidroxiestearato. Se describe para su preparación un novedoso método de inversión de fase, libre de disolventes. En este proceso todos los componentes del sistema se mezclan dentro de la fase acuosa, y se aplican una serie de ciclos de temperatura[79]. Una etapa final de enfriamiento rápido-dilución permite la formación de las LNC con tamaño de partícula promedio entre 20 y 100 nm [80].

[77] **Preetz, C., et al.**, *Preparation and characterization of biocompatible oil-loaded polyelectrolyte nanocapsules.* Nanomedicine: Nanotechnology, Biology, and Medicine, 2008. **4**(2): p. 106-114.

[78] **Heurtault, B., et al.**, *A Novel Phase Inversion-Based Process for the Preparation of Lipid Nanocarriers.* Pharm. Res., 2002. **19**(6): p. 875-880.

[79] **Anton, N., et al.**, *Nano-emulsions and nanocapsules by the PIT method: An investigation on the role of the temperature cycling on the emulsion phase inversion.* International Journal of Pharmaceutics, 2007. **344**(1-2): p. 44-52.

[80] **Heurtault, B., et al.**, *Physico-chemical stability of colloidal lipid particles.* Biomaterials, 2003. **24**(23): p. 4283-4300.

NANOCÁPSULAS EN LA TERAPIA DEL CÁNCER

Las nanocápsulas, como plataforma tecnológica, ofrecen una importante solución a la formulación de fármacos antitumorales, generalmente hidrofóbicos y/o inestables [81]. Su tamaño y la gran relación tamaño-volumen de las nanocápsulas facilitan su acumulación en el tumor debido al previamente descrito efecto EPR [82] y su fluidez favorece la interacción con membranas celulares e internalización [83-84].

Además, es importante considerar la posibilidad, particularmente en las nanocápsulas, de encapsular más de un principio activo y de asociar agentes de imagen a los sistemas para permitir una monitorización de los sistemas a través del tiempo en el organismo.

Existen numerosos trabajos relacionados con el uso de nanocápsulas en la formulación de fármacos antitumorales (Tabla 5). Por ejemplo, Leanerts y colaboradores [85] encapsularon ftalocianinas, agentes de gran importancia en la terapia fotodinámica, en nanocápsulas de PACA modificadas con poloxámeros. Se demostró que la presencia de determinados poloxámeros en la superficie de las nanocápsulas disminuyó significativamente su captura por órganos ricos en células fagocíticas, y

[81] **Couvreur, P., et al.**, *Nanocapsule technology: A review.* Critical Reviews in Therapeutic Drug Carrier Systems, 2002. **19**(2): p. 99-134.

[82] **Fang, J., et al.**, *The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect.* Advanced Drug Delivery Reviews, 2011. **63**(3): p. 136-151.

[83] **Roger, M., et al.**, *Ferrociphenol lipid nanocapsule delivery by mesenchymal stromal cells in brain tumor therapy.* International Journal of Pharmaceutics, 2012. **423**(1): p. 63-68.

[84] **Teixeira, Z., et al.**, *Retinyl palmitate flexible polymeric nanocapsules: Characterization and permeation studies.* Colloids and Surfaces B: Biointerfaces, 2010. **81**(1): p. 374-380.

[85] **Lenaerts, V., et al.**, *Nanocapsules with a reduced liver uptake: Targeting of phthalocyanines to EMT-6 mouse mammary tumour in vivo.* European Journal of Pharmaceutics and Biopharmaceutics, 1995. **41**(1): p. 38-43.

por el contrario, se incrementó su acumulación en tumor en torno a 200 veces.

En nuestro grupo de investigación, se ha explorado la capacidad de nanocápsulas de quitosano para promover liberación intracelular de docetaxel[86] y mejorar su efecto antitumoral. Este tipo de nanocápsulas permitió la encapsulación de elevadas cantidades de docetaxel en el núcleo, además de mostrar una rápida internalización del fármaco. Tras su administración intratumoral, hasta valores similares a la formulación comercial, mostrando un efecto más duradero [87].

[86] **Lozano, M.V., et al.**, *Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers.* Biomacromolecules, 2008. **9**(8): p. 2186-2193.

[87] **Torrecilla, D., et al.**, *Anti-tumor efficacy of chitosan-g-poly(ethyleneglycol) nanocapsules containing docetaxel: anti-TMEFF-2 functionalized nanocapsules vs non-functionalyzed nanocapsules.* Biomaterials, Submitted.

Tabla 5. Nanocápsulas investigadas en la terapia del cáncer.

Tipo NC	*Fármaco*	*Tumor/Target*	*Vía Ad.*	*Ref.*
PACA	DNA y RNA	-	I.V.	[88]
PACA	Ftalocinanias	Imaging	I.T.	[89]
	siRNA, ODNs	-	I.T	[67]
Quitosano	Docetaxel	MCF7	-	[90]
LNC	Paclitaxel	Carcinoma hepatocelular	Oral	[91]
	Paclitaxel	Glioma	I.V.	[92]
	Docetaxel	Biodistribución	I.V.	[93]
	DNA	HEK293β3	I.V.	[94]
	Ferrocifenol	9L-Glioma	I.V.	[95]
PEG/albumina	Paclitaxel	MCF7/Hph1	I.V.	[96]
PLGA	Docetaxel	Carcinoma (WRC 256)	Oral	[97]
PCL	Indometacina	Glioblastoma	I.P.	[98]

[88] **Bouclier, C., et al.**, *Physicochemical characteristics and preliminary in vivo biological evaluation of nanocapsules loaded with siRNA targeting estrogen receptor alpha*. Biomacromolecules, 2008. **9**(10): p. 2881-2890.

[89] **Lenaerts, V., et al.**, *Nanocapsules with a reduced liver uptake: Targeting of phthalocyanines to EMT-6 mouse mammary tumour in vivo*. European Journal of Pharmaceutics and Biopharmaceutics, 1995. **41**(1): p. 38-43.

[90] **Lozano, M.V., et al.**, *Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers.* Biomacromolecules, 2008. **9**(8): p. 2186-2193.

[91] **Lacoeuille, F., et al.**, *In vivo evaluation of lipid nanocapsules as a promising colloidal carrier for paclitaxel.* International Journal of Pharmaceutics, 2007. **344**(1-2): p. 143-149.

[92] **Garcion, E., et al.**, *A new generation of anticancer, drug-loaded, colloidal vectors reverses multidrug resistance in glioma and reduces tumor progression in rats.* Molecular Cancer Therapeutics, 2006. **5**(7): p. 1710-1722.

[93] **Khalid, M.N., et al.**, *Long circulating poly(ethylene glycol)-decorated lipid nanocapsules deliver docetaxel to solid tumors.* Pharm. Res., 2006. **23**(4): p. 752-758.

[94] **Morille, M., et al.**, *Tumor transfection after systemic injection of DNA lipid nanocapsules.* Biomaterials, 2011. **32**(9): p. 2327-2333.

[95] **Allard, E., et al.**, *Dose effect activity of ferrocifen-loaded lipid nanocapsules on a 9L-glioma model.* International Journal of Pharmaceutics, 2009. **379**(2): p. 317-323.

[96] **Lee, J.Y., et al.**, *Intracellular delivery of paclitaxel using oil-free, shell cross-linked HSA – Multi-armed PEG nanocapsules.* Biomaterials, 2011. **32**(33): p. 8635-8644.

[97] **Nassar, T., et al.**, *High Plasma Levels and Effective Lymphatic Uptake of Docetaxel in an Orally Available Nanotransporter Formulation.* Cancer Research, 2011. **71**(8): p. 3018-3028.

[98] **Bernardi, A., et al.**, *Indomethacin-loaded nanocapsules treatment reduces in vivo glioblastoma growth in a rat glioma model.* Cancer Letters, 2009. **281**(1): p. 53-63.

En un reciente estudio, se evaluaron nanocápsulas con cubierta de PEG-albumina con una superficie modificada con el péptido Hph1 como vehículo transportador de paclitaxel [99]. El Hph1 es un péptido de penetración celular, derivado de un factor humano de transcripción, cuyo papel en los nanosistemas sería el de facilitar su captura intracelular. Los resultados mostraron un aumento significativo en la efectividad antitumoral en un modelo *in vivo* obtenido tras la implantación de células MCF-7.

Bernardi y col [100] desarrollaron nanocápsulas de PCL para la encapsulación de indometacina para aplicación tumoral. Tras su evaluación *in vivo* en un modelo de glioma en ratas, estas nanocápsulas demostraron tras administración I.V., ser capaces de disminuir de manera considerable el tamaño del tumor, además de aumentar el tiempo de supervivencia al ser comparadas con el fármaco en solución (Figura 5).

Finalmente, en distintos estudios se ha demostrado la capacidad de las LNC para la encapsulación y vehiculización de taxanos. Específicamente, la encapsulación de paclitaxel en LNC condujo a un aumento significativo en la concentración del fármaco en el tejido tumoral tras administración I.V.[101]. Además, se consiguió una reducción significativa en el volumen tumoral en un modelo *in vivo* tras su administración I.V. en comparación a la formulación comercial Taxol®. Las LNC han demostrado también, ser capaces de mejorar al triple la biodisponibilidad oral del paclitaxel [102]. Con docetaxel, mostraron un incremento en la acumulación en tumor en

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[99] **Lee, J.Y., et al.**, *Intracellular delivery of paclitaxel using oil-free, shell cross-linked HSA – Multi-armed PEG nanocapsules.* Biomaterials, 2011. **32**(33): p. 8635-8644.

[100] **Bernardi, A., et al.**, *Indomethacin-loaded nanocapsules treatment reduces in vivo glioblastoma growth in a rat glioma model.* Cancer Letters, 2009. **281**(1): p. 53-63.

[101] **Lacoeuille, F., et al.**, *Lipid nanocapsules for intracellular drug delivery of anticancer drugs.* Journal of Nanoscience and Nanotechnology, 2007. **7**(12): p. 4612-4617.

[102] **Peltier, S., et al.**, *Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules.* Pharm. Res., 2006. **23**(6): p. 1243-1250.

ratón, reflejado en un aumento de 5 veces el área bajo la curva concentración en tumor vs tiempo, en comparación a la formulación comercial.

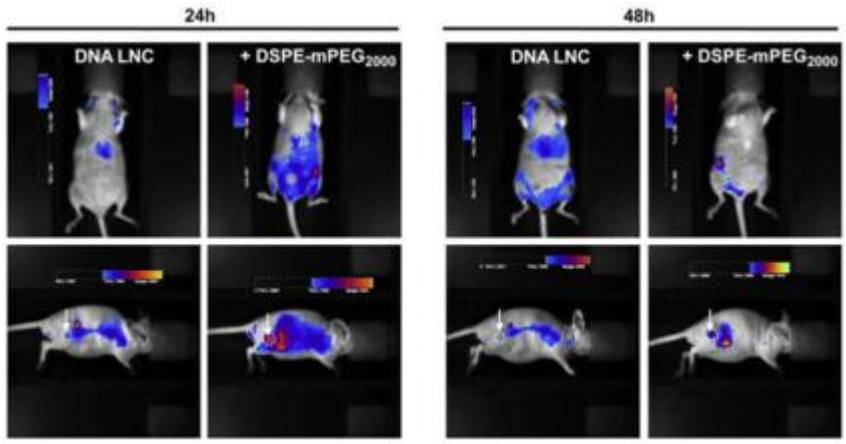


Figura 5. Imágenes de fluorescencia *in vivo* de la biodistribución en tiempo real (24 y 48 h) de LNC cargadas con una sonda fluorescente tras su administración I.V, en ratones desnudos implantados con un tumor U87MG. La barra lateral o superior indica la intensidad de fluorescencia, factor proporcional a la concentración de la sonda. Se especifica la zona donde se encuentra el tumor por medio de una flecha blanca.

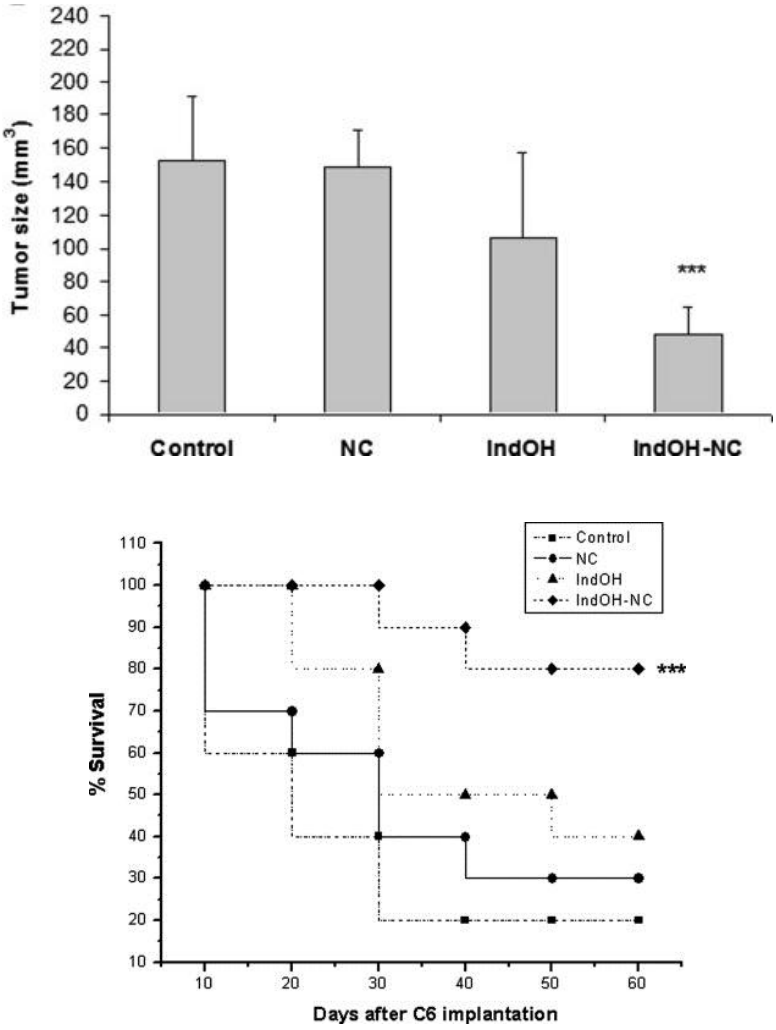


Figura 6. Arriba: Efecto *in vivo* de nanocápsulas de PCL cargadas con indometacina sobre el crecimiento de un glioblastoma en rata (control= suero salino; NC= nanocápsulas blancas; IndOH= solución de indometacina e IndOH-NC = nanocápsulas cargadas con indometacina) *** $p<0.01$ q. Abajo: Grafico de probabilidad de supervivencia para ratones tratados con las distintas formulaciones.

En un reciente trabajo, se evaluó la distribución mediante técnicas de *imagi-ng* de LNC pegiladas, conteniendo DNA complejado con lípidos catiónicos[103]. Estas LNC demostraron larga permanencia en plasma, consiguiendo además, mediante la pegilación, una acumulación selectiva en tumor (Figura 6).

OTROS POLÍMEROS DE ÍNTERES PARA LA FORMACIÓN DE NANOCÁPSULAS

Como se ha mencionado anteriormente, el éxito de un nanosistema reside en gran medida en la correcta selección de sus componentes, así como del impacto y la función de cada uno de estos dentro de la formulación. En el caso de las nanocápsulas, además de una buena selección del agente oleoso utilizado como reservorio, y de los tensoactivos que garanticen un bajo perfil de toxicidad del sistema, un factor crucial es la selección adecuada de un polímero versátil, seguro, biodegradable y sobre todo con la posibilidad de dotar de funciones específicas al sistema (larga permanencia, capacidad interacción, capacidad de penetración...etc).

Además de los materiales descritos previamente, merecen especial mención dentro de los biopolímeros, los poliaminoácidos y las proteínas

POLIAMINOÁCIDOS

Los poliaminoácidos son polímeros iónicos, constituidos por largas cadenas de aminoácidos. Difieren de los polipéptidos por la variedad de aminoácidos que presentan en su estructura, y difieren de las proteínas por el tipo de estructura y conformación que presentan. Se conocen tres

[103] **Morille, M., et al.**, *Long-circulating DNA lipid nanocapsules as new vector for passive tumor targeting.* Biomaterials, 2010. **31**(2): p. 321-329.

poliaminoácidos "naturales" que se encuentran normalmente en la naturaleza, la cianoficina, un copolímero de ácido aspártico y arginina, la poli-ε-lisina y el ácido poli-D-glutámico [104]. Sin embargo, actualmente gracias a los grandes avances en biotecnología es posible contar con poliaminoácidos conteniendo cualquier combinación y número de residuos.

En el desarrollo de nanosistemas se han utilizado poliaminoácidos por ejemplo, para otorgar propiedades de larga permanencia en el organismo, como es el caso del ácido poli-L-glutámico [105], o para dar propiedades de mucoadhesión y penetración a los sistemas, como es el caso de la poliarginina [106].

POLIASPARAGINA

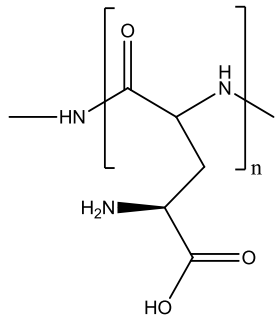
La poli-L-asparagina, emerge como uno de los polímeros naturales con mayor futuro en el desarrollo de sistemas nanométricos especialmente en los dirigidos a la terapia del cáncer.

Es un poliaminoácido constituido por monómeros del aminoácido esencial L-asparagina (Figura 7). La L-asparagina, a su vez, es un aminoácido esencial involucrado en el desarrollo celular, ya que es el principal precursor de la síntesis de proteínas. Su potencial interés en el tratamiento del cáncer radica en la extrema necesidad que presentan las células cancerosas por este aminoácido. Mientras que las células normales pueden abastecerse por sí mismas sintetizándolo, las células del tejido tumoral no tienen capacidad para sintetizarlo en cantidad suficiente, y por

[104] **Nair, L.S. and Laurencin, C.T.**, *Biodegradable polymers as biomaterials.* Progress in Polymer Science, 2007. **32**(8–9): p. 762-798.
[105] **Jack W, S.**, *Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane.* J Control Release, 2005. **109**(1–3): p. 120-126.
[106] **Lozano M. V., et al.**, *Polyarginine nanocapsules: a new platform for intracellular drug delivery.* Submitted.

lo tanto lo buscan a partir de nutrientes periféricos o directamente de células circundantes, siendo éste uno de los procesos básicos de la metástasis [107].

De hecho, una de las terapias tumorales está basada en la administración del enzima que metaboliza a la asparagina, la asparaginasa. Al administrar dicho enzima, se consigue una disminución de la concentración del aminoácido, lo que impide que las células sinteticen DNA y otras proteínas esenciales para su supervivencia. Dicha formulación conteniendo L-asparaginasa, está comercializada como Oncaspar® o Elspar®[108].



Poly-L-asparagine

Figura 7. Estructura molecular de la poli-L-asparagina

La avidez por la asparagina, puede hacer que polímeros como la poliasparagina, aporten a los nanosistemas una cierta especificidad tumoral, por el reconocimiento en superficie de la asparagina, originando un mecanismo de vehiculización activa.

[107] **Allan, J.D., et al.**, *L-asparagine L-asparaginase and cancer.* Lancet, 1967. **1**(7491): p. 682-&.

[108] **Verma, N., et al.**, *L-Asparaginase: A Promising Chemotherapeutic Agent.* Critical Reviews in Biotechnology, 2007. **27**(1): p. 45-62.

Junto con esta especificidad, los polímeros a base de este aminoácido han demostrado tener características de hidrofilia, estructurales y fisicoquímicas análogas a PEG [109], que dan lugar recubrimientos de nanosistemas que alargan la vida media del fármaco, y mejoran su biodistribución [110].

Finalmente, la poliasparagina puede unirse covalentemente a otros polímeros, incrementando su capacidad para incorporarse en sistemas coloidales, como es el caso del copolímero poliasparagina-policaprolactona sintetizado para el desarrollo de compuestos micelares [111].

PROTEÍNAS

Al ser el principal componente de muchos tejidos naturales, las proteínas han sido ampliamente investigadas y utilizadas como materiales para suturas, agentes hemostáticos, *scaffolds* en ingeniería de tejidos [112], excipientes farmacéuticos y como componentes de sistemas de liberación de fármacos [113].

Son generalmente biocompatibles y biodegradables. Muchas de ellas están aprobadas por la FDA para su uso como excipientes en formas farmacéuticas para uso humano. De las proteínas más utilizadas en el

[109] **Romberg, B., et al.**, *Poly(amino acid)s: Promising enzymatically degradable stealth coatings for liposomes.* International Journal of Pharmaceutics, 2007. **331**(2): p. 186-189.

[110] **Romberg, B., et al.**, *Pharmacokinetics of poly(hydroxyethyl-l-asparagine)-coated liposomes is superior over that of PEG-coated liposomes at low lipid dose and upon repeated administration.* Biochimica et Biophysica Acta - Biomembranes, 2007. **1768**(3): p. 737-743.

[111] **Jae, H.J., et al.**, *Biodegradable poly(asparagine) grafted with poly(caprolactone) and the effect of substitution on self-aggregation.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2005. **264**(1-3): p. 187-194.

[112] **Gebauer, M. and Skerra, A.**, *Engineered protein scaffolds as next-generation antibody therapeutics.* Current Opinion in Chemical Biology, 2009. **13**(3): p. 245-255.

[113] **Hawkins, M.J., et al.**, *Protein nanoparticles as drug carriers in clinical medicine.* Advanced Drug Delivery Reviews, 2008. **60**(8): p. 876-885.

desarrollo de sistemas coloidales podemos citar el colágeno [114], el mayor componente proteico del organismo, la caseína [115], principal componente del suero de la leche, y la albumina [116], entre otras.

La albumina es de las proteínas con mayor relevancia en el desarrollo de nanosistemas en la terapia del cáncer. Como se mencionó anteriormente, el Abraxane®, un sistema de nanoparticular a base de albumina, fue el primer sistema nanoparticular en obtener su aprobación para uso en humanos por parte de la FDA [117]. Actualmente, esta misma estrategia se desarrolla para conseguir la vehiculización de otros tipos de fármacos diferentes al paclitaxel.

CASEÍNAS

Las caseínas son un grupo de fosfoproteínas de suero de leche que representan su mayor componente proteínico. Existen principalmente cuatro tipos diferentes de caseínas, diferenciadas en su punto isoeléctrico y su naturaleza, estas son la α_{s1}, α_{s2}, β y κ [118]. Al conjunto de cuatro proteínas se le conoce como caseinato y es una mezcla aprobada por la FDA como excipiente para formas farmacéuticas orales para uso humano.

Muchas de las características físico-químicas de las caseínas hacen de éstas polímeros interesantes para el desarrollo de sistemas de liberación de fármacos. Gracias a su naturaleza anfifílica, las caseínas pueden ser

[114] **Lee, C.H., et al.**, *Biomedical applications of collagen.* International Journal of Pharmaceutics, 2001. **221**(1–2): p. 1-22.

[115] **Elzoghby, A.O., et al.**, *Casein-based formulations as promising controlled release drug delivery systems.* Journal of controlled release : official journal of the Controlled Release Society, 2011. **153**(3): p. 206-16.

[116] **Elsadek, B. and Kratz, F.**, *Impact of albumin on drug delivery - New applications on the horizon.* Journal of controlled release : official journal of the Controlled Release Society, 2011(0).

[117] http://www.abraxane.com/dtc/

[118] **Horne, D.S.**, *Casein structure, self-assembly and gelation.* Current Opinion in Colloid & Interface Science, 2002. **7**(5-6): p. 456-461.

consideradas como tensoactivos, y de hecho, este uso es el que generalmente se encuentra asociado a esta proteína en la industria tanto alimenticia como farmacéutica. Las caseínas se encuentran generalmente asociadas en sistemas micelares [119] y constituyen nanovehículos naturales de nutrientes. Así en algunos estudios, se ha evaluado su uso como vehículos para otro tipo de nutrientes o de fármacos como lo son la vitamina D [120], insulina [121], triclosan [122] y algunos antitumorales como el mitoxantrone [123].

Las caseínas se encuentran generalmente asociadas en sistemas micelares [124] y constituyen nanovehículos naturales de nutrientes. Así en algunos estudios, se ha evaluado su uso como vehículos para otro tipo de nutrientes o de fármacos como lo son la vitamina D [125], insulina [126], triclosan [127] y algunos antitumorales como el mitoxantrone [128].

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[119] **Dalgleish, D.G.**, *Casein Micelles as Colloids: Surface Structures and Stabilities.* Journal of Dairy Science, 1998. **81**(11): p. 3013-3018.

[120] **Semo, E., et al.**, *Casein micelle as a natural nano-capsular vehicle for nutraceuticals.* Food Hydrocolloids, 2007. **21**(5-6): p. 936-942.

[121] **Morçöl, T., et al.**, *Calcium phosphate-PEG-insulin-casein (CAPIC) particles as oral delivery systems for insulin.* International Journal of Pharmaceutics, 2004. **277**(1-2): p. 91-97.

[122] **Roach, A., et al.**, *Association of Triclosan to Casein Proteins Through Solvent-Mediated High-Pressure Homogenization.* Journal of Food Science, 2009. **74**(2): p. N23-N29.

[123] **Shapira, A., et al.**, *[beta]-casein-based nanovehicles for oral delivery of chemotherapeutic drugs: drug-protein interactions and mitoxantrone loading capacity.* Nanomedicine: Nanotechnology, Biology and Medicine, 2010. **6**(4): p. 547-555.

[124] **Dalgleish, D.G.**, *Casein Micelles as Colloids: Surface Structures and Stabilities.* Journal of Dairy Science, 1998. **81**(11): p. 3013-3018.

[125] **Semo, E., et al.**, *Casein micelle as a natural nano-capsular vehicle for nutraceuticals.* Food Hydrocolloids, 2007. **21**(5-6): p. 936-942.

[126] **Morçöl, T., et al.**, *Calcium phosphate-PEG-insulin-casein (CAPIC) particles as oral delivery systems for insulin.* International Journal of Pharmaceutics, 2004. **277**(1-2): p. 91-97.

[127] **Roach, A., et al.**, *Association of Triclosan to Casein Proteins Through Solvent-Mediated High-Pressure Homogenization.* Journal of Food Science, 2009. **74**(2): p. N23-N29.

[128] **Shapira, A., et al.**, *[beta]-casein-based nanovehicles for oral delivery of chemotherapeutic drugs: drug-protein interactions and mitoxantrone loading capacity.* Nanomedicine: Nanotechnology, Biology and Medicine, 2010. **6**(4): p. 547-555.

ANTECEDENTES, HIPÓTESIS Y OBJETIVOS

ANTECEDENTES

1. Dentro de los avances experimentados en la terapia del cáncer, la nanotecnología ha aportado mejoras a la hora de prolongar el escaso tiempo de permanencia en circulación y modificar la biodistribución indiscriminada de los fármacos antitumorales.

2. Las nanocápsulas, constituidas por un núcleo oleoso y una cubierta polimérica, ofrecen un gran potencial como vehículos de fármacos de naturaleza hidrofóbica [1, 2, 3]. Su estructura no solo permite conseguir una encapsulación adecuada del fármaco, y protegerlo del entorno, sino que ofrece una fluidez que facilita el contacto y la interacción con membranas celulares.

3. Los poliaminoácidos se presentan como interesantes materiales de cubierta capaces de dotar a las nanoestructuras de propiedades específicas para cumplir distintos objetivos en la terapia antitumoral. Así, por ejemplo, nuestro grupo de investigación, ha diseñado nanocápsulas elaboradas a base poliaminoácidos

[1] **Peltier, S., et al.**, *Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules.* Pharm. Res., 2006. **23**(6): p. 1243-1250.

[2] **Bae, K.H., et al.**, *Oil-encapsulating PEO-PPO-PEO/PEG shell cross-linked nanocapsules for target-specific delivery of paclitaxel.* Biomacromolecules, 2007. **8**(2): p. 650-656.

[3] **Lozano, M.V., et al.**, *Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers.* Biomacromolecules, 2008. **9**(8): p. 2186-2193.

catiónicos como la poliarginina que han demostrado ser transportadores intracelulares eficaces de fármacos antitumorales[4].

3. Poliaminoácidos neutros como poli-l-asparagina ofrecen la posibilidad de formar cubiertas que dotan de propiedades de larga permanencia en plasma a los nanosistemas[5]. Este poliaminoácido presenta además propiedades específicas para lograr el *targeting* hacia células tumorales debido a la bien documentada necesidad de l-asparagina que presentan dichas células

4. La caseína, es una proteína cuyo carácter anfifílico le permite formar distintas estructuras micelares y formar parte de cubiertas con propiedades específicas de retención y liberación del fármaco en función de las condiciones del medio. Es considerada como material seguro para la vía oral, y presenta capacidad mucoadhesiva[6].

5. Los taxanos, como el docetaxel, son actualmente el tratamiento antitumoral de primera elección, sin embargo, además de presentar los inconvenientes asociados a la terapia antitumoral, presentan el inconveniente de su elevada hidrofobicidad. Así, se hace necesario

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[4] **Lozano M. V., et al.**, *Polyarginine nanocapsules: a new platform for intracellular drug delivery.* Submitted.

[5] **Romberg, B., et al.**, *Pharmacokinetics of poly(hydroxyethyl-l-asparagine)-coated liposomes is superior over that of PEG-coated liposomes at low lipid dose and upon repeated administration.* Biochimica et Biophysica Acta - Biomembranes, 2007. **1768**(3): p. 737-743.

[6] **Sokolovski, M., et al.**, *Membrane interactions and lipid binding of casein oligomers and early aggregates.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2008. **1778**(10): p. 2341-2349.

el uso de disolventes y excipientes, en su formulación endovenosa, que dan lugar a importantes reacciones adversas [7,8].

HIPÓTESIS

1. Los sistemas nanocapsulares, constituidos por un núcleo oleoso y una cubierta a base de poliasparagina, serán capaces de ofrecer propiedades de larga permanencia plasmática. Este tiempo prolongado de circulación facilitará el acceso de antitumorales hidrofóbicos, como el docetaxel, al target tumoral.

2. El recubrimiento con poliasparagina podrá además promover su acumulación y captura por células tumorales debido a la avidez por la l-asparagina que presentan dichas células.

3. El diseño y desarrollo de nuevos sistemas nanocapsulares con una cubierta a base de proteínas lácteas, como la caseína, permitirá obtener nanosistemas capaces de encapsular fármacos de carácter hidrofóbico, protegerlos de las condiciones del tracto gastrointestinal, y vehiculizarlos hacia el epitelio intestinal, pudiendo constituir nanovehículos que mejoren la biodisponibilidad oral de fármacos hidrofóbicos como el docetaxel.

[7] **Engels, F.K., et al.**, *Alternative drug formulations of docetaxel: a review.* Anti-Cancer Drugs, 2007. **18**(2): p. 95-103.

[8] **Ten Tije, A.J., et al.**, *Pharmacological Effects of Formulation Vehicles: Implications for Cancer Chemotherapy.* Clin. Pharmacokinet., 2003. **42**(7): p. 665-685.

OBJETIVOS

Teniendo en cuenta los antecedentes expuestos y las hipótesis planteadas, el objetivo general de la presente memoria ha sido el diseño de nuevos nanosistemas, en forma de nanocápsulas, capaces de proporcionar una aproximación terapéutica innovadora al tratamiento del cáncer. Esta aproximación se basa en la capacidad de las nanocápsulas diseñadas para vehiculizar y promover la internalización celular de fármacos antitumorales de carácter hidrofóbico, como el docetaxel. Para lograr este objetivo se han seguido las siguientes etapas:

1. **Desarrollo de nuevos sistemas constituidos por nanocápsulas de poliasparagina y evaluación *in vitro* de su potencial en la terapia del cáncer.**

Esta parte de la memoria se ha dirigido al diseño y caracterización de sistemas de tipo nanocapsular con cubierta de poliasparagina, así como al estudio de su capacidad para encapsular y liberar eficazmente el fármaco citóstatico docetaxel. Por otro lado, se ha pretendido profundizar en el estudio de la interacción de las nanocápsulas con células tumorales, así como en el potencial de estos vehículos para favorecer la liberación intracelular del fármaco.

Los resultados de ésta parte de la memoria se recogen en el capítulo 2. "Novel Polyasparagine nanocapsules can efficiently deliver anticancer drug intracellularly".

2. **Evaluación *in vivo* del perfil farmacocinético de nanocápsulas de poliasparagina fluorescentes y del efecto antitumoral del docetaxel incluido en las nanocápsulas en un modelo de glioma U87MG en ratón.**

Los estudios realizados en esta etapa se han dirigido al estudio del comportamiento *in vivo* de las nanocápsulas de poliasparagina. Así se evaluó tanto su perfil plasmático, a través de sus parámetros farmacocinéticos, tras el marcaje con una molécula fluorescente, como la actividad antitumoral del docetaxel, al ser vehiculizado en los nanosistemas, y administrado en ratones portadores del modelo de glioma U87MG.

Los resultados de este apartado se recogen en el capítulo 3: "Docetaxel Polyasparagine loaded nanocapsules: mechanism of action, efficacy and toxicity."

3. **Desarrollo de nuevos sistemas constituídos por nanocápsulas de caseína y evaluación de su potencial como vehículos orales de fármacos hidrofóbicos.**

El objetivo de esta etapa ha consistido en el diseño y desarrollo de nuevos sistemas nanocapsulares elaborados a partir de polímeros naturales, como son las caseínas, así como al estudio de su capacidad para encapsular y liberar eficazmente el fármaco docetaxel. Por otro lado, se ha pretendido profundizar en el estudio de la interacción de las nanocápsulas con diferentes fluidos biológicos para evaluar su potencial como vehículos de administración oral de fármacos hidrofóbicos.

CAPÍTULO 2. Novel Polyasparagine nanocapsules can efficiently deliver anticancer drug intracellularly

CAPÍTULO 2. NOVEL POLY-L-ASPARAGINE NANOCAPSULES CAN EFFICIENTLY DELIVER ANTICANCER DRUG INTRACELLULARLY

ABSTRACT

This work describes the preparation and characterization of a new drug nanocarrier, named as poly-L-asparagine (PASN) nanocapsules, as well as the evaluation of their potential as anticancer drug delivery vehicle. PASN nanocapsules were designed to mimic the characteristics of PEG-coatings exhibiting long circulating properties and also to explore their capacity of specifically target certain tumors. Nanovehicles are composed by a biodegradable and biocompatible lipid core surrounded by a PASN shell. They were obtained by the solvent displacement technique in which the polymeric coating is formed as a consequence of the ionic-dipolar interactions between a cationic surfactant included in the lipid core and the polymer. PASN nanocapsules showed sizes of around 170-200 nm and zeta potential values around -40mV. These nanocapsules were shown to be efficient vehicles for the encapsulation of the model anticancer drug docetaxel (encapsulation efficiency values of ≈80%), and retained it upon dilution in a simulated biological medium. In vitro studies carried out in the NCI-H460 human cancer cell line indicated a significant increase in the cytotoxicity capacity of the antitumor drug upon its association to the nanocarrier. This enhancement of the antitumor effect has to be attributed to the internalization of nanocapsules and the promotion of the intracellular delivery of docetaxel. This ability of PASN nanocapsules together with their stability during storage conditions and the possibility to be converted in a freeze-dried powder makes these systems an attractive platform for anticancer drug delivery.

INTRODUCTION

Nanotechnology is a revolutionary field in medicine which is expected to elicit significant therapeutic benefits in cancer diagnosis and treatment in the next few decades. Advances in protein engineering and materials science have contributed to approaches that may bring new hope to cancer patients. Nanocarriers can exploit the characteristic tumor microenvironment as in the case of EPR (enhanced and permeation retention) effect [1, 2], or by specific interactions between the diseased tissue and the modified surface of nanocarriers [3] enhancing the specificity of the therapy. However, the extensive opsonization of the nanocarriers and therefore their rapid clearance by macrophages of the mononuclear phagocyte system (MPS) after their intravenous administration hinders that they can reach the tumor area. Thus, even though different therapeutic nanocarriers have been approved for clinical use in several diseases [4-6], to date, only very few have been clinically approved to selectively bind or target cancer cells [7, 8].

One of the most common strategies, and probably the most effective until now, in order to avoid the rapid elimination of nanosystems is their surface modification with poly(ethylene glycol) (PEG). In fact, PEGylation, term employed to PEG modifications, has been widely used to obtain long-circulating nanostructures [9-12], that circulate in the bloodstream for a prolonged period of time, enabling their extravasation into solid tumors. Besides PEG, a variety of hydrophilic polymers, such as poly (amino acids) and polysaccharides, have demonstrated to be able, to prolong circulation times of nanostructures. Among these polymers, the poly (amino acid) poly-L-asparagine (PASN), fulfills this objective and offers the advantage in comparison to PEG of being degradable by lysosomal proteases, allowing for a complete elimination from the body [12, 13]. Furthermore, PASN-coating can provide targeting moieties by

means of its monomers, L-asparagine residues. This amino acid is involved in the rapid malignant growth of certain cancer cells that need the serum asparagine for survival, being however not required by healthy cells [14]. Thus, taking advantage of the preferences of cancer cells, we can hypothesize the construction of a "Trojan horse" vehicle specifically oriented.

Within this frame the use of nanocapsules represents a promising strategy because of their versatility. First, the shell of the nanocapsules, may impair long circulating and even targeting properties; second, the oily core may accommodate high amounts of lipophilic drugs (most anticancer drugs are lipophilic); third, both the core and the shell may contribute to the preservation of the stability of the encapsulated drug. The properties and mechanisms of encapsulation, release as well as the efficacy profile of several drugs incorporated in polymeric nanocapsules have been extensively studied by our group with promising results [15-21].

Based on the above information, the long-term goal of the current study is to develop a rationally designed versatile drug delivery platform comprising hydrophobic anticancer drugs, such as docetaxel, entrapped within PASN nanocapsules. This technology, incorporating PASN, may represent a completely new strategy for surface modification of nanocapsules for use in cancer targeting.

MATERIALS AND METHODS

CHEMICALS

Docetaxel (from Flucka), Poloxamer 188 (Pluronic® F68), benzalkonium chloride (BKC), cetrimonium bromide (CTAB), cetylpiridinium chloride (CPC), oleylamine (OLE), trehalose dehydrate and the polymer Poly-L-asparagine (PASN) were purchased from Sigma-Aldrich (Spain). Miglyol® 812, neutral oil formed by esters of carpylic and capric fatty acids and glycerol, was kindly provided by Sasol Germany GmbH (Germany). The surfactant lecithin (Epikuron 145v, a phosphatidylcholine enriched fraction of soybean lecithin) was donated by Cargill (Spain). The N-(fluorescein-5-thiocarbamoyl)-1.2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (fluorescein-DHPE) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (Did) were obtained from Molecular Probes-Invitrogen (USA).

CONSTRUCTION OF PASN NANOCAPSULES

Blank PASN nanocapsules were prepared by a modification of the solvent displacement technique previously described by our group [15, 22]. We used two different variations of the method, the first named “one-stage procedure” was based on a dipolar-ionic interaction between PASN dissolved in an aqueous phase and the cationic surfactant dissolved in an organic phase, which contains also the oil. Briefly, an organic phase was formed by dissolving 20-40 mg of lecithin in 0.5 ml of ethanol, followed by the addition of 100-250 µl of Mygliol 812 and 9 ml of the cationic surfactant acetone solution. This organic phase was immediately poured over 20 ml of a solution of poloxamer (0.25 % w/v) and PASN (1-10 mg/ml). When the PASN interacts with the cationic surfactant, it forms a

polymer corona at the oil /water interface, thus originating the PASN nanocapsules. Finally, solvents are eliminated from the suspension by under vacuum to constant volume (10 ml).

The second method modification, called "two-stage procedure" consisted of incubating a previously prepared cationic nanoemulsion into a PASN solution. The volume ratio used was 4:1 (cationic nanoemulsion: PASN solution) and the incubation period was 30 min. This cationic nanoemulsion was prepared by the same method above described but without adding polymer in the aqueous phase.

The incorporation of the hydrophobic molecules, docetaxel or fluorescent probes fluorescein-DHPE and DiD, was carried out by adding aliquots of the stock ethanol solutions to the organic phase, and then following the same procedure.

CHARACTERIZATION OF PASN NANOCAPSULES

PASN nanocapsules were characterized with regard to size, zeta potential and morphology as follows:

Particle size and polydispersion index were determined by photon correlation spectroscopy. Samples were diluted to the appropriate concentration with KCl. Each analysis was carried out at 25ºC with an angle detection of 173º. The zeta potential values were calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry. Samples were diluted with KCl 1mM and placed in the electrophoretic cell. Analyses were performed in triplicate using a NanoZS® (Malvern Instruments, Malvern, UK).

The morphological examination of the nanocapsules was performed by transmission electron microscopy (TEM, CM12 Philips, The

Netherlands). Samples were stained with 2% w/v phosphotungstic acid solution, and placed on cupper grids with Formvar® films for analysis.

ENCAPSULATION OF DOCETAXEL INTO THE NANOCAPSULES

Docetaxel encapsulation efficiency in PASN nanocapsules was determined indirectly by the difference between the total amount of docetaxel in the formulation and the free drug measured in the infranatant of the nanocapsules. Therefore, the total amount of drug was estimated by dissolving an aliquot of non-isolated docetaxel loaded PASN nanocapsules with acetonitrile. This sample was centrifuged during 20 min at 4000xg and the supernatant was measured with a high-performance liquid chromatography (HPLC) system. The non-encapsulated drug was determined by the same method following separation of the PASN nanocapsules from the aqueous medium by ultracentrifugation at 20 000 g.

Docetaxel was assayed by a slightly modified version of the method proposed by Lee et al. [23]. The HPLC system consisted of an Agilent 1100 Series instrument equipped with a UV detector set at 227 nm and a reverse phase Zorbax Eclipse® XDB-C8 column (4.6 x 150 mm i.d., pore size 5 µm Agilent USA). The mobile phase consisted of a mixture of acetonitrile and 0.1% v/v ortophosphoric acid (55:45 v/v) and the flow rate was 1 ml/min.

The encapsulation efficiency (E.E.) was calculated as follows:

$$E.E(\%) = \frac{A - B}{A} \times 100$$

Where A is the experimental total drug concentration and B is the drug concentration measured in the external aqueous medium, corresponding to unloaded drug.

STABILITY STUDY AT STORAGE CONDITIONS

Stability study at storage conditions

The suspension stability of PASN nanocapsules prepared with the CTAB surfactant was evaluated according to terms of time and temperature of storage. Therefore, aliquots of the nanocapsules suspension without dilution were placed in sealed tubes at 4 and 37°C for storage. Size and polydispersity index of the nanocarriers were measured for a period of three months, meanwhile zeta potential values were controlled at the end of the study. Each sample corresponds to a different PASN nanocapsules batch.

Freeze-drying of PASN nanocapsules

Different concentrations of PASN nanocapsules (0.125, 0.25 and 0.5 % w/v) and trehalose (0, 0.25, 0.5 and 1 % w/v) were considered the variables included in the lyophilization study. Therefore, 1 ml dilutions of PASN nanocapsules placed in volume glass vials and were quickly frozen in liquid nitrogen. The lyophilization procedure consisted in an initial drying step for 60h at -35ºC, followed by a secondary drying for 24h in a high vacuum atmosphere. Finally, temperature was slowly increased up to 20ºC till the end of the process (Labconco Corp., USA).

PASN nanocapsules were recovered by adding 1ml of ultrapure water to the freeze dried powders followed by manual resuspension and were characterized as explained above.

IN VITRO CELL CULTURE STUDIES

Citoxicity assays

Human non-small lung cancer cell line NCI-H460 was cultured in RPMI-1640 medium (ATCC), supplemented with 10% (v/v) fetal bovine serum (FBS) at 37ºC in a humidified atmosphere containing 5% carbon dioxide. Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide (MTT, Acros Organics) was used for mitochondrial activity evaluation. Briefly, cells were planted onto 96-wells plates, with a seeding density of 15x103 cells/well in 100 µl culture medium. After 24h, medium was removed and dilutions of docetaxel solution, docetaxel loaded PASN nanocapsules and blank PASN nanocapsules in the medium were added to the wells. Finally, after 48h of incubation cell survival was measured by the MTT assay. Briefly, medium was removed and the wells were washed twice with 100 µl Hank's Balanced Salt Serum (HBSS). Then, 20 µl of a MTT (5 mg/ml in PBS) and 100 µl HBSS were added to the wells and maintained at 37ºC in an atmosphere with 5% CO2 for 4h. Afterwards, buffers were removed and 100 µl DMSO were added to each well and maintained at 37ºC in an atmosphere with 5% CO2 overnight. Absorbance (λ=490 nm) was measured in a BioRad 680 spectrophotometer removing background absorbance (λ=655 nm).

The percentage of cell viability was calculated by the absorbance measurements of control growth and test growth in the presence of the formulations at various concentration levels.

IC50 values were obtained by fitting the data with non-linear regression, with Prism 2.1 software (GraphPad, San Diego, CA).

Uptake studies

Cells were plated in a multiwell-12 plate (Falcon) at 15.7x104 cells/well in supplemented medium for 24 h. Next, medium was removed and dilutions of the fluorescent dispersion, fluorescent nanoemulsion and fluorescent PASN nanocapsules were added to the wells. After 2 h of incubation, cells were washed with acidic phosphate saline buffer (PBS, SIGMA) trypsinized and reconstituted in PBS supplemented with 3% (v/v) of FBS.

Living cells suspensions were analyzed for green fluorescence by flow cytometry in a FACScan (Becton Dickinson).

For the uptake studies after the 2 h of cell incubation with the formulations, cells were washed three times with cold acidic phosphate buffer (PBS, SIGMA). Next, they were fixed with paraformaldehyde 4 % for 10 min, washed and counterstained with Bodipy® phalloidin during 30 min in darkness. Finally, after adding a drop of fluorescent mounting medium on the surface of the holders, covers were placed on them for confocal microscope analysis.

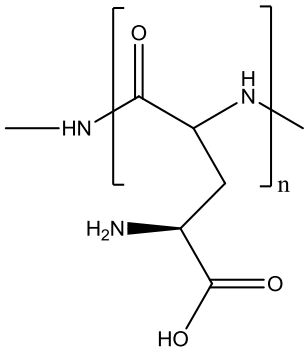
STATISTICAL ANALYSIS

Cell culture results were evaluated in order to determine the statistical significance between the different formulations studied. The statistical evaluation of the cell viability results was performed by an ANOVA test followed by a multiple comparison analysis (SigmaStat Program, Jandel Scientific, version 3.5). Differences were considered by means of a t-test for independent samples using Statgraphics Plus XV (Statpoint Technologies Inc. USA).

RESULTS AND DISCUSSION

This article describes for the first time the development of a new drug nanocarrier consisting of an oily core surrounded by a poly-l-asparagine polymeric shell. The rationale behind the design of this nanocarrier, named PASN nanocapsules, was as follows: the oily core is intended to allocate significant amounts of lipophilic drugs whereas the external polymer shell is expected to have three differentiated roles: (i) to prolong the circulation time in the body; (ii) to facilitate the interaction and internalization of the nanocarrier with cancer cells and (iii) to improve the stability of the encapsulated drug versus biological media and during the storage.

The PASN (Figure 1) was chosen as coating polymer because of its physical, chemical and targeting properties [24]. Moreover, used as nanocarrier coating has demonstrated to prolong system circulation to a similar extent as PEG [12]. Furthermore, PASN-coating can provide targeting moieties [14].



Poly-L-asparagine

Figure 1. Molecular structure of poly-L-asparagine.

The construction of PASN nanocapsules.

Preliminary studies were intended to determine the range of conditions suitable for the formation of PASN nanocapsules. As a first step, we studied the possibility to form cationic nanoemulsions with different surfactants as a previous stage to obtain nanocapsules. The formulation parameters selected were the type and the concentration of the cationic surfactant. The surfactants used were benzalkonium chloride (BKC), cetyltrimethylammonium bromide (CTAB), cetylpyridinium chloride (CPC) and oleylamine (OLE), all of them molecules with a high positive charge due to their ionized amino groups. The tested surfactant concentrations were in the range 0.01 and 0.1 %. Under the conditions assayed, all the surfactants studied allowed the formation of stable cationic nanoemulsions, showing a nanometric size around 165-350 nm, and a positive surface charge around 30 - 40 mV (results not shown). Therefore, all of these new cationic nanoemulsions resulted adequate for the preparation of PASN nanocapsules, selecting 0.02 and 0.04 % as the optimal concentrations to obtain a more homogeneous population of particles with a lower size (Table 1).

Table 1.- Physicochemical characteristics of nanoemulsions prepared with the different cationic surfactants. PI: polydispersity index. Values are given as mean ± S.D; n=3

Cationic Surfactant (C.S.)	**% (w/v) C.S.**	**Size (nm)**	**P.I.**	**ζ Potential (mV)**
BKC	0.04	208 ± 3	0.1	33 ± 1
CPC	0.04	233 ± 10	0.1	34 ± 2
CTAB	0.02	165 ± 6	0.1	41 ± 3
OLE	0.02	244 ± 5	0.2	39 ± 1

CTAB and BKC were finally the surfactants chosen for further studies since they led to the formation of stable nanoemulsions showing the lowest sizes.

For the adequate construction of the PASN nanocapsules, the influence of the concentration of polymer was studied as a critical variable that determine the characteristics of nanocapsules made with the selected surfactants. For this purpose we performed the incubation of cationic nanoemulsions with different concentrations of PASN solution, from 0.1 to 1.2 mg/ml. Figure 2 shows the size and surface charge variation on function of the concentration values for the CTAB-formulation, whose behavior was very similar that shown by that made with BCK (results not shown). Four important phases could be distinguished depending on the PASN concentration. In a first step (from 0.1 to 0.2 mg/ml), we can observe a decrease in the positive value of the zeta potential and an increase in the particle size of the nanoemulsion due to the attachment of PASN to the positively charged surface and the consequent shielding of the surface charge. In the area of polymer concentration between 0.2 and 0.4 mg/ml, the aggregation of the nanocapsules occurs due to the neutralization of the total charge in the surface of the nanosystems. At polymer concentrations higher than 0.4 mg/ml, the inversion of the positive surface charge occurs, evidencing the effective adsorption of the PASN to the surface. Finally, after values concentrations of 0.6 mg/ml, the size and the zeta potential showed only small changes until values concentration of 1 mg/ml. At this point, it could be assumed that the saturation in the polymer deposition has been reached, being the size and the zeta potential independent of the polymer concentration.

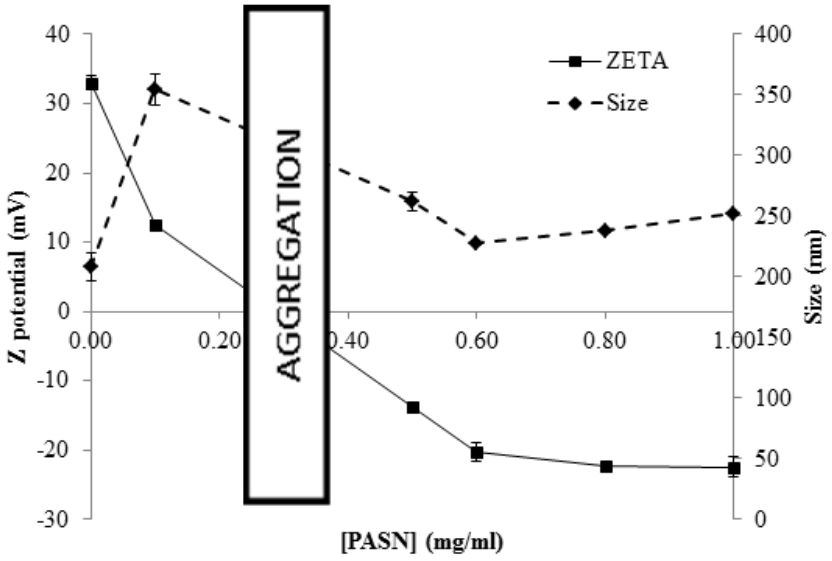


Figure 2.- Influence of the PASN concentration on the size (dash line) and zeta potential of the systems. Each point represents the mean of three experiments ± S.D.

The proposed mechanism for nanocapsule's formation is summarized in Figure 5. Where in the first place the formation of an initial pre-emulsion in acetone/ethanol phase is given by adding the surfactants mixture, this pre-emulsion consist of minidroplets of Miglyol surrounded by lecithin and cationic surfactant in a semidispersion form. The deposition of the PASN onto the nanoemulsion surface was conducted through the ionic-dipolar interaction between amino group of the cationic surfactants on the nanoemulsion surface and the polar carboxilic groups of the PASN as was previously described by Roscigno et al (Figure 3) [25].

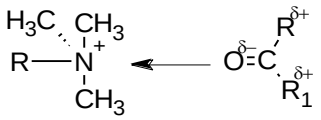


Figure 3.- Cationic surfactant - PASN interaction.

In order to promote this interaction the use of a non-ionic surfactant was necessary, the poloxamer 188. In this sense, Roscigno also demonstrated that non-ionic surfactants improve significantly the force of the interaction between cationic surfactants and PASN [25].

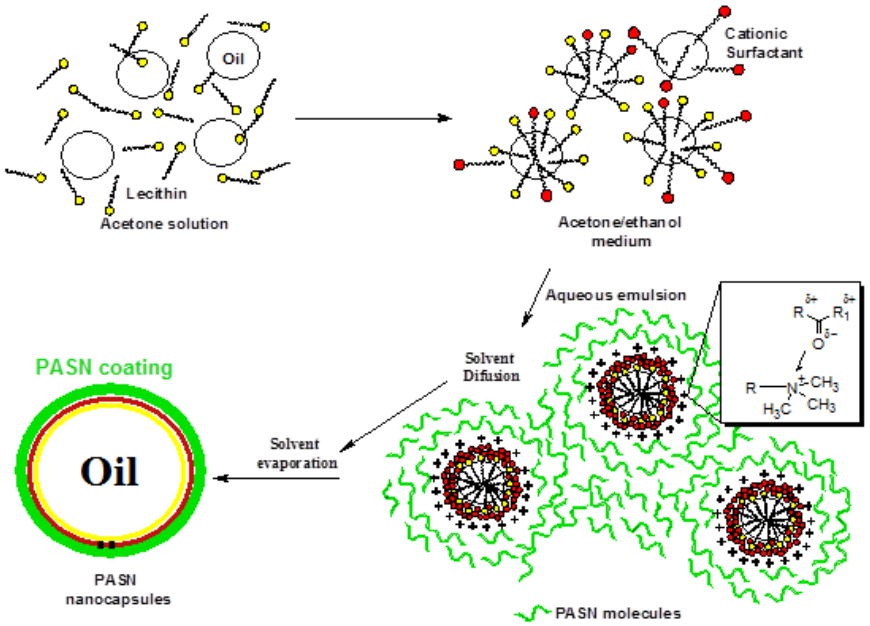


Figure 4.- Schematic diagram of the mechanism of formation of PASN nanocapsules by solvent displacement technique by one stage procedure.

The physicochemical characteristics of the PASN nanocapsules developed at above conditions are showed in the Table 2. PASN nanocapsules have a mean size of 170 nm. These nanocarriers exhibit a negative surface charge due to cationic charge shielding after the deposition of the PASN shell. The zeta potential was around -30 mV. The transmission electron microscopy (TEM) images illustrated that both formulations have a low polydispersion and exhibit spherical shape (Figure 5).

Table 2.- Physicochemical characteristics of PASN nanocapsules prepared by one step method. Blank and docetaxel loaded nanocapsules are shown.

System	Size (nm)	P.I.	z Potential (mV)	Enc. Eff. (%)
PASN/CTAB blank NCs	173.9 ± 5	0.1	(-)24± 3	-
PASN/BKC blank NCs	162.0 ± 2	0.1	(-)20± 6	-
PASN/CTAB docetaxel NCs	186.5 ± 3	0.1	(-)32 ± 8	76 ± 3
PASN/BKC docetaxel NCs	166.0 ± 6	0.1	(-)21 ± 7	73 ± 5
PASN/CTAB DiD-loaded NCs	184.8 ± 4	0.1	(-)34 ± 11	78.5 ± 6

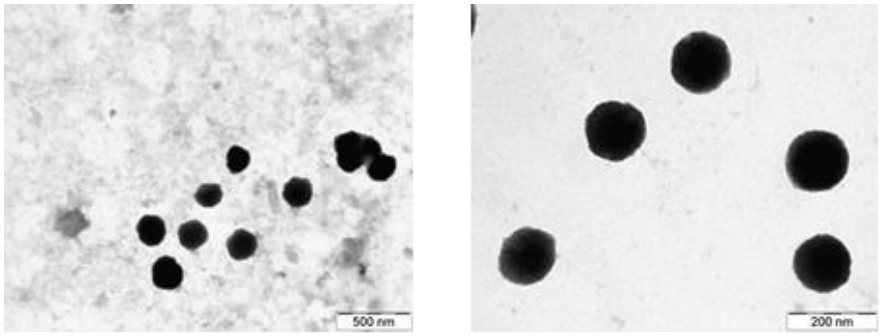


Figure 5.- Transmission electron micrographs of PASN nanocapsules obtained by one step method. Left: PASN nanocapsules prepared with BKC; Right: PASN nanocapsules prepared with CTAB.

Encapsulation and release of docetaxel from PASN nanocapsules

The high efficacy of docetaxel in the treatment of a wide range of solid tumors [26], together with its hydrophobic character makes this molecule an attractive candidate for the inclusion in the developed nanocapsules. Among the tested blank nanocapsules formulations, we selected those prepared with a PASN concentration of 1mg/ml for the further studies with loaded nanocapsules. Table 2 shows that docetaxel could be efficiently encapsulated in the PASN nanocapsules prepared either with BKC or CTAB, hardly being affected the characteristics of the blank formulations. These data correlate well with the previously published results on docetaxel encapsulation in the lipid core of nanocapsules , this high encapsulation efficiently being attributed to the affinity of the drug for the oily components of the core [15, 27, 28].

In an additional experiment we evaluated the release behavior of the encapsulated docetaxel upon incubation of the loaded-nanocapsules prepared with BKC or CTAB in simulated physiological medium based on phosphate buffer pH=7.4. The results showed that the drug was similarly released from nanocapsules regardless of the surfactant type as was described by Oyarzún et al [28] for nanocarriers with the same oily core. The release follows a biphasic profile, characterized by an initial burst effect (releasing around 55%), and followed by a second stage in which no further changes were observed. This profile is typically observed in these type of systems [15, 19] and can be attributed to the dilution of the nanocapsules in the incubation medium and the subsequent partition of the drug between the oily core and the external aqueous phase. The absence of release in the second stage confirms the high affinity of docetaxel by the oil core.

In vitro efficacy of docetaxel-loaded PASN nanocapsules.

Cell toxicity studies were performed in order to assess the efficacy of docetaxel-loaded PASN nanocapsules in the NCI-H460 non-small lung cancer cell line. Figures 6 and 7 show the cellular viability profiles upon exposure for 2 and 48 h, to the docetaxel-loaded PASN nanocapsules, prepared with both surfactants CTAB or CBK. These results indicate that the encapsulation of docetaxel in PASN nanocapsules led to a significant increase of its cytotoxicity. From the IC50 values, summarized in Table 4, we can notice that the potency of docetaxel was increased up to more than 3 times upon its encapsulation into PASN nanocapsules, either after 2 or 48 h of contact with the cells.

Table 4.- IC50 Values (drug concentration resulting in a 50% of cell viability) expressed in nM. Mean values of 4 independent experiments. *: P<0.01 respect IC50 of docetaxel (One-way ANOVA test, post-hoc Tukey test)

Formulation	**IC50 Values (nM)**	
	2 h Exposure	**48 h Exposure**
Free docetaxel solution	105 ± 9	36.4 ± 4
Docetaxel-PASN CTAB NCs	30.9 ± 6.4*	10.51 ± 1.3*
Docetaxel- PASN BKC NCs	26.2 ± 9.3*	13.25 ± 0.6*

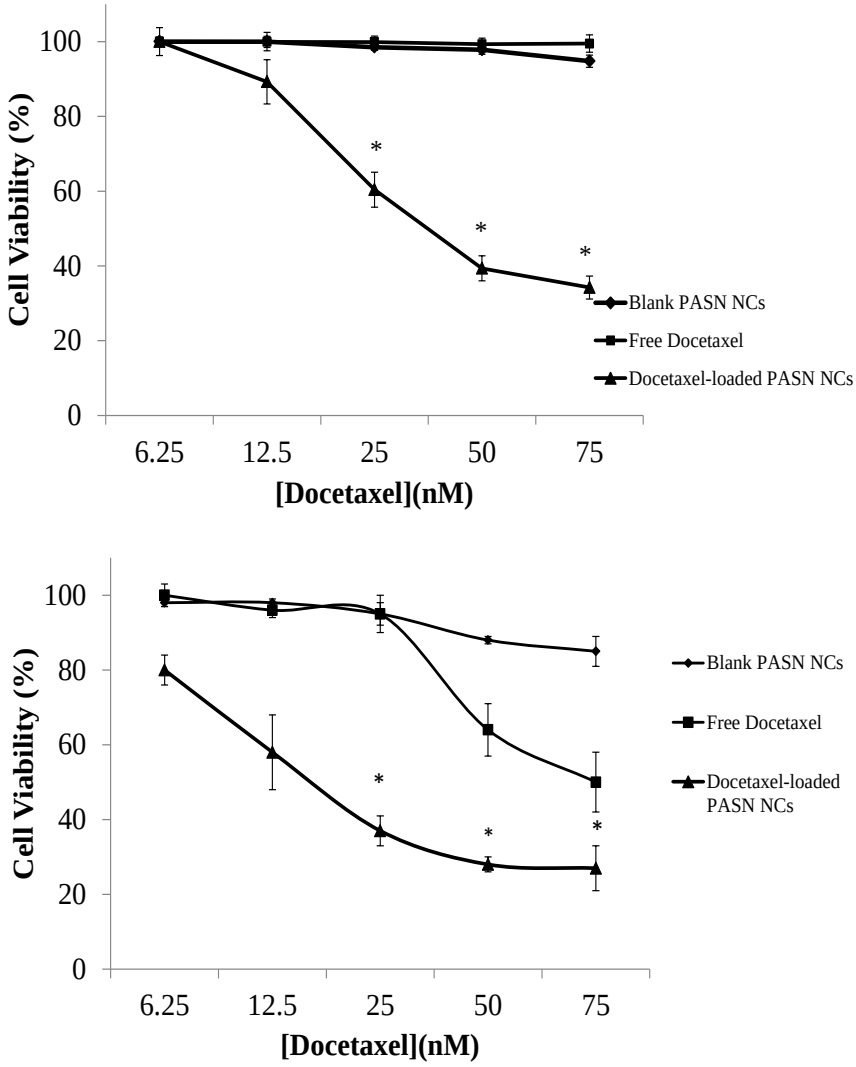


Figure 6.- Effect on H460 cell viability of PASN nanocapsules prepared with the surfactant CTAB (1.8 mg) after 2 h (a) or 48 h (b) of contact with the cells: (♦) Blank PASN nanocapsules, (■) free docetaxel and (▲) docetaxel-loaded PASN nanocapsules (n=4).* p< 0.05: docetaxel- loaded PASN nanocapsules vs. free docetaxel.

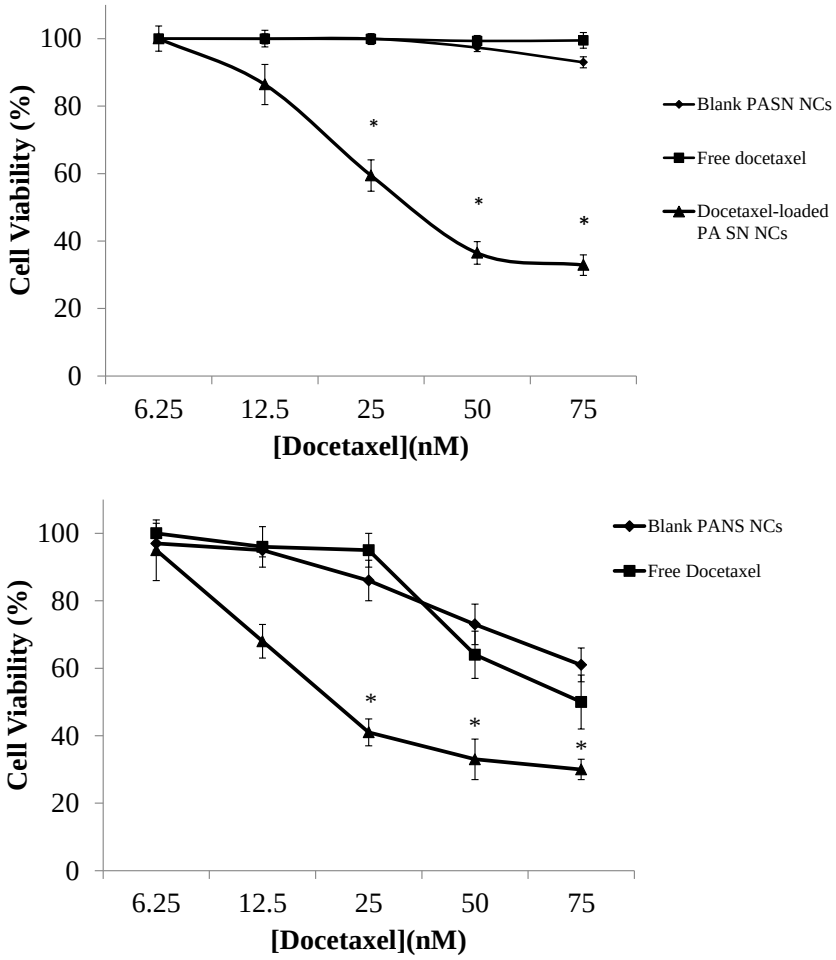


Figure 7.- Effect on H460 cell viability of PASN nanocapsules prepared with the surfactant BCK (4 mg) after 2 h (a) or 48 h (b) of contact with the cells: (♦) Blank PASN nanocapsules, (■) free docetaxel and (▲) docetaxel- loaded PASN nanocapsules (n=4). * $p<0.05$: docetaxel- loaded PASN nanocapsules vs. free docetaxel.

The IC50 values after 2 h of cell contact inform us about the accelerated uptake of the encapsulated docetaxel, in comparison with the free drug. This rapid uptake could be related to a favored interaction of the PASN nanocapsules with the cancer cells, and further internalization.

However, we cannot exclude as a possible facilitating factor for this uptake, the well-known asparagine-needs of certain type of cells, including the lung cancer cells.

The significant improvement in the docetaxel cytotoxicity observed for PASN nanocapsules is in agreement with the results previously observed with other nanocapsules coated with PEG [29], chitosan [15], polyarginine or hyaluronan [28] tested in a variety of cell lines. This improvement has been attributed to the internalization of the nanocapsules in association with a potential reversion of the multidrug resistance effect.

It could be noted from the observation of the viability profiles that blank PASN nanocapsules prepared with CTAB did not cause any perceptible damage to cells, however a certain reduction of cell viability was observed at the higher concentrations of blank nanocapsules prepared with BCK. This effect could be attributed to the higher toxicity of BKC over CTAB [28] and to the need of using higher amounts of BKC to obtain stable nanocapsule formulations.

Afterwards, in order to understand more about the mechanism of action of the nanocapsules, we incubated them with the NCI-H460 cells and studied their uptake by the cells by means of confocal microscopy. With this purpose, nanocapsules were loaded with the fluorescent probe DiD (Table 3), showing similar particle size and ζ potential than those previously reported formulations. Strong fluorescent signals could be detected inside the majority of the cells, upon treatment with fluorescent PASN nanocapsules for up to 2 h (Figure 8) however this fluorescence intensity was less appreciable in the case of the uncoated negative nanoemulsion, used as a control. These results evidence the favorable uptake of PASN nanocapsules by cancer cells.

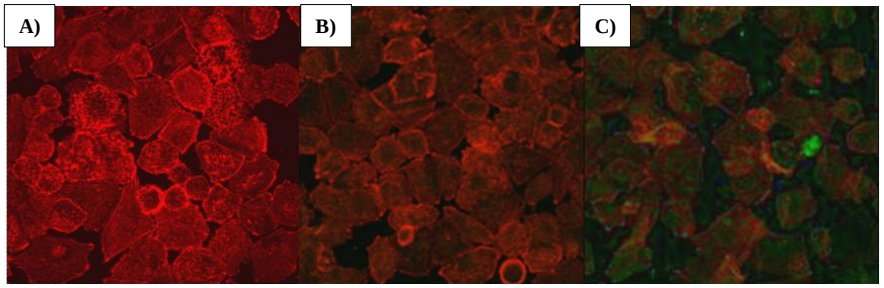


Figure 8.- Confocal microscopic images of (a) NCI-H460 cells, after incubation for 2 h with DiD-loaded nanoemulsion (b) and Did-loaded PASN nanocapsules (c). Images are projection of x-y sections, at a magnification of 63x. Green channel: fluorescent nanoemulsion and nanocapsules. Red channel: actin filaments counterstained with Bodipy ® phalloidin.

In addition, we studied the internalization of fluorescently labeled nanocarriers into the NCI-H460 cells by FACS analysis. For the correct evaluation of the contribution of PASN coating to the uptake of nanocarriers, we used the uncoated negative nanoemulsion and a dispersion of the fluorescent probe DiD as controls. The results in Figure 9 clearly illustrate that the PASN shell has a critical role in the internalization of PASN nanocapsules by NCI-H460 cells. Indeed, when fluorescent PASN nanocapsules were in contact with the cells, fluorescence could be detected in the cells at a intensity 6 times higher than in the case of the nanoemulsion or the fluorescent probe dispersion. Taking these results together, they confirm those previously observed in the same cell line for other nanocapsules coatings made of polymers such as chitosan, polyarginine [30], which evidence the favorable uptake of polymer-based nanocarriers by cancer cells. Moreover, in both cases, it has clearly shown that the polymer coating promotes by itself the entry in the cells, in comparison with the uncoated nanoemulsion.

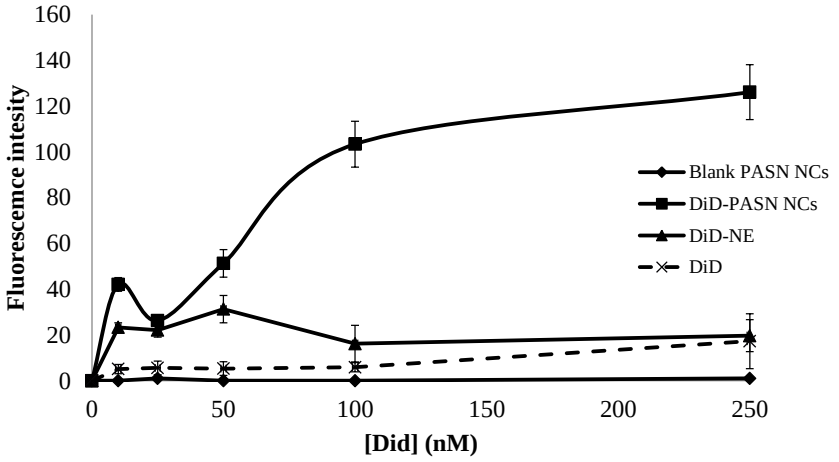


Figure 9: Uptake studies of fluorescent PASN nanocapsules in NCI-H460 cells assessed by FACS. Mean fluorescence intensity of DiD-PASN nanocapsules (■), blank PASN nanocapsules (♦), fluorescent DiD-nanoemulsion (▲) and fluorescent probe (DiD) dispersion (×). * $p < 0.05$, DiD PASN nanocapsules vs DiD dispersion and fluorescent Did-nanoemulsion.

Stability studies

Stability is a critical issue in the development of any pharmaceutical formulation. Variations on temperature are known to importantly compromise the stability of colloidal systems, and show evident importance during storage. Is known that surface charge of colloidal systems, as nanocarriers, usually plays a significant role in its stability: highly charged particles normally suffer aggregation due to the repulsion between particles [31]. In the present study we evaluated the CTAB-PASN nanocapsules suspension stability under storage at 4ºC and 37ºC, during a period of two months. The results indicated that there was not effect of the temperature and time on the particle size evolution, neither on the zeta potential of the nanocapsules, which maintained their original values throughout the study. This prolonged stability could be due to both

factors, surface charge and a non-ionic surfactant presence. The magnitude of the zeta potential, ± 30 mV, is exactly in the range observed previously for such steric stabilization. In the other hand, it has been demonstrated that the use of a non-ionic surfactant as the poloxamer 188 improve significantly the stability of the interactions between a cationic surfactant and the PASN [25].

Formation of a lyophilized product

One of the most common strategies to avoid stability problems is the freeze-drying of the systems [32]. Nevertheless, this process becomes quite complex in the case of nanocapsules due to the fluidity of the polymer shell and also due to the presence of the oil core, which is susceptible of leakage [33]. In order to facilitate the lyophilization of the nanocarriers and avoid their collapse, the use of cryoprotectant agents is necessary [34]. The first evidence that polymeric nanocapsules could be effectively protected during the lyophilization process, by cryoprotectants derived from sugars, was carried out by Calvo in 1997 [16]. More concretely, trehalose has demonstrated many advantages in comparison with other sugars such as less hygroscopicity and higher glass transition temperature [35].

Figure 10 shows the size of PASN nanocapsules containing CTAB upon reconstitution of the freeze-dried product. Overall, the results indicate that at relatively high concentrations of nanocapsules (1% w/v) it is possible to resuspended the dried product, by using a trehalose concentration of 5% w/v without altering the size of the nanocapsules.

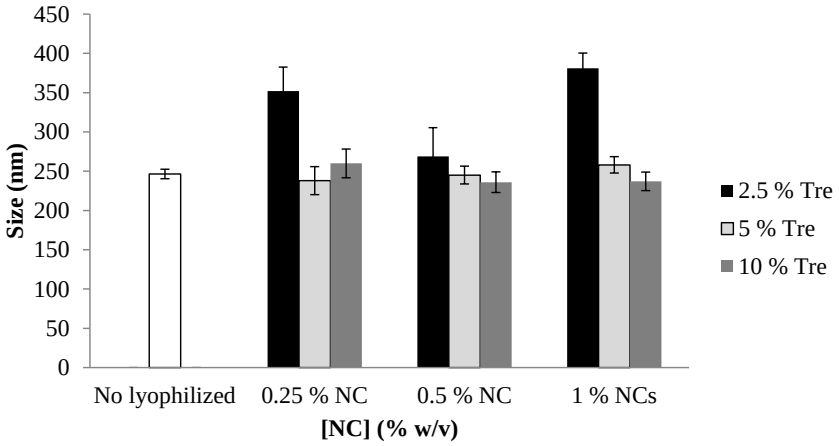


Figure 10.- Particle size of the reconstituted freeze dried PASN nanocapsules prepared with CTAB. Different concentrations (w/v) of nanocapsules were lyophilized using trehalose at 10% (black bars), 5% w/v (grey bars) or 2.5% w/v. (mean ± S.D.; n=3).

CONCLUSIONS

In this paper we report the formation and characterization of a new type of drug nanocarriers named as PASN nanocapsules. Because of their hydrophobic core, these nanocarriers are able to successfully encapsulate the hydrophobic drug docetaxel. In addition, thanks to their PASN hydrophilic coating, these nanocarriers have the capacity to interact with NCI-H460 cancer cells and to be internalizing by them, thus improving the intracellular effect of the model drug docetaxel. In fact, PASN nanocapsules were able to reduce the IC50 values in a 3 factor in comparison with the free drug. Finally, pharmaceutical parameters such as drug release, stability during storage and reconstitution of freeze-dried PASN nanocapsules render these novel systems promising platforms for the intracellular delivery of anticancer drugs.

ACKNOWLEDGEMENTS:

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CAPÍTULO 3. Docetaxel loaded Polyasparagine nanocapsules: mechanism of action, efficacy and toxicity.

CAPÍTULO 3. DOCETAXEL POLYASPARAGINE LOADED NANOCAPSULES: MECHANISM OF ACTION, EFFICACY AND TOXICITY

ABSTRACT

Here, we report an in vivo proof of-concept of a new nanocarrier, Poly-l-asparagine (PASN) nanocapsules, consisting of an oily core and a PASN shell, as an anticancer drug delivery vehicle. For this purpose of this study PASN nanocapsules were loaded with the anticancer drug docetaxel (DCX) and also with the fluorescent marker, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD), using the solvent displacement technique. The resulting nanocapsules had a homogeneous particle size distribution with a mean a size of 170 nm and a negative surface charge (-30 mV). They also exhibited a very high drug encapsulation capacity (encapsulation efficiency ≈90%). In a first instance, the fluorescent nanocapsules were administered to mice in order to determine their pharmacokinetic and biodistribution parameters. The results indicated that the PASN shell around the oily nanodroplets led to a significant prolongation of their blood circulation, as noted by the increase in the parameters MRT, AUC and t1/2. In a second set of experiment, DCX-loaded nanocapsules were administered to tumour-bearing mice for the evaluation of their efficacy/toxicity profile. The results of these studies indicated that this new formulation was as efficacious as Taxotere®, with the additional advantage of having a reduced toxicity (increment in survival rates in tumor bearing mice). All together these results illustrate the promising potential of this new drug nanocarrier for anticancer therapy.

INTRODUCTION

Nanomedicine has already shown a great potential to intervene with cancer at molecular scale and deliver therapeutic agents to cancer cells with improved specificity and reduced toxicity [1]. In fact, the advances in nano-oncology over the last half a century, have led to the clinical development and commercialization of a significant number of anticancer nanomedicines [2, 3]. In addition, the hope is that the use of such technologies will accelerate the progress in identifying the differences between normal and tumor cells, which in turn will lead to the development of new targeted cancer therapies [4].

Among the variety of nanocarriers described so far to deliver anticancer drugs, nanocapsules, consisting in an oily core enveloped with a polymer coating, represent promising strategy. The value of this technology, extensively studied by our group, relies in their versatility [5]. First, the shell of the nanocapsules may impair long circulating and even targeting properties; second, the core may accommodate great amounts of lipophilic drugs (most anticancer drugs are lipophilic); third, both the core and the shell may contribute to the preservation of the stability of the encapsulated drug.

Irrespective of the nature of the drug delivery nanocarriers, historically, a challenge has been to avoid their immediate uptake by the mononuclear phagocyte system (MPS) and, thus, their rapid elimination after intravenous administration. So far this has been mainly achieved by the use of polyethylene glycol (PEG) [6]. However, the same ability has also been described for other hydrophilic polymer such as polysaccharides [7] and polyaminoacids [8]. Within this frame, the polyaminoacid, poly-l-asparagine, is a promising biomaterial which has only been slightly explored as a hydrophilic coating material for liposomes [9, 10].

In addition to the long circulating properties, poly-l-asparagine (PASN) exhibits the advantages of being degraded by the lysosomal proteases [8]. Moreover, it is known that the amino acid l-asparagine (ASN), a required nutrient for protein synthesis, is produced by normal cells in sufficient amounts for their development, whereas this amount does not fulfill the high requirement of tumor cells in order to keep up with their rapid malignant growth [11]. Thus, taking advantage of the avidity of this amino acid by cancer cells we have hypothesized that this would be highly a valuable targeting molecule.

Bearing in mind the above information, we recently reported the development of a new type of nanocapsules made with a shell of PASN with the idea of targeting cancer tissues [12]. This nanocarrier could be prepared by a variety of emulsification techniques and accommodate hydrophobic anticancer drug docetaxel (DCX). Moreover, using in vitro cell culture studies (NCI-H460, human lung cancer cell line) we could show the cytostatic activity of encapsulated DCX was around 3-fold that of the free drug solution. This great improvement of the cytostatic activity was attributed to the internalization of the nanocarrier by the tumor cells and the subsequent intracellular release of the encapsulated drug.

In line with our previous development and findings of PASN nanocapsules, the main purpose of current study was to validate the potential of this technology for anticancer drug delivery in vivo. More precisely, we analyzed the biodistribution and blood kinetics profiles in normal mice, as well as the antitumoral effect and toxicity of these nanocapsules after a single intravenous injection to tumor glioma-bearing model mice.

MATERIALS AND METHODS

Chemicals

Docetaxel (from Flucka), Poloxamer 188 (Pluronic® F68), benzalkonium chloride (BKC) and the polymer Poly-l-asparagine (PASN) were purchased from Sigma-Aldrich (Spain). Miglyol® 812, neutral oil formed by esters of caprylic and capric fatty acids and glycerol, was kindly provided by Sasol Germany GmbH (Germany). The surfactant Epikuron 170, a phosphatidylcholine enriched fraction of soybean lecithin, was donated by Cargill (Spain). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) were obtained from Molecular Probes-Invitrogen (USA).

Preparation of Nanocapsules

In order to achieve the incorporation of the hydrophobic molecules, docetaxel and fluorescent probe DiD, aliquots of ethanol solutions were prepared, with a final concentration of drugs in formulation of 2 mg/ml and 10µg/ml for DCX and DiD respectively.

PASN nanocapsules were obtained by a modification of the solvent displacement technique based on a dipolar-ionic interaction between PASN and cationic surfactants after solvents diffusion [13]. Briefly, an organic phase was formed by dissolving 30 mg of Epikuron 170 in 0.5 ml of drug ethanol solution, followed by 100-250 µl of Miglyol 812 and 9 ml of a cationic surfactant in acetone solution at 0.22 mg/ml. This organic phase was immediately poured over 20 ml of a mix solution of poloxamer (0.25 % w/v) and PASN (2 mg/ml). Finally, solvents were eliminated from the suspension by under vacuum to constant volume.

Two different nanoemulsions, with anionic and cationic surfaces, were prepared and used as controls. Both nanoemulsions were prepared

follow the above described procedure. However, for the anionic nanoemulsion the ingredients in the organic phase were only Epikuron 170 and Miglyol and in the aqueous phase 0.25% w/w of poloxamer, formulation without either cationic surfactant or polymer. Compared with this anionic nanoemulsion, the cationic one presents also 2 mg of benzalkonium chloride in organic phase added in acetone solution.

Characterization of PASN nanocapsules

PASN nanocapsules were characterized regarding their size, zeta potential and morphology as follows:

Nanocapsules were diluted 1:100 (v/v) in KCl 1mM, the measurements were performed at 25 ºC. Particle size and polydispersion index were determined by photon correlation spectroscopy. Each analysis was carried out at 25ºC with an angle detection of 173º. In other hand, the zeta potential values were calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry. Analyses were performed in triplicate using a zetasizer NanoZS® (Malvern Instruments, Malvern, UK).

Docetaxel encapsulation efficiency

DCX encapsulation efficiency in PASN nanocapsules was determined indirectly by the difference between the total amount of DCX in the formulation and the free drug measured in the infranatant of the nanocapsules. Therefore, the total amount of drug was estimated by dissolving an aliquot of non-isolated DCX-loaded PASN nanocapsules with acetonitrile. This sample was centrifuged during 20 min at 4000 x g and the supernatant was measured with a high performance liquid chromatography (HPLC) system. The no-encapsulated drug was determined by the same method following separation of the PASN

nanocapsules from the aqueous medium by ultracentrifugation at 20 000 x g for 1h at 15ºC.

DCX was assayed by a slightly modified version of the method proposed by Lee et al. [14]. The HPLC system consisted of an Agilent 1100 Series instrument equipped with a UV detector set at 227 nm and a reverse phase Zorbax Eclipse® XDB-C8 column (4.6 x 150 mm i.d., pore size 5 µm Agilent USA). The mobile phase consisted of a mixture of acetonitrile and 0.1% v/v ortophosphoric acid (55:45 v/v) and the flow rate was 1 ml/min.

The encapsulation efficiency (E.E.) was calculated as follows:

$$E.E(\%) = \frac{A - B}{A} \times 100$$

Where A is the experimental total drug concentration and B is the drug concentration measured in the external aqueous medium, corresponding to unloaded drug.

Encapsulation efficiency of fluorescent probe DiD, into the nanocapsules

DiD encapsulation efficiency in PASN nanocapsules was determined indirectly by a spectrophotometric method UV at λ=646 nm. The DiD was measured from the infranatant after the ultracentrifugation, infranatant was diluted and detected in acetonitrile. The encapsulation efficiency was calculated with the same formula than in DCX.

Hemolysis studies

- *Preparation of RBC suspension*

The blood of female Wistar rats was obtained by cardiac puncture and then placed in a tube containing heparin. The 2% w/v suspension of red blood cells (RBCs) was prepared as follows. First, the blood was centrifuged at 4000 r.p.m. at 4 °C for 15 min and the plasma and the top 2–3 mm of the pellet removed. The pellet was resuspended in PBS, pH 7.4, which had been chilled to 4 °C. The suspension was then subjected to further centrifugation as before. This washing process was repeated three times. The final concentration of the resuspended pellet was 2% (w/v) in PBS, pH 7.4, solution and stored at 4 °C.

- *Lysis quantification*

A series of nanocapsule dilutions was prepared in PBS, pH 7.4. Dilutions of each concentration were mixed with the same volume of 2 % w/v RBC suspension. The pH of each solution was adjusted to 7.4 by adding HCl and then incubated for 30 min at 37 °C. Each solution was centrifuged at 4000 r.p.m. at 4 °C for 15 min and the concentration of the released hemoglobin in the supernatant measured by spectrophotometry at 550 nm. Triton X-100 (1 % (v/v)) and PBS, pH 7.4, were used to set the two reference hemolysis values, 100% and 0%, respectively. Percentage hemolysis was calculated as follows:

$$lysis(\%) = \frac{A_{nanocapsules} - A_{blank}}{A_{Triton\ X-100} - A_{blank}} \times 100$$

where $A_{nanocapsules}$ is the absorbance of nanocapsules and RBC mixture, $A_{Triton\ X\text{-}100}$ is the absorbance of Triton X-100 and RBC solution and A_{blank} is the absorbance of the PBS and RBC solution.

IN VIVO STUDIES

Blood kinetic study

Animal care was administered in strict accordance to French Ministry of Agriculture regulations. The pharmacokinetic study was carried out according to Morille et al [15] as follows: one hundred and 150 □l of fluorescent PASN nanocapsules were injected in the tail vein of six-week old female Swiss mice (20-22 g) (Ets Janvier, Le Genest-St-ile, France). The fluorescence was measured at time 30, 60, 120, 180, 240 min and 24 h. At each time blood sampling was performed by cardiac puncture on 3 mice and each sample was centrifuged for 10 min at 2000g in a venous blood collection tube (Vacutainer, SST II Advance, 5 ml, Becton Dickinson France SAS, France). One hundred and 150 µl of the supernatant were deposited in a black, 96-well plate (Greiner Bio-one, Germany). Empty samples were constituted by the supernatant of centrifuged blood taken from 3 mice injected with an isotonic solution (150 nM NaCl), representing the residual fluorescence of the plasma. DiD fluorescence (emission wavelength of 665 nm with an excitation wavelength of 644 nm) was counted by a Fluorscan (Ascent FL, Thermo Fischer Scientific, France) and the results were analyzed with the Ascent software for Fluorscan (Thermo Fischer Scientific, France). The blood concentration of the different systems at the various times was calculated on the assumption that blood represents 7.5 % of mouse body weight [16]. Fluorescence was expressed in fluorescence units (FU) and was calculated as: FU sample – FU empty. 100% of fluorescence was considered as the value of fluorescence in ex vivo blood DiD-labeled nanocapsules mixture.

Pharmacokinetic data were treated by non-compartmental analysis of the percentage of the injected dose versus time profiles with Kinetica 5.1 software (Thermo Fischer Scientific, France). The half-lives were calculated as following:

$$t_{1/2} = \frac{\log(2)}{Lz}$$

The Lz was determined from linear regression using defined intervals (1 h and 24 h for $t_{1/2}$ distribution [0-1 h] and $t_{1/2}$ elimination [1-24 h] respectively). The trapezoidal rule was used to calculate the area under the curve (AUC) during the whole experimental period (AUC [0-24 h]) without extrapolation, as well as the area under the first moment curve (AUMC). The mean residence time was calculated from 0 to 24 h, from the following equation:

$$MRT[0-24h] = \frac{AUMC[0-24h]}{AUC[0-24h]}$$

In vivo antitumor efficacy study

Tumor bearing mice were prepared by injecting subcutaneously a suspension of 1 x 106 U87MG glioma cell line (ATCC, Manassas, VA) in 150 µl of Hanks Balanced Saline Solution (HBSS) into the right flank of athymic nude mice (6 weeks old females, 20-24 g, purchased from Charles Rivers, Wilgmintong, Ma). Tumor growth was tracked by regularly measuring the length and width of tumors with a caliper. The tumor volume (v) was estimated by the mathematical ellipsoid formula:

$$V = \left(\frac{\pi}{6}\right) \times (width)^2 \times (length)$$

When tumors reached a calculated average volume of approximately 200mm3, the mice were randomized into three groups to ensure that the initial tumor volumes on the day of treatment were not significantly different among groups. In order to evaluate the antitumor efficacy of treatments PASN nanocapsules were loaded with DCX at 2 mg/ml concentration. Animals were treated (Day 0) by a single intravenous injection of different treatments (200 µl) via lateral tail vein as follows: physiological saline solution (0.9% NaCl), PASN loaded nanocapsules (2 mg/Kg mouse weight) and Taxotere® (2 mg/Kg mouse weight).

The different treated groups were compared in terms of median and mean survival time in days after tumor implantation. The percentage increase in survival time (% IST) mi was determined relative to the median and mean survival of untreated controls as presented in the following equation:

$$\%IST = \frac{[Median_T(Mean_T) - Median_C(Mean_C)}{Median_C(Mean_C)} \times 100$$

where MedianT/MeanT was the median/mean of survival time of the treated group and MedianC/MeanC was the median/mean of the survival time of the control group [17].

Statistical analysis

Data from in vitro, in vivo experiments are presented as a mean ± SD and statistical analysis among groups was conducted with the two-tailed Student t-test ($p<0.05$ was considered to be statistically significant). The Kaplan-Meier method was used to plot animal survival. Statistical significance was calculated using the log-rank test (Mantel-Cox test). SPSS software version 16.0 (SPSS Inc.) was used for that purpose and tests were considered as significant with p values <0.05. The different

treatment groups were compared in terms of range, median, and mean survival time (days), long term survivors (%) and increase in survival time (IST_{median} and IST_{mean} %)

RESULTS AND DISCUSSION

The main goal of this study was to evaluate in vivo the potential of PASN nanocapsules as drug delivery vehicles in cancer therapy. As indicated in the introduction, our group has previously developed these nanocapsules and shown their ability to improve the in vitro antitumor efficacy of DCX using the NCI-H460 cancer cell line. We selected this polyaminoacid on the basis of its biodegradability [9] as well as its long circulating properties [17]. Moreover, taking into account the avidity of cancer tumor cells by ASN [18], we have speculated about its potential targeting properties. Here we report a preliminary in vivo proof-of-concept of PASN nanocapsules, which includes the biodistribution and blood kinetics of the nanocapsules as well as the efficacy and toxicity of DCX-loaded nanocapsules, as a nanomedicine prototype.

Characterization of DiD-labeled and DCX-loaded nanocapsules

Two sets of nanocapsules containing, either DiD or DCX were prepared according to the solvent displacement technique and characterized for their size, zeta potential and encapsulation efficiency. The results presented in Table 1 indicate that PASN nanocapsules form homogeneous populations with a mean particle size of around 170 nm and a negative surface charge (-30 mV). The encapsulation efficiency of both hydrophobic molecules, DCX and DiD, was high (90% and 70% respectively), with final concentrations in isolated formulation of around 4 mg/ml and 80 µg/ml respectively. Interestingly, the formation of the polyaminoacid shell around the oily nanodroplets led to a decrease in their size, as compared to that of the control emulsion, which was accompanied

of an increase in the loading efficiency. Furthermore, after the encapsulation of hydrophobic molecules, the size of the nanocapsules was slightly reduced, probably due to the compaction of the nucleus caused by the in hydrophobicity increase.

On the other hand, the encapsulation of DCX did not significantly affect the zeta potential of the nanocapsules, however, the encapsulation of the fluorescent probe, DiD, led to a small reduction of the negative surface charge. This could be attributed to the amphiphilic character of the DiD molecules and their localization at the surface of the oily droplets in association to the polymer coating. This small reduction (around 10mV) is not expected to affect the biodistribution of the nanocarrier.

Table 1. Physicochemical characteristics of PASN nanocapsules. PdI.: polydispersity index; DCX: Docetaxel; DiD: Fluorescent probe. Values are given as mean ± SD; n=3

Formulation	**Size (nm)**	**PdI**	**ζ potential (mV)**	**Enc. Eff.(%)**
PASN nanocapsules	187 ± 7	0.1	-38 ± 3	-
DiD loaded nanoemulsion	192 ± 3	0.1	-20 ± 7	60.3 ± 5
DiD loaded PASN nanocapsules	163 ± 3	0.1	-29 ± 6	74.4 ± 4
DCX loaded PASN nanocapsules	176 ± 3	0.1	-43 ± 6	90.1 ± 3

Hemolytic activity of PASN nanocapsules

A key parameter for the acceptability of a formulation for i.v. administration is the hemolytic activity. Drug-induced hemolysis in vitro is considered to be a simple and reliable estimation of membrane damage

caused by drugs in vivo [19]. Taking into account the presence of a cationic surfactant in the nanocapsule's composition, we found it particularly important the assessment of the blood compatibility of PASN nanocapsules. The release of hemoglobin was used to quantify the possible membrane-damaging properties of the PASN nanocapsules. As 100% and 0% values we used Triton X-100 and PBS-treated erythrocytes, respectively. Erythrocytes were incubated for 1h with five different nanocapsule concentrations (1-10 mg nanocapsules/ml of blood), which corresponded to nanocapsules-blood dilutions of 1:20 to 1:1 formulation : blood (v/v) (Figure 1). The results indicated that the high hemolytic activity of the control cationic nanoemulsion (100% hemolysis) was almost totally reduced in the case of the nanocapsules. In fact, the hemolytic activity exhibited by the nanocapsules varied from 7% (for the highly concentrated nanoparticles) to negligible values (for the 1:20 dilution of nanocapsules). Therefore, the conclusion is that PASN nanocapsules did not cause a disturbance of the red blood cell membranes. These positive results persuaded us to move ahead towards the pharmacokinetic evaluation of the blank nanocapsules.

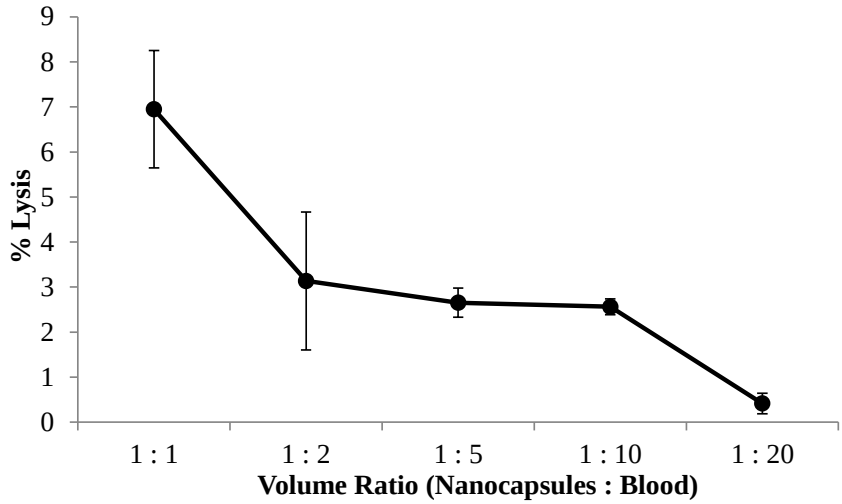

Figure 1.- Percentage of lytic activity versus concentration of PASN nanocapsules after incubation for 1 h. Cells were incubated at 37 °C with 1–10 mg/ml of PASN in blood (1:20 – 1:1 (v : v) formulation : blood).

Blood kinetics and biodistribution of PASN nanocapsules

For this study fluorescent DiD-labeled PASN nanocapsules, were injected into the tail vein of Swiss mice at a probe dose of 1 mg/kg of animal weight. Afterwards, blood samples were collected from 30 min to 24 h, and the plasma was dosed for its fluorescent content. The fluorescence of a mixture of labeled nanocapsules with blood was taken as the 100 % of the injected dose. Figure 2 illustrates the plasma pharmacokinetic behavior of the fluorescent nanocapsules and also that of an anionic nanoemulsion used as a control. Following the administration of the control emulsion, a very rapid decrease in the fluorescence concentration was observed reaching the value of 15% of total fluorescence after 30 min. On the contrary, the fluorescence associated to the nanocapsules remained at high concentrations (about 40% of the initial dose) for at least 3 h post-injection. Finally, approximately 10% of the

injected dose of both anionic nanoemulsion and PASN nanocapsules remained in circulation after 24 h after the injection.

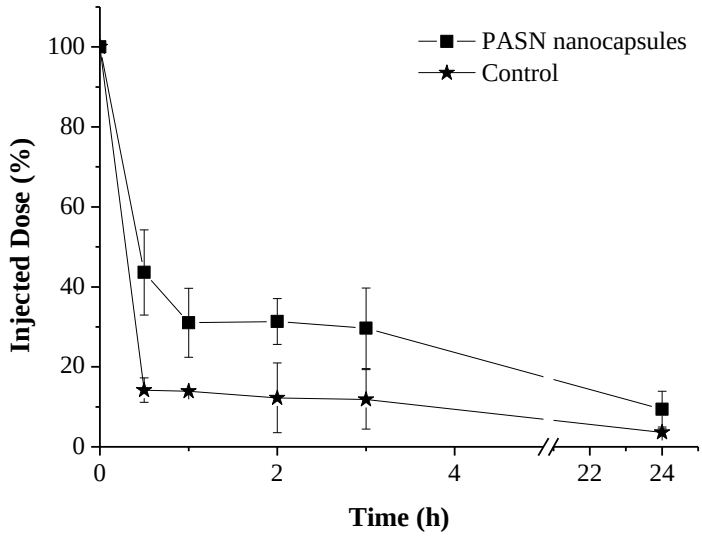

Figure 2. Kinetic blood profiles of PASN nanocapsules following systemic injection in Swiss mice. The percentage of injected dose ([Did] = 1 mg/Kg of animal weight) remaining in plasma following a single bolus injection is displayed as a function of time. Administration of an anionic nanoemulsion is showed as a control. Each data point represents the group mean ± SD of the percent injected dose.

A more illustrative way of presenting the blood kinetics profiles is through the blood kinetic parameters. As shown in (**¡Error! No se encuentra el origen de la referencia.**), PASN nanocapsules exhibited a 50 % longer half-life respect to the control emulsion, being the values for $t_{1/2}$ of 12.64 ± 0.2 h and 8.17 ± 0.3 for PASN nanocapsules and anionic control nanoemulsion respectively. On the other hand, the values of mean residence times (MRT) and area under the curve (AUC) were significantly higher for the nanocapsules than for the control emulsion. Namely, PASN

the MRT value of the nanocapsules was 12.75 ± 0.4 h, 2 folds higher than that the control, while the AUC was extended around the 138% with respect to that of the anionic nanoemulsion (from 11.43 ± 2.1 h to 34.83 ± 2.3 mg/ml * h).

Overall, the results of the blood kinetic profiles represent the proof-of-concept of the long-circulating properties of PASN nanocapsules. These results are in good agreement with those reported by Romberg *et al.* for PASN-coated liposomes. These authors showed that the PASN coating provided a better protection of the nanocarrier than PEG coating, thus leading to as important increase in the half-life and AUC of the encapsulated drug paclitaxel . Even though, it would be no reasonable to conclude from these studies the superiority of PASN *vs* PEG in terms of nanocarrier protection ability, we could at least affirm that the use of PASN coatings could be an interesting alternative technology for designing long-circulating nanocarriers. Moreover, as indicated above, the use of PASN could bring targeting properties to nanocarriers intended to reach the cancer cells.

Table 2.- The main pharmacokinetic characteristics of PASN nanocapsules and its control after a single I.V. injection in Swiss mice. Plasma clearance of PASN nanocapsules was measured over a 24 h period in animals treated with 1 mg of DiD/Kg of mouse weight. Each data point represents the group mean ± SD

Formulation	$t_{1/2}$ **distribution [0-1h] (h)**	$t_{1/2}$ **elimination [1-24h](h)**	**MRT [0-24h] (h)**	**AUC[0-24h] (mg/ml*h)**
PASN-nanocapsules	0.58 ± 0.1	12.64 ± 0.2	12.75 ± 0.4	34.83 ± 2.3
Nanoemulsion	0.34 ± 0.3	8.17 ± 0.3	6.59 ± 0.1	11.43 ± 2.1

As a complementary study, the body distribution profile of PASN nanocapsules and the control emulsion was tested in mice by monitoring the real-time NIR fluorescence intensity in the whole body. Near-infrared (NIR)-absorbing dyes represent a very interesting way to obtain information form living models since they can be monitored with safe, non-invasive optical imaging/constrasting techniques. The advantages of imaging in the NIR region are numerous: the significant reduction of background absorption, fluorescence and light scattering along with high sensitivity, the availability of low-cost sources of excitation and the versatility that allows the large catalog of existing probes.

In agreement with the results of the blood kinetic parameters (Figure 3 and Table 2), the nanoemulsion was rapidly eliminated from the blood compartment and localized in the liver and kidney. In contrast, as noted by the diffused large NIR signal observed following administration of PASN nanocapsules, a significant amount of fluorescence remained in circulation for at least 24 hours. The prolonged circulation times showed by PASN nanocapsules are also in accordance with the previously reported stealth properties of PASN and its derivatives [20, 22].

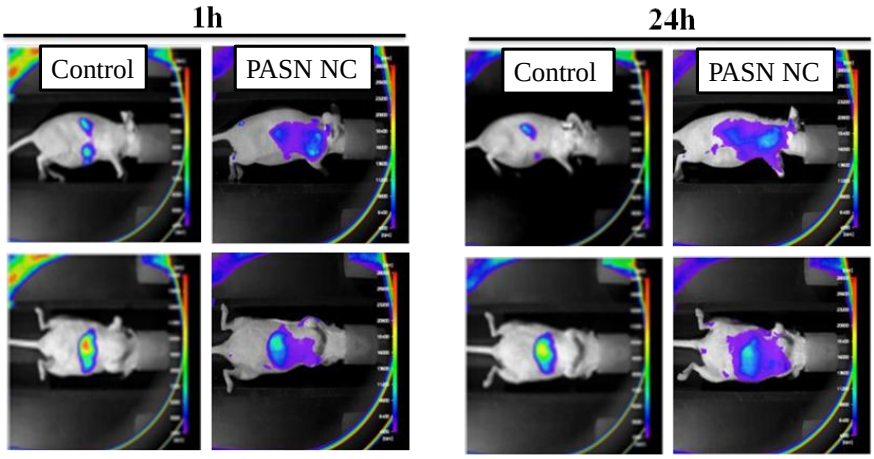

Figure 3. *in vivo* fluorescence imaging of athymic nude mice after intravenous injection of an anionic nanoemulsion (control) and PASN nanocapsules after 1 hour (left) and 24 hours (right) after the fluorescent nanocapsules injection.

In vivo antitumor activity of DCX-loaded PASN nanocapsules

We used a U87MG subcutaneous glioma model in order to evaluate tumor reduction efficacy after treatment with PASN nanocapsules in comparison with the commercial docetaxel form, Taxotere®. When tumors were grown up to about 200 mm3, we performed the comparative antitumor efficacy studies for which animals were divided in three groups according to the treatment received.

The results presented in Figure 4 show the tumor growth during the experiment (a) and the tumor volume at times 18 days and 21 days after treatment relative to the original one (b) respectively. It can be noted that tumor in control groups receiving serum grew very quickly, reaching a relative tumor volume of about 6x103 mm3 after 21 days. On the contrary, the intravenous administration with both DCX-loaded PASN nanocapsules and Taxotere®, significantly reduced the final tumor volumes as compared to the control group ($p<0.01$). It is also interesting

to note that the tumor volume reached a plateau at day 18 in animals exposed to the treatment with DCX-loaded nanocapsules, whereas it continued to grow in the non-treated animals and also in those treated with taxotere®. This result led us to speculate on the expected sustained release behavior of DCX from PASN at nanocapsules once they have reached the target tissue.

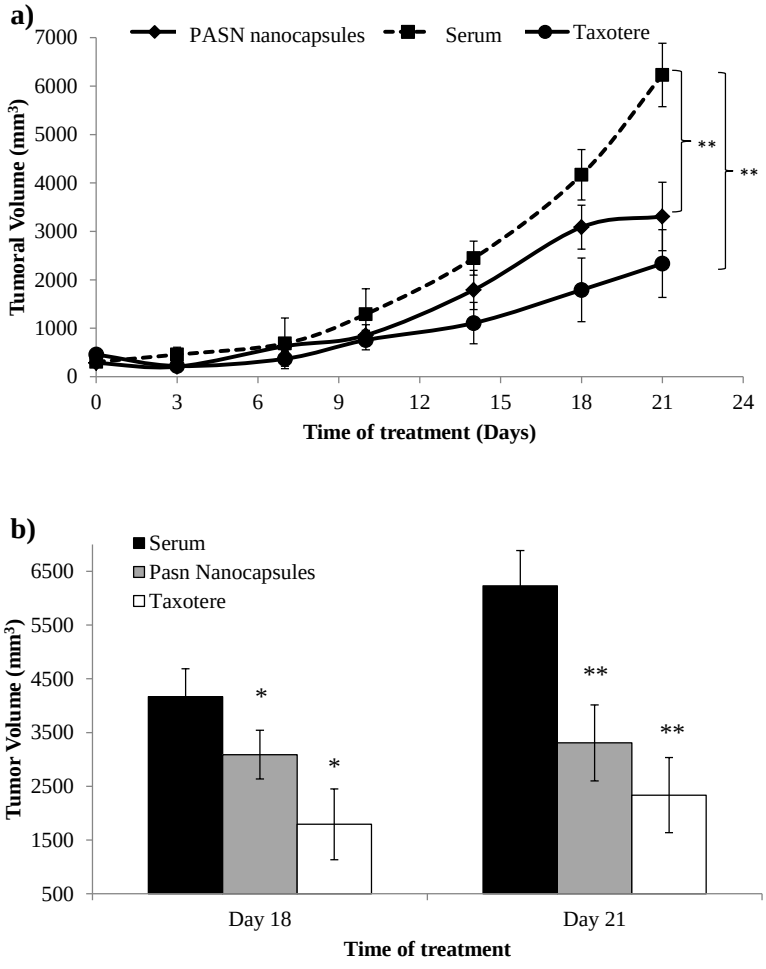

Figure 4.- Antitumor effect of DCX loaded-PASN nanocapsules and Taxotere after a single intravenous treatment as compared to control groups receiving 0.9% NaCl (serum) solution in a subcutaneous U87MG glioma mouse model. a) Evolution of the tumor size over the time; b) Tumor growth at day 18 and 21 post-administration, relative to the initial size prior treatment.. Statistical analysis was done at day 18 and 21 for PASN

nanocapsules and Taxotere® treatment compared to serum-received animals. *P < 0.05 **P < 0.01 t-Student test. All data are reported as means ± SEs; n = 6

As a complement of the antitumoral activity of DCX-loaded nanocapsules we studied the toxicity of this treatment as compared to that of the commercial one (Taxotere ®) by monitoring the mice survival time. The Kaplan-Meier survival plots are displayed in Figure 5. For the calculation of the mean and median survival times, a censured model was used, which takes as a censure event an excessive tumor volume (more than 10% of mouse weight). The results in Figure 8 and Table 3 indicate that the treatment with PASN nanocapsules led to a significant improvement of the mice survival, this improvement being significantly higher than that corresponding to the commercial formulation, Taxotere®. In fact PASN nanocapsules showed an increased mean survival time of 67% when compared to control, whereas Taxotere® increased the mean survival time in 50% respect to the controls. The differences between these two groups (PASN nanocapsules and Taxotere®) appeared to be significant (p=0.05). Consequently, overall, these results provide evidence of the reduced toxicity of DCX-loaded nanocapsules as compared to that of the commercial formulation.

Taking together the toxicity and efficacy results as well as those of the blood kinetic profiles, we could speculate about a more adequate biodistribution profile for the nanocapsules as compared to the commercial formulation. This improved biodistribution profile would imply a facilitated accumulation of the drug in the tumor cells and a reduced accumulation in other vital organs. This targeting capacity of these novel nanocarriers could be a simple passive targeting similar to that reported for variety of PEGylated nanocarriers loaded with taxanes [21, 23-25]. However, we cannot discard the possibility for the PASN coating

to provide a certain active targeting capacity based on the avidity of cancer cells for the ASN amino acid [26].

Table 3.- Survival time of implanted, U87MG tumor bearing mice that received an intravenous injection of PASN nanocapsules or Taxotere® solution at 200 mm3 reaching tumor. Compared to mice injected with physiological serum group.

Treatment	Survival time (days)			Increase survival time (%)		
	Range	Median	Mean ± SD	IST_{median}	IST_{mean}	*p*-value vs serum
PASN nanocapsules	14-21	0	18.7 ± 2	0	66.96	0.036
Taxotere	11-21	21	16.8 ± 3	50	50	0.056
Serum	6 - 14	14	11.2 ± 2	-	-	

% IST percentage of increase in survival time relative to that of the serum control

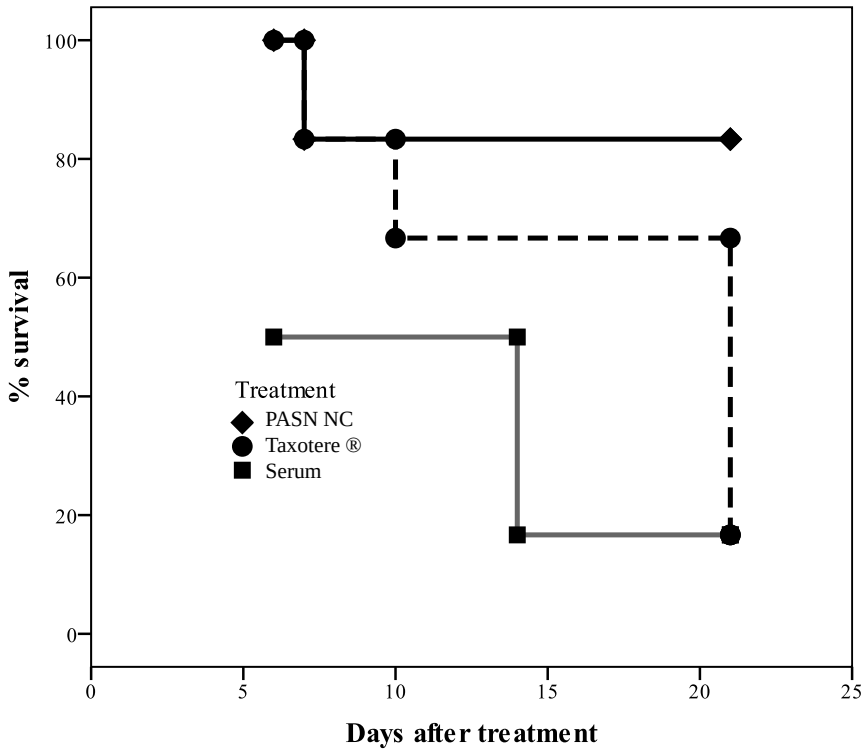

Figure 5.- Kaplan-Meier survival curves of subcutaneous U87MG-glioma tumor bearing mice showed significant enhanced median survival after DCX loaded PASN nanocapsules (vs. Taxotere-treated and control mice)

CONCLUSION

Here we present the first proof-of-principle of the in vivo efficacy of PASN nanocapsules as nanovehicles for anticancer drugs as docetaxel. PASN nanocapsules exhibited a prolonged blood circulation and reduced accumulation in liver and kidney. Also, the treatment with PASN nanocapsules containing docetaxel of U87MG glioma tumor bearing mice showed a significantly improvement over the survival rates compared with Taxotere, and an important suppress of tumor growth compared to

control, after a single intravenous administration. Overall, this novel nanocarrier is presented as a new anticancer drug delivery targeting vehicle with potential advantages over the PEG-coated classical nanocarriers.

ACKNOWLEDGEMENTS:

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CAPÍTULO 4. Casein nanocapsules: a new platform for oral drug delivery

CAPÍTULO 4. CASEIN NANOCAPSULES: A NEW PLATFORM FOR ORAL DRUG DELIVERY

ABSTRACT

In this work, we present for the first time a new drug delivery system, named as casein nanocapsules, consisting of an oil core (Miglyol® 812 and lecithin) and a shell made of two different types of milk proteins, β-casein and κ-casein. These new nanocapsules were specifically designed to resist the gastric pass, interact with the intestinal epithelium and release the cargo in the absorption site. Following an adequate selection of the formulation parameters, the nanocapsules could be obtained using the solvent displacement technique. The resulting prototypes of nanocapsules had sizes in the range of 170 and 250 nm and a negative surface charge (from -40 up to -70 mV), depending on the formulation conditions. Interestingly, due to the stabilizing role of casein, these nanocapsules exhibited very good stability patters. Namely, (i) they were stable upon incubation in both, simulated gastric and intestinal fluids; (ii) they presented a long-term (more than 6 months) stability profile during storage at 37ºC and (iii) they could be freeze-dried and reconstituted without alteration of their properties. Finally, these nanocapsules have shown the capacity to accommodate significant amounts of lipophilic drugs, i.e. docetaxel, in their core and deliver it in a controlled manner. Overall, because of their interesting pharmaceutical and known safety profile, casein nanocapsules are presented here as a promising vehicle for oral drug delivery.

INTRODUCTION

One of the current challenges of nanomedicine is the development of nanocarriers intended for the oral administration of complex molecules [1, 2]. Various delivery carriers have been investigated with regard to their potential for the oral administration of drugs, among them the so-called nanocapsules [3]. A potential key advantage of these nanocarriers relies in their versatility, as they are able to associate different active agents in their core, coating or both. In addition, they offer opportunities to confront specific biological barriers by modifying the nature of their coating [3, 4]. We have contributed to this field by designing a few prototypes of nanocapsules consisting of biopolymers such as polysaccharides, i.e. chitosan [5] and polyaminoacids, i.e. polyarginine [6] and polyasparagine [7]. With regard to their potential for oral drug delivery, we have already shown that chitosan nanocapsules are able to enhance and prolong the intestinal absorption of the peptide salmon calcitonin [8, 9].

In this work, we propose an alternative composition for oral drug delivery based on the use of casein, a FDA GRAS (Generally Recognized as Safe) biomaterial, approved by human use [10] due to its non-toxic profile [11] and biodegradability [12]. Casein is a protein found in cow's milk along with whey proteins; it makes up approximately 80 percent of its protein content. In addition, this protein is naturally present in mammalians, in the form of micelles, associated to essential micronutrients [13]. Furthermore, because of its structure with hydrophilic and hydrophobic domains, this protein works as a surfactant molecule [14] and exhibits as well as pH-responsive gel swelling behavior [15]. Consequently, casein represents a very promising biomaterial in the development of nanocarriers for oral drug delivery.

Interestingly, despite this interesting casein profile, there are only a few articles reporting its presentation in a micro/nanometric form for oral drug delivery. In a particular study, Morçöl et al [16] observed that coating calcium phosphate-insulin micro-complexes with casein it is possible to protect insulin from degradation in the gastrointestinal tract and increase its intestinal absorption. In other instances casein micelles has been used to improve the stability, the solubility and the bioavailability of low molecular weight drugs such as triclosan [17], curcumin [18], paclitaxel and vinblastine [19]. Within this context, particularly worth of mentioning is a clinical trial phase I that is currently being conducted in order to evaluate the improvement in the vitamin D bioavailability after the encapsulation within casein micelles [20].

Based on the above information, the main goal of the current work was the development of a new drug nanocarrier, named as casein nanocapsules, consisting of an oily core and a shell made of casein. For this, two different types of casein were explored, β-casein (β-CN) and κ-casein (κ-CN). In order to assess its pharmaceutical potential for oral drug delivery, this new nanocarrier was fully characterized and its stability in simulated biological media and during storage was monitored. Moreover, the potential of this nanocarrier for the delivery of hydrophobic drugs was explored using docetaxel as model drug.

MATERIALS AND METHODS

Chemicals

Docetaxel (from Flucka), trehalose dehydrate, β-casein (β-CN) and κ-casein (κ-CN) were purchased from Sigma-Aldrich (Spain). Miglyol® 812, neutral oil formed by esters of caprylic and capric fatty acids and glycerol, was kindly provided by Sasol Germany GmbH (Germany). The

surfactant Epikuron 145v, a phosphatidylcholine enriched fraction of soybean lecithin, was donated by Cargill (Spain).

Preparation of Nanocapsules

Blank casein nanocapsules were obtained by the previously reported solvent displacement technique [5] using two different molecules of casein, κ-casein and β-casein. Briefly, an organic phase was formed by dissolving 20-40 mg of Epikuron 145v in 0.5 ml of ethanol, followed by 100-250 µl of Miglyol® 812 and 9 ml of acetone. This organic phase was immediately poured over 20 ml of a or -CN solution (1 % w/v) in 10 mM phosphate buffer (pH 7.4). Finally, solvents were eliminated from the suspension by under vacuum to constant volume (10 ml).

In order to achieve the incorporation of hydrophobic molecules, such as docetaxel, the ethanol was replaced with 0.25 ml aliquots of ethanol drug solutions in range concentrations of 100 – 250 µg/ml and the same procedure was follows.

Characterization of casein nanocapsules

Casein nanocapsules were characterized with regard to their size, zeta potential and morphology as follows.

Particle size and polydispersion index were determined by photon correlation spectroscopy. Samples were diluted 1:50 with KCl. Each analysis was carried out at 25ºC with an angle detection of 173º. The zeta potential values were calculated from the mean electrophoretic mobility values, which were determined by laser doppler anemometry. Samples were diluted with KCl 1mM and placed in an electrophoretic cell. Analyses were performed in triplicate using a NanoZS® (Malvern Instruments, Malvern, UK).

The morphological examination of the nanocapsules was performed by transmission electron microscopy (TEM, CM12 Philips, The Netherlands). Samples were stained with 2% w/v phosphotungstic acid solution, and placed on cupper grids with Formvar® films for analysis.

Encapsulation of docetaxel

The incorporation of the anticancer drug docetaxel in casein nanocapsules was achieved as previously reported by our group [21], adding 50 µl of a drug solution in ethanol to organic phase in order to obtain a final drug concentration of 10 µg/ml.

Docetaxel encapsulation efficiency in CN nanocapsules was determined indirectly by the difference between the total amount of docetaxel in the formulation and the free drug measured in the infranant of the nanocapsules. Therefore, the total amount of drug was estimated by dissolving an aliquot of non-isolated docetaxel loaded CN nanocapsules with acetonitrile. This sample was centrifuged during 20 min at 4000 x g and the supernatant was measured with a high-performance liquid chromatography (HPLC) system. The non-encapsulated drug was determined by the same method following separation of the casein nanocapsules from the aqueous medium by ultracentrifugation at 20 000 x g during 1 h at 15ºC.

Docetaxel was assayed by a slightly modified version of the method proposed by Lee et al [22]. The HPLC system consisted of an Agilent 1100 Series instrument equipped with a UV detector set at 227 nm and a reverse phase Zorbax Eclipse® XDB-C8 column (4.6 x 150 mm i.d., pore size 5 µm Agilent USA). The mobile phase consisted of a mixture of acetonitrile and 0.1% v/v ortophosphoric acid (55:45 v/v) and the flow rate was 1 ml/min.

The encapsulation efficiency (E.E.) was calculated as follows:

$$E.E(\%) = \frac{A - B}{A} \times 100$$

Where A is the experimental total drug concentration and B is the drug concentration measure in the external aqueous medium, corresponding to unloaded drug.

Stability studies

The stability of casein nanocapsules was evaluated upon storage for different periods. The storage conditions were for pH: 1.2, 6.8 and 7.4 and for temperature: 4 and 37°C for storage. Size and polydispersity index of the nanocarriers were measured for a period of three months, meanwhile zeta potential values were controlled at the end of the study. Each sample corresponds to a different casein nanocapsules batch.

Drug release studies

The release studies of docetaxel from CN nanocapsules were performed by incubating a sample of the formulation at 37ºC in both gastric and intestinal simulated medium without enzymes at an appropriate drug concentration to assure sink conditions. The vials were placed in an incubator at 37ºC with horizontal shaking. 4 ml of the suspension were collected and centrifuged at different time intervals. The DCX released was calculated indirectly by determining the amount of drug remaining in the system by processing the isolated CN nanocapsules with acetonitrile before HPLC analysis.

The HPLC method and the drug quantification were performed as was described in the encapsulation efficiency study.

Freeze-drying of casein nanocapsules

Different concentrations of CN nanocapsules (0.25, 0.5 and 1.0 % w/v) and trehalose (10 % w/v) were considered the variables for the lyophilization study. Therefore, 1 ml dilutions of CN nanocapsules were placed in volume glass vials and were quickly frozen in liquid nitrogen. The lyophilization procedure consisted in an initial drying step for 60h at -35ºC, followed by a secondary drying for 24h in a high vacuum atmosphere. Finally, temperature was slowly increased up to 20ºC until the end of the process (Labconco Corp., USA).

CN nanocapsules were reconstituted by adding 1ml of ultrapure water to the freeze-dried powders followed by manual resuspension and were characterized as explained above.

Statistical analysis

The design of experiments was formulated using the statistical software Statgraphics Centurion XV (Statpoint, Inc, USA). Particle size and zeta potential were statistically analyzed by the analysis of variance (ANOVA) combined with a lineal regression using the same software. Differences were considered to be significant at level of $p < 0.05$.

RESULTS AND DISCUSSION

This article describes for the first time the development of a new type of nanocarriers, named as casein nanocapsules, consisting of an oily core surrounded and stabilized by a shell made of a milk protein, casein. The rationale behind the design of this system was as follows: the oily core is intended to allocate lipophilic active ingredients and the protein shell is expected to have three differentiated roles: (i) to facilitate the interaction

of the nanocapsules with the intestinal epithelium, based on its known mucoadhesive behavior [23]. (ii) To provide an adequate stability in the gastrointestinal medium, and during the storage thanks to its amphiphilic nature and gastro-resistant properties [24] and (iii) to associate and deliver hydrophobic compounds (encapsulated into the oily core) and hydrosoluble macromolecules (associated to the casein shell).

There are four types of casein molecules in mammalian milk, α1-CN, α2-CN, β-CN or κ-CN [25], differenced in their molecular weight, isoelectric point values, protein conformation and hydrophilicity. We selected β-CN with an isoelectric point around 4.83 – 5.07 because it is the most lipophilic one and, thus, it is preferentially adsorbed onto hydrophobic surfaces. i.e. oily droplets [26]. We also selected, κ-CN, with an isoelectric point of 5.45 – 5.7 [27], because it is the one responsible for the great stability of casein micelles in milk [28]. In fact κ-CN molecules form a brush-like coating on the periphery of the nanostructures, which helps improving their stability by electrostatic and steric repulsion forces [29]. This casein has been particularly used as a stabilizing agent for the oral administration of nutraceuticals, i.e. vitamin D [13] or Omega-3 polyunsaturated fatty acids [30].

Development and characterization of blank casein nanocapsules

Based on the use of the two selected caseins, we performed initial experiments intended to define the optimal conditions for the formation of the nanocapsules. Namely, we evaluated the influence of lecithin concentration in organic phase, the oil (Miglyol®) amount in the organic phase and the casein concentration in the aqueous phase, on the morphology, stability and drug loading capacity of the nanocapsules.

Blank and loaded casein nanocapsules were obtained by a modified solvent displacement technique, which has previously been applied by our

group to the formation of other types of polymeric nanocapsules, such as polyaminoacid [6] or polysaccharide nanocapsules [31]. The formation of the nanocapsules is based on the hydrophobic interaction between the lipid core, composed by Miglyol® and lecithin, and the hydrophobic domains that casein molecules exhibit under certain conditions of pH and temperature [24]. These conditions generally are in the pH range of 2.0 – 3.0 and 5.5 – 12.0 and 20 – 40 ºC as has been described by Liu et al. [32] and Aschi et al [33]. On the other hand, it was assumed that the casein hydrophilic regions remain located onto the surface of the nanocapsules forming the casein shell.

The analysis of the physico-chemical properties of the nanocapsules indicated that all the formulations displayed a nanometric size between 150 and 250 nm and a negative zeta potential between -40 and -70 mV, depending on the values of the variables selected (Figure 1-4). Figure 1 and 2 display the surface response showing the simultaneous influence of the variables selected on the size of casein nanocapsules. The first observation was that the size of the nanocapsules with either β-casein or κ-casein was not affected by the concentration of protein (Figure 1B and 2B). Specifically, the results obtained for κ-casein nanocapsules when the protein concentration was modified showed that the particle size was constant in 175 ± 10 nm or 270 ± 10 nm, depending of lecithin concentration, until all the range of protein concentrations evaluated (Figure 2B) independently of the changes in other components. Something similar occurs with the formulation with β-casein (Figure 1B).

In addition, the results indicated that the concentration of either, lecithin or Miglyol®, had a distinct influence on the particle size, depending on the type of casein. Concretely, the results obtained for β-casein nanocapsules (Figure 1A-B) indicate that the particle size increases with the lecithin concentration (statistically significance $p<0.05$), whereas

the Miglyol® volume has only a minor effect for the lowest lecithin concentration. On the other hand, the results obtained for κ-casein nanocapsules indicated that their particle size increases significantly ($p<0.05$) with both Miglyol® and lecithin amounts (Figure 3) but, the effect of Miglyol® was only noted for the highest lecithin concentration.

Despite oil concentration in the formulation, from 9.8 mg/ml to 28.2 mg/ml of formulation, did not show significant influence over the size, it caused a slightly variation in this parameter, this is in agreement with previously studies that described the role of oil amounts in nanocapsule sizes, as in the case of chitosan [34], PLA [35] and PTC/DETA nanocapsules [36]. The low-influence of oil concentration, combined with the significant lecithin influence on the size, lead us to suppose that the size evolution varying the components proportion could be due to a special molecular arrangement in the casein nanocapsules compared with systems previously mentioned, mainly in the formation process of droplets in the system. So, is know that he interfacial tension between oil and water phases has a preponderant role in determining nanocapsule size [35] and also is well-know that due to the surfactant character of the caseins, they are directly related with the interfacial tension in this process. A decrease of o/w interfacial tension is related to a diminution in the size of the capsules thus, if the casein molecules are in this interface we can suppose that this effect on the interface reduce the effect of the oil concentration on the size.

Contrary to other nanocapsules where nanodroplets are previously pre-formed by the emulsification of oil with phospholipids in aqueous phase [37], in this case we propose that the formation of casein nanocapsules was achieved in only one-step with the incorporation of the casein into the phospholipid bilayer. This, due to the dual role of casein, as surfactant and polymer, in the emulsification and the specific well

described arrangement of casein and lecithin into colloidal systems. In this way, is known that, in emulsified systems, casein is linked to lecithin during the emulsification process and hydrophobic regions are allocated inside the bilayer of the phospholipid in the particle [38].

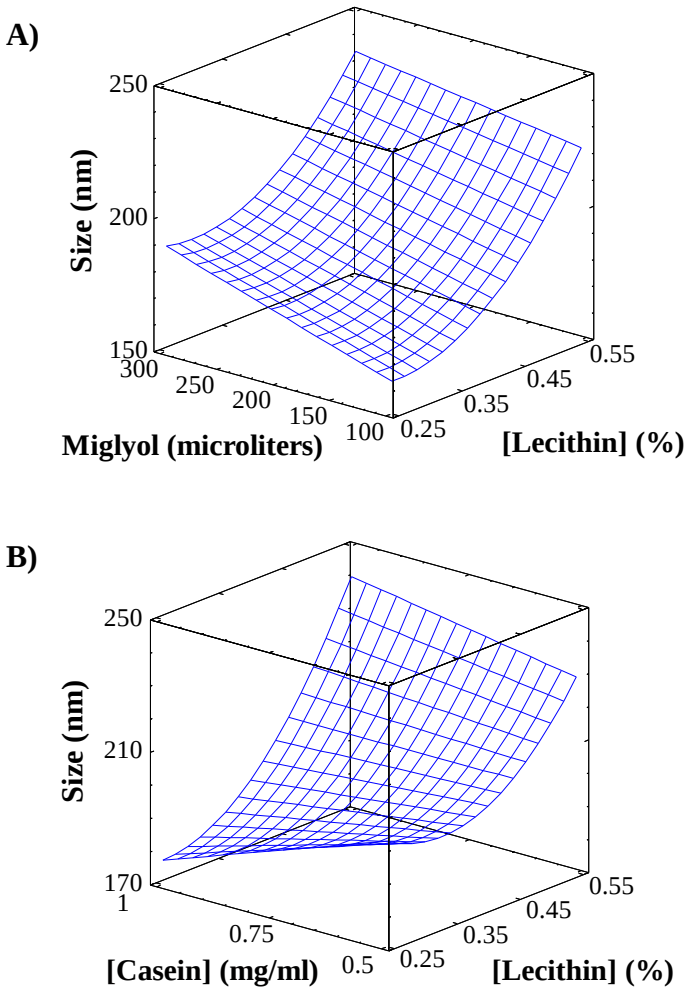

Figure 1.- A) Response surface showing the effect of lecithin concentration (%) and Miglyol® volume (µl) on the particle size of blank (beta) β-CN nanocapsules with the higher casein concentration assayed (1mg/ml). B) Response surface showing the effect of lecithin concentration (%) and casein concentration (mg/ml) on the particle size of blank β-CN nanocapsules with Miglyol volume constant of 150 µl.

Mean Particle Size=225.33-518.04Lecithin+0.355Miglyol - 48.664Casein + 702.85Lecithin^2-0.25 Lecithin x Miglyol+240.8 Lecithin x Casein-0.208 Miglyol x Casein; | R2 = 96.2%

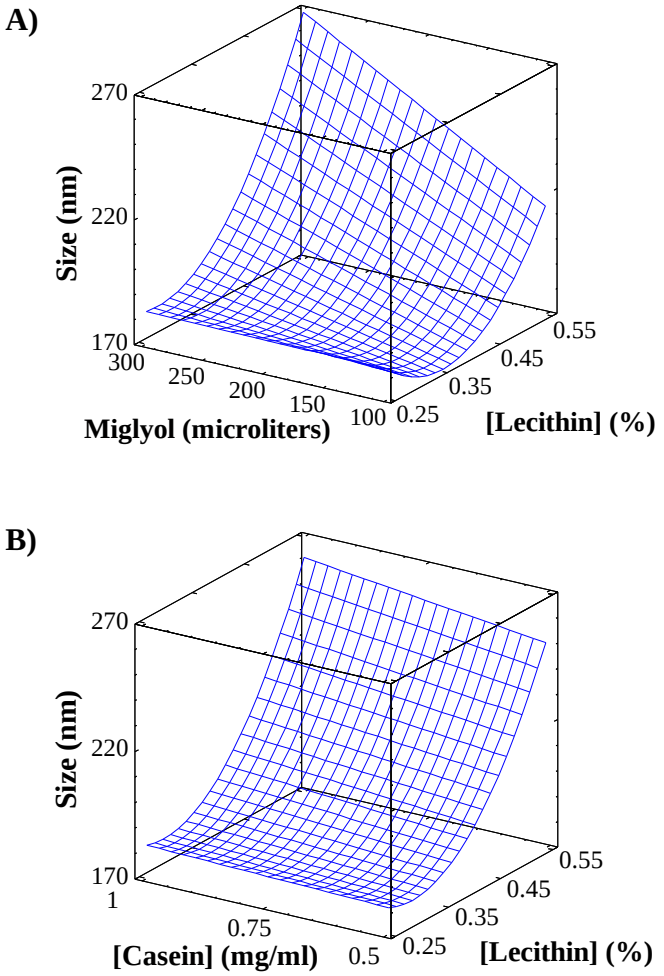

Figure 2.- A) Response surface showing the effect of lecithin concentration (%) and Miglyol® volume (µl) on the particle size of blank (kappa) κ-CN nanocapsules with the higher casein concentration assayed (1mg/ml). B) Response surface showing the effect of lecithin concentration (%) and casein concentration (mg/ml) on the particle size of blank κ-CN nanocapsules with Miglyol volume constant of 150 µl.

Mean Particle Size: 323.346 - 887.243 x Lecithin - 0.220098 x Miglyol + 12.2933 x Casein + 1108.42 x Lecithin^2+ 0.90475 x Lecithin x Miglyol; R2 = 96.2%

In terms of electrical surface charge, the results in figures 3 and 4 show that zeta potential values were significantly affected by the lecithin concentration. Concretely, as expected, the negative zeta potential increased in intensity with the lecithin concentration (statistically significance $p<0.05$) (Figure 3A and 4A). This result could be attributed to the negative charge of lecithin, which is expected to be assembled together with casein in the nanocapsules shell. In contrast, the statistical analysis showed that, neither casein or Miglyol® concentrations cause a statistically significant change in the surface charge of the nanocapsules (Figure 3B and 4B).

Finally, is remarkable the differences in conditional formation parameters among proteins, we suppose that this is related with the differences in conformation and in number of reactive groups along the hydrophilic regions of the caseins, as will be discussed later.

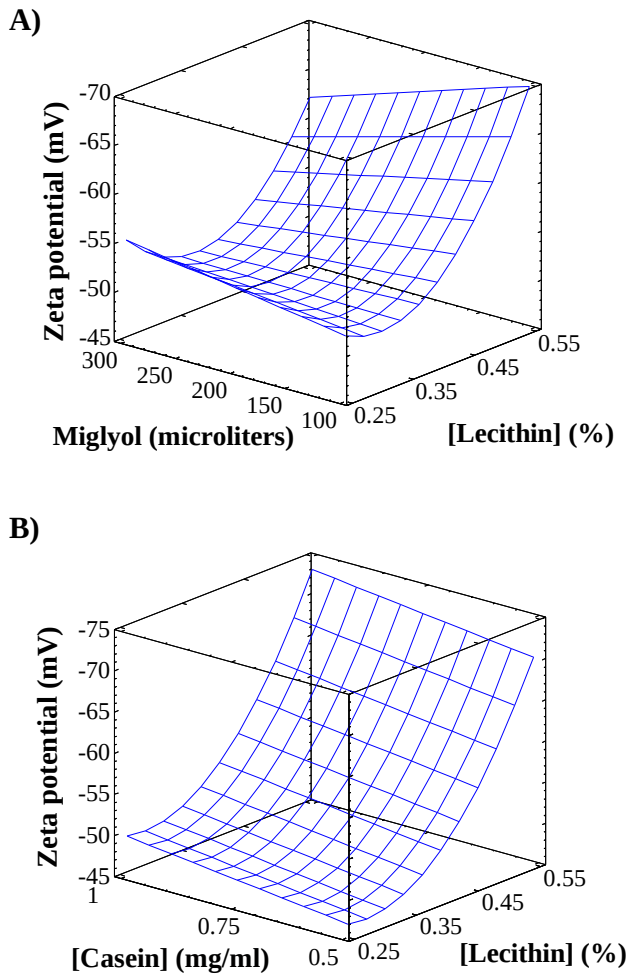

Figure 3. A) Response surface showing the effect of lecithin concentration (%) and Miglyol® volume (µl) on the particle surface charge of blank β-CN nanocapsules with the higher casein concentration assayed (1mg/ml). B) Response surface showing the effect of lecithin concentration (%) and casein concentration (mg/ml) on the particle surface charge of blank β-CN nanocapsules with Miglyol volume constant of 150 µl.

ξ potential = -83.1494 + 40.0 x Lecithin + 0.0312708 x Miglyol + 69.925 x Casein + 0.1815 x Lecithin x Miglyol - 136.6 x Lecithin x Casein - 0.126333 x Miglyol x Casein; n | ξ-potential: R-squared 2 = 95.38 %

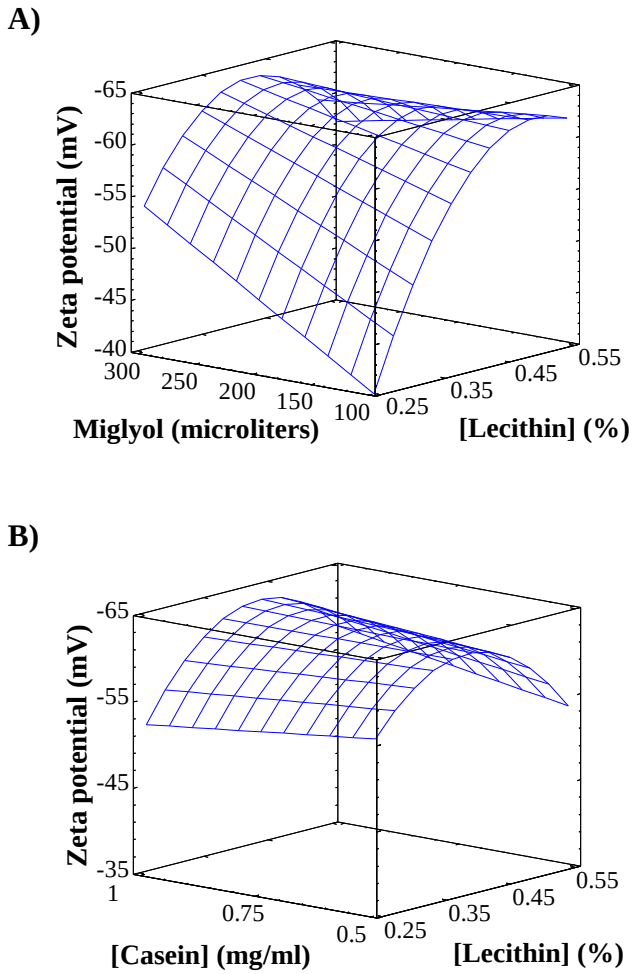

Figure 4. A) Response surface showing the effect of lecithin concentration (%) and Miglyol® volume (µl) on the particle surface charge of blank κ-CN nanocapsules with the higher casein concentration assayed (1mg/ml). B) Response surface showing the effect of lecithin concentration (%) and casein concentration (mg/ml) on the particle surface charge of blank κ-CN nanocapsules with Miglyol volume constant of 150 µl.

ξ potential= 29.8196 - 338.58 x Lecithin - 0.158979 x Miglyol + 9.95833 x Casein + 357.04 x Lecithin^2 + 0.3075 x Lecithin x Miglyol - 50.6 x Lecithin x Casein + 0.0155 x Miglyol x Casein; R2 = 84.4 %

Taking into account these preliminary studies, we selected for further experiments, the conditions that allowed us to obtain the smallest nanocapsules. These conditions were casein concentration: 1 mg/ml; Miglyol® volume: 150 µl and final lecithin concentration in the organic phase: 0.3 % (w/v).

The optimized physicochemical characteristics of β-CN and κ-CN nanocapsules with the composition described below are show in Table 1. Using this experimental approach, we obtained mono-dispersed populations of casein nanocapsules with a mean size in the range of 165-178 nm, this size being slightly affected by the type of casein, around 10 nm. As described above, these nanocarriers exhibit a high negative surface charge due mainly to the influence of lecithin electrical charge, attributed to the pH of the formulation in which the lecithin, due its isoelectrical point around 6.7, is highly negative charged [39]. Both casein molecules could also contribute to this negative charge, as their isoelectric point is around 4.98 for β-CN and 5.5 for κ-CN, and, thus, would be negatively charged at the formulation pH (7.4), but as was described previously this effect is lower and short relevant [40].

Table 1. Physicochemical characteristics of blank and docetaxel-loaded casein nanocapsules.

	Size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)
Control nanoemulsion[1]	198 ± 16	0.1	-30 ± 6	-
β-CN nanocapsules	165 ± 11	0.1	-51 ± 3	-
κ-CN nanocapsules	178 ± 11	0.1	-39 ± 1	-
DCX loaded β-CN nanocapsules	168 ± 7	0.1	-49 ± 6	70 ± 6
DCX loaded κ-CN nanocapsules	181 ± 16	0.1	-38 ± 5	84 ± 3

[1]Containing the same ingredients that nanocapsules but without casein

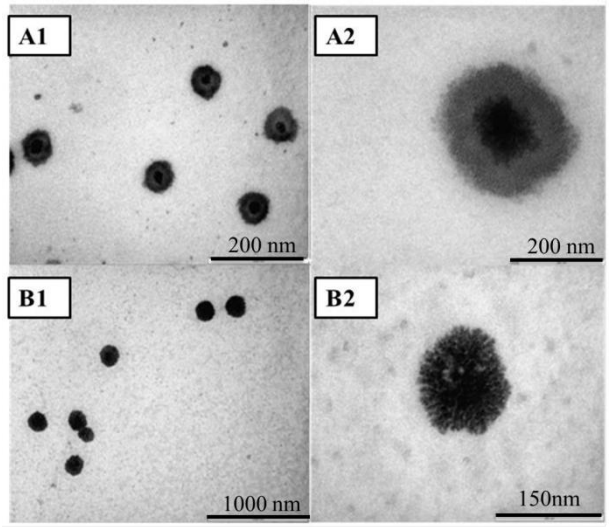

Figure 5. Transmission electron micrographs of: A1-A2) β-Casein nanocapsules, B1-B2) κ-Casein nanocapsules.

The morphological appearance of the nanocapsules was observed by transmission electron microscopy. In the micrograph presented in Figure 5 it can be noted that both types of casein nanocapsules have a round shape

and a size of less than 200 nm, similar to the values obtained by photonic correlation spectroscopy. In addition, the micrograph shows a slight difference in the morphology of the shell in nanocapsules. As illustrated in Figure 6, based on Figure 5, we have speculated that κ-casein nanocapsules would present a "brush-like" shell, according to the topology already described for casein micelles in milk [29] and κ-CN coatings in other type of nanoparticles [16]. In contrast, we hypothesized that the shell of β-casein nanocapsules would adopt the so-called "mushroom" configuration previously described for other type of polymers [41].

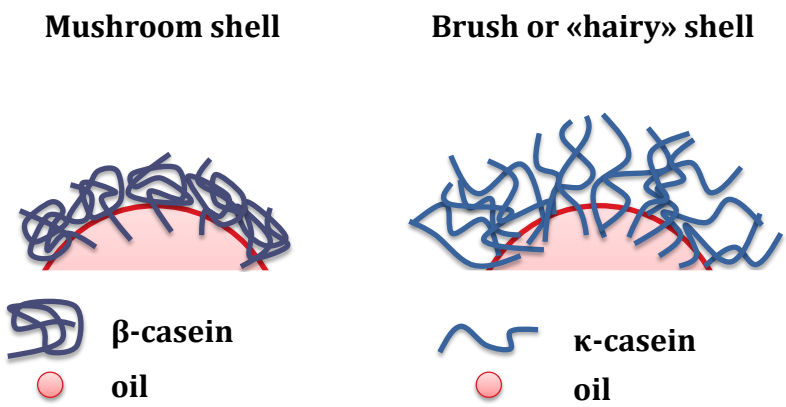

Figure 6.- Schematic illustration of casein configurations on the nanocapsule's surface. On the left, the disposition of β-casein molecules, disulfide bridge between phosphoserine residues leads to a type of "mushroom" configuration. On the right, the small hydrophilic chains of κ-CN lead to a type of "brush" configuration where most of the chains are extended away from the nanocapsule's core.

Despite of conformation of caseins is not currently available we suppose based on molecular modeling predictions [42, 43] that the difference in the coating conformation could be due mainly to the presence and disposition of different amino acid residues along protein

structures (Figure 7). In this way, we suppose that disulfide bridges among phosphoserine residues in the polar region of β-casein, cause a folding of the protein attracting the hydrophilic chains to core. Nevertheless, another type of interactions among hydrophilic regions could be present having the influence over the conformation. Furthermore, the disposition of hydrophobic regions in the β-casein chain also contributed with hydrophobic interactions among them, enhancing the folding of the protein over the surface of nanocapsules.

On the other hand, if we considering the same conditions for κ-casein coatings in micelles and in the new nanocapsules, we can suppose that due to the high-density charge, produced by the disposition of hydrophilic residues κ-casein the hydrophilic region of this protein is disposed over the nanocapsule's surface as extended chains. κ-casein has 63 of its 169 amino acids outside of the nanocapsules, while other 106, which are more hydrophobic are inside the core of the system. In the exterior part, there are 15 charged groups that bring a high density charge in the region exposed to the aqueous medium [28].

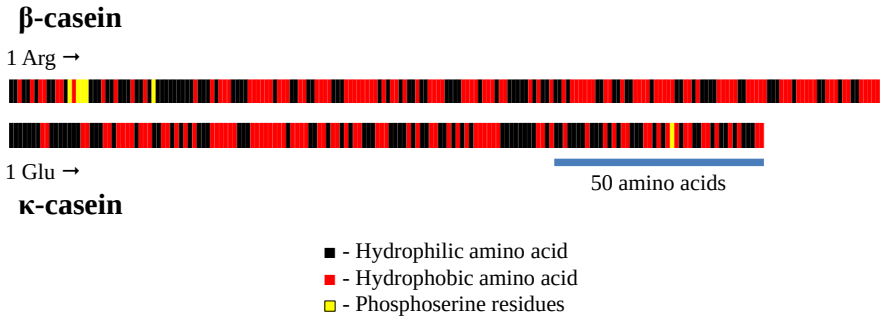

Figure 7.- Schematic representation of the amino acid distribution in both β-casein and κ-casein, considering a primary protein structure. The amino acids are represented according to their hydrophobic/hydrophilic nature. Based in the work published in [42, 43]

Stability studies

To preserve the stability of orally administered nanocarriers in the gastrointestinal track (GIT) is critical for protecting the encapsulated drug and preventing its premature release [44]. Furthermore, the nanocarrier's stability is also important in order to achieve the adequate interaction of the nanocarrier with the absorptive epithelium and, thus, the promotion of the absorption of the associated drug [45]. Taking this into account, we monitored the stability of β-casein and κ-casein nanocapsules in different GIT media over the time by incubation at 37 ºC. Their stability was compared with that of a nanoemulsion, in order to corroborate the influence of the protein shell in the stability of the nanocapsules.

Stability in simulated gastric fluids

The results in Figure 8 indicate that β-casein nanocapsules are stable in the gastric fluids for at least three hrs. A lower stability was noted for κ-casein nanocapsules, although their size increase upon incubation was minor for up to two hrs. This difference in stability could be related with the well described model of acid-gelation of casein micelles [46]. Interestingly, both types of casein led to different degrees of stabilization under simulated gastric conditions, being the nanocapsules formulated with β–CN the most stable system. Lower stability displayed by κ–CN nanocapsules cannot be attribute to the net charge of the protein, as considering the iep of both proteins, κ–CN is the protein with the highest charge in gastric fluids, but to the lower association of this protein to the lipidic core. As has been commented above, NCs formulated with β–CN presented higher accumulation of casein than those prepared with κ–CN due to the hydrophobic interactions between the nanoemulsion and the protein. Based on this, the lower accumulation of κ–CN onto the negative

nanoemulsion will lead to particles with lower positive net charge in the acid pH of the gastric fluid in comparison with β–CN nanocapsules, and then to particles with lower colloidal stability.

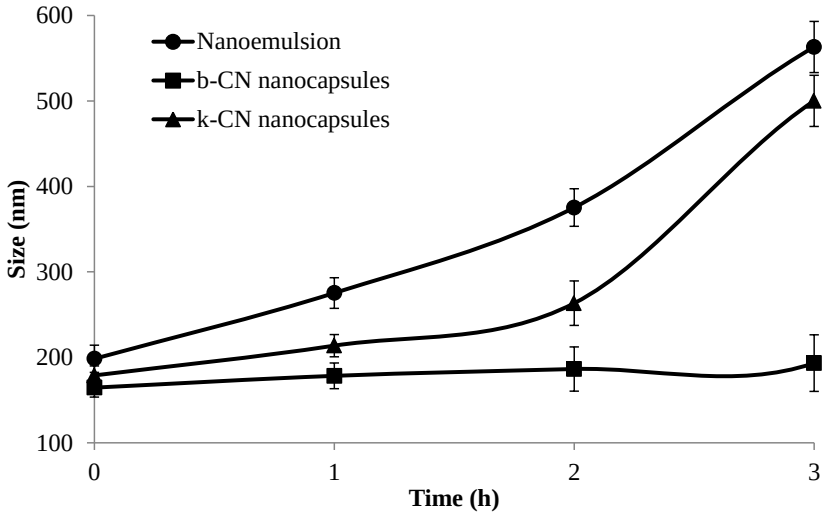

Figure 8.- Evolution of the particle size of casein nanocapsules following incubation at simulated gastric medium without enzymes (pH 1.2 and 37 °C) (mean ± SD; n=3)

Stability in simulated intestinal fluids

The results shown in Figure 9 indicate that the two types of casein nanocapsules are totally stables upon incubation in the intestinal fluids for at least 3 hrs. In contrast, the non-coated emulsions, showed a slight size increase after the first hour. This can be attributed to the different superficial charge displayed by the different systems.. As is shown in Table 1 protein coated systems presented higher superficial charge than the nanoemulsion, which helps to prevent the slight aggregation process observed for the non/coated system.

Overall, the good stability presented by both types of casein nanocapsules in biological simulated fluids make them a good platform as oral drug delivery carriers, assuming that could protect the cargo under this conditions, and control the release of this.

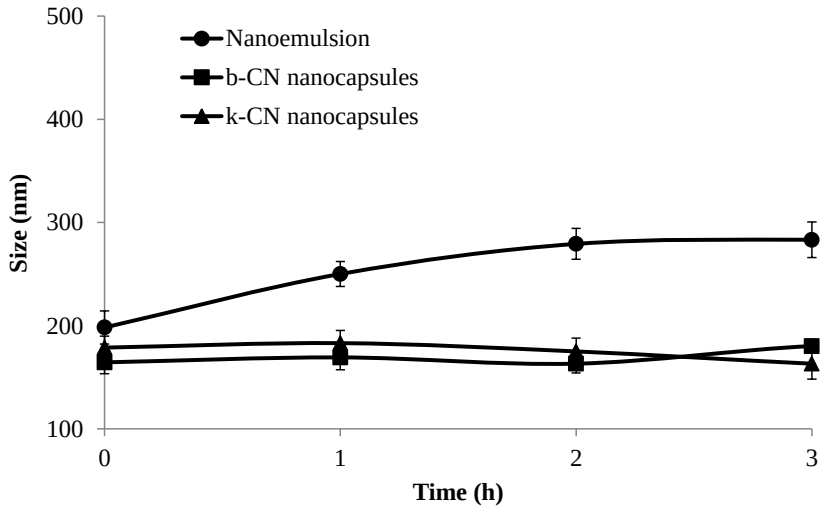

Figure 9.- Particle size of casein nanocapsules following incubation in simulated intestinal medium without enzymes (pH 6.8 and 37 ºC) (mean ± SD; n=3)

Encapsulation and release of docetaxel from casein nanocapsules

One of the reported advantages of nanocapsules is their versatility for the association of different molecules. In this study we have found that DCX could be efficiently encapsulated within the core of either β-CN or κ-CN nanocapsules, with encapsulation efficiencies of 70 and 80 % respectively, without altering the original size and zeta potential values of the nanocapsules.

In an additional experiment, we evaluated the release behavior of the encapsulated DCX upon incubation of DCX-loaded nanocapsules, under "sink conditions", in simulated intestinal medium (pH= 6.8). The results presented in (Figure 10. indicate that DCX is released from the nanocapsules according a biphasic profile, characterized by a rapid initial release phase (around 30% during the first hour), followed by a second phase of slow and continuous drug release (30% in three hours). The initial release phase, typically observed for nanocapsules [9, 21], is attributed to the partition of the poorly entrapped drug molecules from the oily phase to the aqueous medium, whereas the second release phase could be associated to the diffusion of the well-encapsulated molecules through the oily core and subsequently transport across the protein shell.

Overall, the sustained release achieved with these nanocarriers suggests the utility of casein nanocapsules for the oral administration of lipophilic compounds.

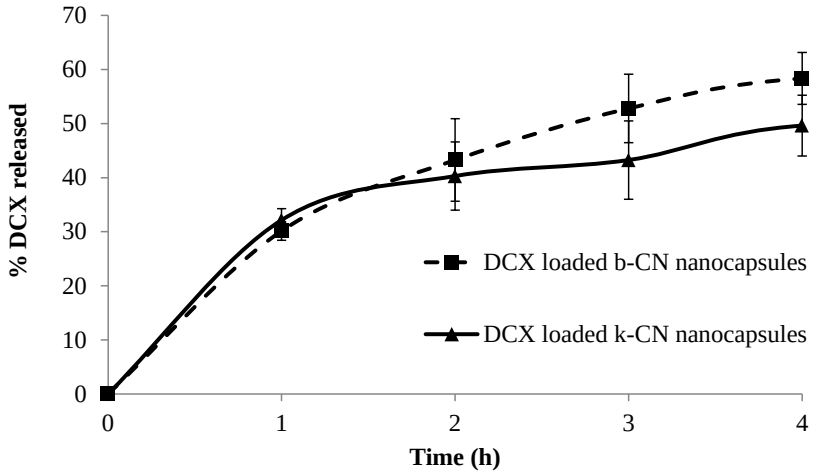

Figure 10.- In vitro release of docetaxel from DCX-loaded β-casein and κ-casein nanocapsules in simulated intestinal medium (pH 6.8) at 37ºC (mean ± S.D.; n=6).

Stability of casein nanocapsules upon long-term storage

Long-term stability during storage is a critical issue in the pharmaceutical development of new nanocarrier formulations. Variations in the temperature are known to significantly compromise the stability of colloidal systems and, therefore, we found it important to investigate the stability of the β-CN and κ-CN nanocapsules in PBS at pH 7.4 and 4º and 37 °C during storage.

The results in Figure 11 indicate that the two prototypes of nanocapsules are stable at 37ºC for at least 6 months. This prolonged stability could be, in part, due to surface repulsion forces, as noted by the high zeta potential values [47], as well as to the steric-stabilizing properties of the protein layer around oil droplets [26].

Surprisingly, despite the good stability profile exhibited by κ-casein nanocapsules at 37ºC, their stability was compromised at 4 ºC. This problem could be related to potential disruption of the hydrophobic interactions at low temperatures [48], a situation that does not apply to β-casein molecules as their gelation and, thus, their stabilizing properties is favored at low temperatures [49] .

In conclusion, this study illustrates the exceptionally good stability profile of casein nanocapsules during storage at high temperatures. Irrespective of these very good results and taking into account the potential instability of possible drug candidates for loading within these nanocapsules, we decided to pursuit a lyophilization study.

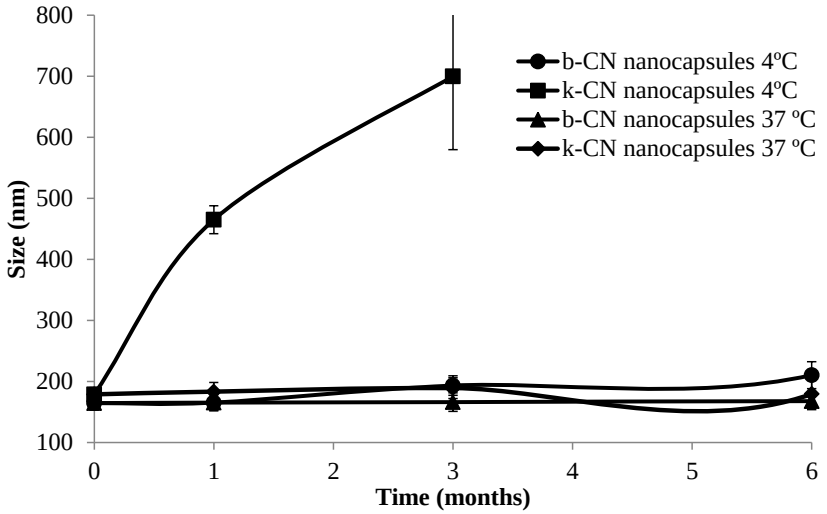

Figure 11.- Particle size evolution of β-casein and κ-casein nanocapsules upon storage for up to 6 months at 4ºC and 37ºC (mean ± SD; n=3).

Lyophilization of casein nanocapsules

Lyophilization process is an interesting alternative to preserve the stability of drug-loaded nanocarriers during storage. This process may become quite complex in the case of nanocapsules due to the fluidity of the polymer shell and to the presence of oil core, which is susceptible of leakage [50]. In this study we have selected trehalose as a cryoprotectant because of its advantages in comparison with other sugars, such as its hygroscopicity and high glass transition temperature [51].

Figure 12 shows the change in particle size of the nanocapsules upon freeze-drying with 10 % of trehalose (w/v) of different concentrations of nanocapsules and further reconstitution in PBS with a pH of 7.4. The results indicate that size of both β-casein and κ-casein nanocapsules remained close to their initial values after the reconstitution for the nanocapsule concentrations of 0.25 and 0.5 % w/v. However, when this concentration increased up to 1 % w/v, a significant increase in the nanocapsule size was noted. The same behavior has already been found for other types of nanocapsules, thus confirming the necessity to properly dilute the nanocapsules in sugar solutions in order to preserve their integrity in a dried form.

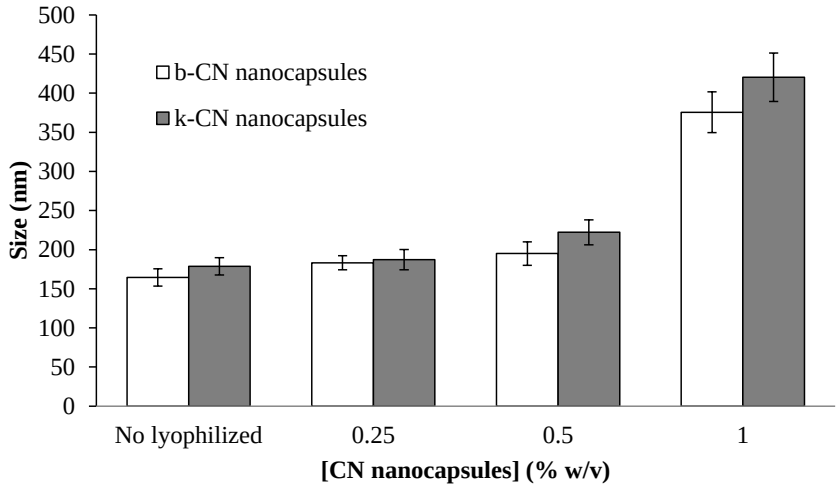

Figure 12.- Mean particle size after the reconstitution of lyophilized β-CN (grey bars) and κ-CN (white bars) nanocapsules with 10% of trehalose (Mean ± SD; n=3).

CONCLUSIONS

This is the first report of a new drug delivery platform, named as casein nanocapsules consisting of an oily core surrounded by a shell made of casein (either β-CN or κ-CN). The nanocapsules are very versatile from the technological point of view as they can be formed under a broad range of formulation conditions. As an example, we have shown that they could accommodate high quantities of hydrophobic drugs and controlled their release. Besides these interesting properties and the acceptable safety profile of this nanocarrier (made of regulatory acceptable materials), these nanocapsules exhibited a good resistance to gastrointestinal conditions, thus being promising carriers for oral drug delivery. Finally, these nanocapsules have shown an exceptionally good stability during long-term storage at high temperatures (37ºC) and can be converted into a dried powder. Consequently, this new delivery nanocarriers fulfills the adequate

pharmaceutical requirements for further development and in vivo proof-of-principle.

ACKNOWLEDGEMENTS:

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DISCUSIÓN GENERAL

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En los últimos años, el desarrollo de sistemas nanométricos para la vehiculización de fármacos antitumorales ha despertado gran interés en el campo farmacéutico [1,2]. Concretamente, las nanocápsulas poliméricas, han sido utilizadas con éxito para la encapsulación de moléculas pequeñas con escasa solubilidad en agua, como los fármacos antitumorales, los cuales poseen en su mayoría estas características [3,4,5]. El núcleo oleoso presente en este tipo de sistemas es de marcado interés en este caso por su elevada capacidad de carga al compararse con otro tipo de nanoestructuras[6].

Nuestro grupo de investigación, ha propuesto la inclusión de docetaxel en nanocápsulas de quitosano [7] y poliarginina [8], confirmando la idoneidad de estos nanosistemas para lograr una encapsulación adecuada del fármaco, y potenciar su vehiculización hacia el interior de células tumorales. Otros

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[8] **Lozano M. V., et al.**, *Polyarginine nanocapsules: a new platform for intracellular drug delivery.* Submitted.

poliaminoácidos como la poliasparagina, [9][10], han permitido prolongar la permanencia plasmática de nanosistemas y ofrecen posibilidades de *targeting* tumoral.

La caseína es un polímero de aceptada seguridad, que ha sido escasamente explorado como formador de nanoestructuras. Su hidrofilia, carga superficial, y capacidad tensoactiva y mucoadhesiva la hacen especialmente adecuada para el desarrollo de nanovehículos orales destinados a mejorar la biodisponibilidad oral de fármacos [11].

En este contexto, en el desarrollo de este trabajo se ha planteado la elaboración de nuevas nanocápsulas poliméricas para la vehiculización de docetaxel. Las formulaciones propuestas comparten la composición del núcleo hidrofóbico básico, variando su composición en la cubierta y vía a la que se dirigen.

Así, la discusión general se ha dividido en dos partes principales para comprender mejor el alcance de cada uno de los sistemas desarrollados:

- Parte I. Nanocápsulas de poliasparagina para la vehiculización selectiva de fármacos antitumorales.
    - Desarrollo, caracterización y evaluación de su eficacia *in vitro* e *in vivo*.
- Parte II. Nanocápsulas de caseína para la vehiculización de fármacos hidrofóbicos por vía oral.

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PARTE I. NANOCÁPSULAS DE POLIASPARAGINA PARA LA VEHICULIZACIÓN SELECTIVA DE FARMACOS ANTITUMORALES.

ESTUDIO PRELIMINAR: FORMACIÓN DE LA NANOEMULSIÓN

De acuerdo con la naturaleza negativo-polar de la poliasparagina [12] , nos hemos planteado un estudio preliminar para la preparación de las nanocápsulas, con el fin de obtener nanoemulsiones con carga positiva y grupos superficiales polares que permitan la interacción de los grupos carboxilo del aminoácido (**Figura 8**).

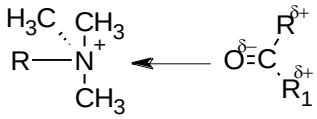

Figura 8. Representación esquemática de la interacción iónico-polar descrita por Roscigno y col [13] entre la poliasparagina y un hipotético tensoactivo catiónico

Para conseguir la carga superficial positiva y los residuos polares necesarios, hemos buscado tensoactivos que contengan una amina terciaria o cuaternaria polar. Se evaluaron cuatro tensoactivos y dos concentraciones para la obtención de las nanoemulsiones catiónicas primarias, con el fin de lograr el menor tamaño de partícula con la menor cantidad de tensoactivo posible. Los compuestos evaluados fueron, el cloruro de benzalconio, el cloruro de cetilpiridinio, el bromuro de

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[13] Ibid.

hexadeciltrimetilamonio (CTAB) y la oleilamina, estudiando un rango de concentraciones entre 0.01 and 0.1 %.

En el proceso de formación, de acuerdo con lo descrito por Roscigno [14], se ha utilizado además, un tensoactivo no iónico, el poloxámero 188, para favorecer la interacción polímero – nanoemulsión catiónica. Las nanoemulsiones catiónicas se obtuvieron mediante una ligera variación de la técnica del desplazamiento de solvente, previamente utilizada por nuestro grupo para la obtención de otros tipos de nanocápsulas [15,16,17]

El proceso consistió en la adición, bajo agitación magnética, de una fase oleosa, conteniendo Miglyol ®, lecitina de soja y el tensoactivo catiónico, sobre una fase acuosa que contiene 0.25% de poloxámero 188. De este modo, se forman pequeñas gotículas nanométricas, estabilizadas en parte por la acción del poloxámero. Debido a su naturaleza, el tensoactivo catiónico queda dispuesto entre la interfaz aceite/agua, con la región hidrofóbica orientada hacia el núcleo oleoso y la amina polar expuesta a la superficie.

La caracterización fisicoquímica de las nanoemulsiones se resume en la **Tabla 6**, en la que se recogen únicamente las formulaciones obtenidas con la concentración mínima de tensoactivo. Se puede observar que en el caso de cualquier tensoactivo evaluado, se obtuvieron nanoemulsiones de

[14] Ibid.

[15] **Calvo, P., et al.**, *Development of positively charged colloidal drug carriers: Chitosan-coated polyester nanocapsules and submicron-emulsions.* Colloid & Polymer Science, 1997. **275**(1): p. 46-53-53.

[16] **Prego, C., et al.**, *Chitosan nanocapsules: a new carrier for nasal peptide delivery.* J. Drug Deliv. Sci. Technol., 2006. **16**(5): p. 331-337.

[17] **Lozano, M.V., et al.**, *Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers.* Biomacromolecules, 2008. **9**(8): p. 2186-2193.

tamaño nanométrico, siendo el cloruro de benzalconio y el CTAB, aquellos que dan lugar a nanoemulsiones de menor tamaño.

Para continuar con el estudio se seleccionaron dos tensoactivos: el CTAB, por dar lugar a nanoemulsiones de menor tamaño medio y también el cloruro de benzalconio, por ofrecer el perfil más confiable de seguridad, al estar aprobado su uso en humanos por vía parenteral por la FDA [18] .

Tabla 6. Propiedades físico-químicas de las nanoemulsiones catiónicas obtenidas con los cuatro tipos de tensoactivos estudiados. (Resultados expresados como media ± D.E.; n ≥ 3)

Tensoactivo	[tensoactivo] (%)	Tamaño (nm)	I.P.	Potencial ζ (mV)
Cloruro benzalconio	0.04	208 ± 3	0.1	33 ± 1
Cloruro cetilpiridinio	0.04	233 ± 10	0.1	34 ± 2
CTAB	0.02	165 ± 6	0.1	41 ± 3
Oleilamina	0.02	244 ± 5	0.2	39 ± 1

I.P.=Indice de polidispersión

CTAB (bromuro de hexadeciltrimetilamonio)

Una vez determinadas las condiciones de formación de la nanoemulsión, se procedió a la preparación de las nanocápsulas. Para ello, se hizo uso de la técnica de incubación o técnica en "dos etapas", aunque se podría recurrir directamente a la técnica en "una etapa", es decir

[18] **FDA**. *Inactive Ingredient Search for Approved Drug Products*. 2012 [cited 2012 24-01-2012]; Available from: http://www.accessdata.fda.gov/.

obteniendo directamente las nanocápsulas sin el procedimiento previo de la formación de la nanoemulsión.

En el proceso de formación, se evaluó la influencia de la concentración de poliasparagina sobre la formación y características de las nanocápsulas. Para ello se incubaron las nanoemulsiones con concentraciones diferentes de polímero, que alcanzaron valores en la dilución final desde 0 a 1 mg/ml, en proporciones de volumen de 4:1, llevándose a cabo el seguimiento del tamaño y potencial zeta. En la Figura 10 se muestra lo que ocurre en el caso de la nanoemulsión de CTAB, representativa de ambos tensoactivos, para los que se observa una evolución muy similar

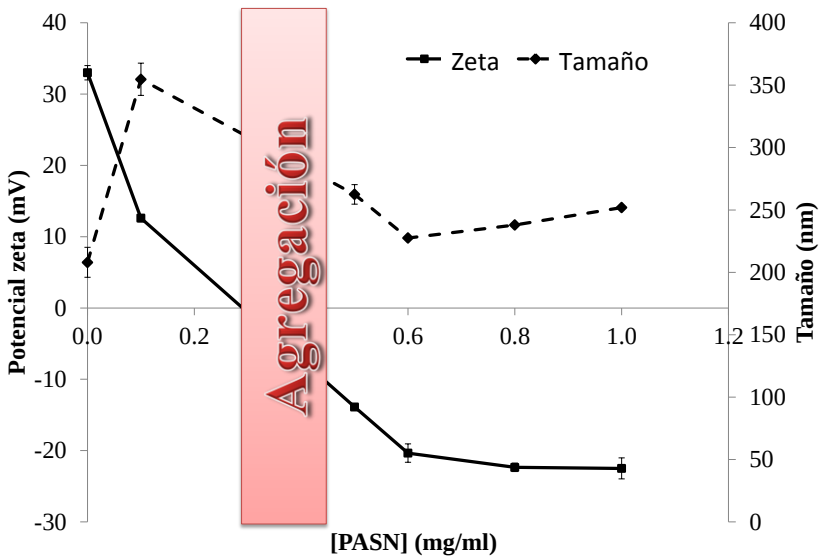

Figura 9.- Evolución del tamaño de partícula y carga superficial de las nanocápsulas de poliasparagina obtenidas por incubación de una nanoemulsión catiónica de CTAB, en función de la concentración de polímero.

La primera evidencia que se aprecia es la existencia de la interacción entre el polímero y la superficie catiónica de las nanoemulsiones, que

produce una inversión de la carga eléctrica superficial. Esta inversión, junto con el aumento en el tamaño de partícula nos indica que el polímero se ha depositado sobre la superficie del sistema, enmascarando las cargas positivas de las aminas expuestas. Esta inversión de potencial ha sido ampliamente descrita para sistemas en los que la cubierta se ha conseguido mediante la interacción electrostática del polímero sobre la superficie [19, 20]. Cuando la densidad de cargas negativa y positiva es idéntica, se produce una agregación del sistema debido a la falta de repulsión electrostática entre las partículas, sucediendo esto alrededor de 0.3 mg/ml, como se aprecia en la representación.

Para concentraciones superiores, vemos que el tamaño va disminuyendo, y la carga superficial va alcanzado valores más negativos, hasta que se estabilizan a partir de 0.6 mg/ml. Esta concentración es la que se utilizó para el desarrollo posterior de las nanocápsulas.

DISEÑO Y CARACTERIZACIÓN DE LAS NANOCÁPSULAS DE POLIASPARAGINA

Las nanocápsulas de poliasparagina se obtuvieron mediante la técnica de desplazamiento de solvente, convenientemente modificada por nuestro grupo de investigación[21, 22, 23]. introduciendo ahora un nuevo cambio,

[19] **Garcia-Fuentes, M., et al.**, *A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery.* Eur J Pharm Sci, 2005. **25**(1): p. 133-143.

[20] **Prego, C., et al.**, *Chitosan nanocapsules: a new carrier for nasal peptide delivery.* J. Drug Deliv. Sci. Technol., 2006. **16**(5): p. 331-337.

[21] **Calvo, P., et al.**, *Development of positively charged colloidal drug carriers: Chitosan-coated polyester nanocapsules and submicron-emulsions.* Colloid & Polymer Science, 1997. **275**(1): p. 46-53-53.

[22] **Prego, C., et al.**, *Chitosan nanocapsules: a new carrier for nasal peptide delivery.* J. Drug Deliv. Sci. Technol., 2006. **16**(5): p. 331-337.

[23] **Lozano, M.V., et al.**, *Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers.* Biomacromolecules, 2008. **9**(8): p. 2186-2193.

consistente en la ya comentada incorporación del tensoactivo catiónico. Para la elaboración de las nanocápsulas hemos elegido la técnica más directa, de preparación en "una etapa". El proceso es idéntico al seguido en la formación de la nanoemulsión: excepto en la incorporación ahora del polímero disuelto en la solución acuosa que contiene poloxámero 188 al 0.25 %. Tras la difusión de los solventes orgánicos en la fase acuosa, se forman pequeñas gotas de tamaño nanométrico a base del aceite y tensoactivos, sobre la que inmediatamente se dispone la poliasparagina, gracias a una interacción iónico-polar entre los grupos carboxilo de esta y las aminas expuestas del tensoactivo catiónico. En la Figura 10 se muestra una representación esquemática sobre el mecanismo de formación propuesto.

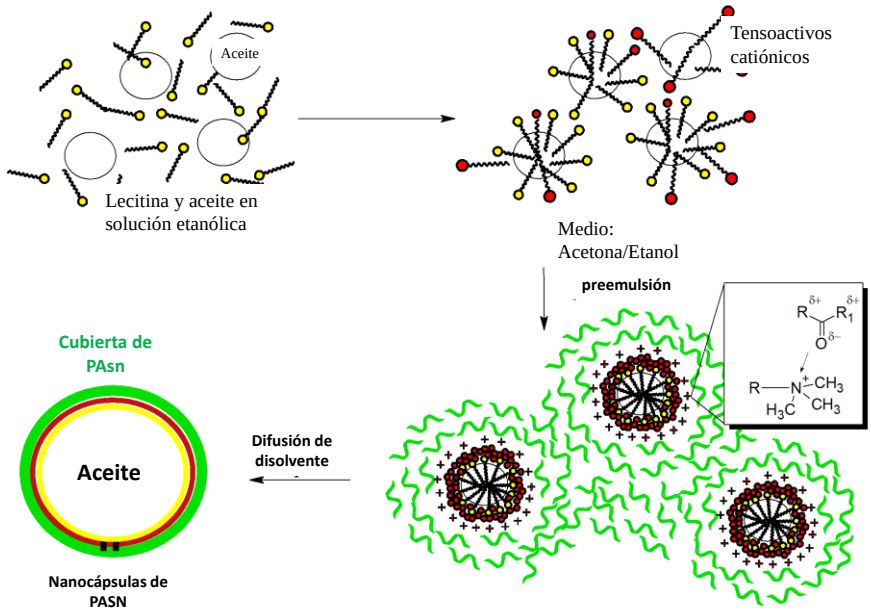

Figura 10.- Representación esquemática del mecanismo de formación de las nanocápsulas de poliasparagina por el método de desplazamiento del solvente en "una etapa".

La encapsulación del docetaxel o del marcador fluorescente DiD en las nanocápsulas, para llevar a cabo los estudios posteriores, se consiguió por medio de la inclusión en la fase orgánica de una alícuota de la solución stock del docetaxel, o DiD en el caso del marcador, en etanol.

La caracterización físico-química de las nanocápsulas de poliasparagina se recoge en la **Tabla 7**, que muestra el tamaño, polidispersión y carga superficial de las nanocápsulas blancas y cargadas con docetaxel o con el marcador fluorescente perclorato de 1,1′-dioctadecil-3,3,3′,3′-tetrametilindodicarbocianina (DiD).

En ella podemos apreciar que los sistemas forman una sola población con un bajo índice de polidispersión, inferior al 0.1, y con un tamaño medio entre 162 y 187 nm, dependiendo del tipo de tensoactivo catiónico y de si contienen alguna molécula activa o marcador, o se trata de nanocápsulas blancas. Como vemos, son mínimas las diferencias atribuibles a uno u otro factor. Además, todos los sistemas presentaron una carga superficial negativa, originada por los residuos de la poliasparagina en la superficie de los sistemas.

Tabla 7.- Características físico-químicas de las nanocápsulas de poliasparagina (PASN) blancas y conteniendo docetaxel o bien el marcador fluorescente DiD, preparadas con los distintos tensoactivos catiónicos (Resultados expresados como media ± D.E.; n ≥ 3)

Formulación	Tamaño (nm)	I.P.	Potencial z (mV)	E.E. (%)
NCs PASN blancas (CTAB)	174 ± 5	0.1	-24 ± 3	-
NCs PASN blancas (CBC)	162 ± 2	0.1	-20 ± 6	-
NCs PASN- docetaxel (CTAB)	187 ± 3	0.1	-32 ± 8	76 ± 3
NCs-docetaxel (CBC)	166 ± 6	0.1	-21 ± 7	73 ± 5
NCs-DiD (CTAB)	185 ± 4	0.1	-34 ± 11	79 ± 6

I.P.=Indice de polidispersión;
CTAB (bromuro de hexadeciltrimetilamonio);
CBC (cloruro de benzalconio)

La morfología de los sistemas se evaluó mediante microscopia electrónica de transmisión. Como se muestra en la **Figura 11**, las nanocápsulas de poliasparagina constituyen una población homogénea de morfología esférica, con un tamaño acorde a lo obtenido previamente.

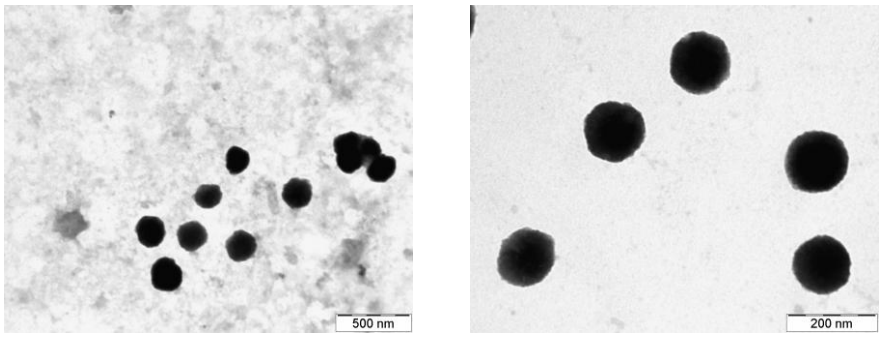

Figura 11.- Imágenes de microscopia electrónica de transmisión de las nanocápsulas de poliasparagina blancas, preparadas con CTAB.

EFICACIA DE ENCAPSULACIÓN Y LIBERACIÓN DE DOCETAXEL A PARTIR DE LAS NANOCÁPSULAS DE POLIASPARAGINA

El docetaxel, fármaco altamente lipofílico, se incorporó eficazmente en el núcleo oleoso de las nanocápsulas, alcanzando valores de eficacia de encapsulación en torno a un 75 % (**Tabla 7**), para una concentración inicial de 10 µg/ml en la formulación. Estos resultados concuerdan con los descritos en trabajos previamente publicados [24, 25], donde la alta eficacia de encapsulación se asocia a la enorme afinidad de esta molécula por componentes oleosos.

La liberación del docetaxel a partir de las nanocápsulas de poliasparagina no se mostró dependiente del tensoactivo utilizado. El perfil obtenido fue de tipo bifásico, acorde con la cinética observada en otros sistemas nanocapsulares [26 , 27] . Se caracterizó por una rápida liberación inicial (55 %) seguida por una segunda etapa de estabilización y liberación más lenta. La liberación rápida al inicio, se relaciona con la dilución de las nanocápsulas en el medio de incubación, llevándose a cabo un reparto del fármaco entre el núcleo oleoso de las nanocápsulas y el medio externo, al estar en condiciones *sink*. La posterior ausencia de liberación en la etapa posterior, confirma la alta afinidad del docetaxel para interaccionar con los componentes del sistema.

En estudios posteriores, la evaluación *in vivo* de las nanocápsulas cargadas con docetaxel hizo necesaria la incorporación de una cantidad

[24] Ibid.

[25] **Khalid, M.N., et al.**, *Long circulating poly(ethylene glycol)-decorated lipid nanocapsules deliver docetaxel to solid tumors.* Pharm. Res., 2006. **23**(4): p. 752-758.

[26] **Lozano M. V., et al.**, *Polyarginine nanocapsules: a new platform for intracellular drug delivery.* Submitted.

[27] **Oyarzun-Ampuero, F., et al.**, *Hyaluronan nanocapsules: a new safe and effective nanocarrier for the intracellular delivery of anticancer drugs.* Submitted.

más elevada de docetaxel, por lo que se incrementó la concentración de fármaco en la solución inicial, obteniendo eficacias de encapsulación del 90 %, y consiguiendo sistemas con 2 mg de docetaxel por ml de formulación.

ESTUDIO DE ESTABILIDAD EN ALMACENAMIENTO DE LAS NANOCÁPSULAS DE POLIASPARAGINA

ESTABILIDAD DE LA SUSPENSIÓN EN ALMACENAMIENTO

La estabilidad es uno de los parámetros críticos en el desarrollo de formulaciones de sistemas coloidales [28]. La carga superficial de los nanosistemas, generalmente desempeña un papel fundamental en este aspecto. Así, comúnmente, partículas fuertemente cargadas experimentan agregación debido a la fuerte repulsión interparticular presentada en el sistema. Por otra parte, partículas cercanas a la neutralidad generalmente experimentan agregación por atracción. Para asegurar una buena estabilidad del coloide, el valor de la carga superficial debería encontrarse alrededor de – 30 y +30 mV [29]. En el presente estudio se ha evaluado la estabilidad de las nanocápsulas desarrolladas, almacenadas en suspensión a 4 y 37 °C.

Los resultados obtenidos indicaron que todas las formulaciones fueron estables por un período de al menos 2 meses, independientemente de las condiciones de temperatura, conservando los sistemas durante este tiempo sus características originales tanto de tamaño como de carga superficial. Esta estabilidad puede ser debida, como se mencionó anteriormente, tanto a la carga superficial presentada, alrededor de -30

[28] **Müller, R.H., et al.**, *Long term stability investigation of solid lipid nanoparticles (SLN).* Eur J Pharm Sci, 1996. **4**(Supplement 1): p. S132-S132.

[29] **Heurtault, B., et al.**, *Physico-chemical stability of colloidal lipid particles.* Biomaterials, 2003. **24**(23): p. 4283-4300.

mV, como a la estabilidad que otorga el tensoactivo no iónico presente en la formulación.

OBTENCIÓN DE UN PRODUCTO LIOFILIZADO

Una de las limitaciones tecnológicas comunes a los sistemas coloidales es la deficiente estabilidad de los mismos durante largos periodos de tiempo. La gelificación, formación de crema, fusión o agregación son algunos de los fenómenos más comunes que causan desestabilización durante el almacenamiento [30].

En este sentido, uno de los métodos más eficaces para preservar la estabilidad de los nanosistemas es someterlos a liofilización. Sin embargo, cuando se aplica a sistemas coloidales de núcleo oleoso, resulta un proceso complejo, debido a la fluidez de la cubierta polimérica y a la presencia del núcleo oleoso, más frágil que las estructuras matriciales [31]. Por ello, el uso de agentes crioprotectores suele ser esencial en el proceso de liofilización de este tipo de sistemas, ya que facilita el proceso y previene el colapso de los nanosistemas.

Así, para conseguir un producto liofilizado se han evaluado tres variables principales, la presencia de un crioprotector, la trehalosa, la concentración del crioprotector, y la concentración de nanocápsulas a liofilizar.

Los resultados obtenidos (Capítulo 2; Fig 10, pág 74), nos llevaron a concluir que la trehalosa es un agente que permite obtener un producto liofilizado adecuado, para concentraciones de nanocápsulas hasta del 1% p/v.

[30] Ibid.

[31] **Choi, M.J., et al.**, *Effect of freeze-drying process conditions on the stability of nanoparticles.* Dry. Technol., 2004. **22**(1-2): p. 335-346.

EFICACIA ANTITUMORAL DE LAS NANOCÁPSULAS DE POLIASPARAGINA CONTENIENDO DOCETAXEL. ESTUDIOS *IN VITRO*

Para evaluar el potencial de las nanocápsulas de poliasparagina cargadas con docetaxel como vehículos para el tratamiento de cáncer, se consideró esencial el estudio de la capacidad antiproliferativa del fármaco encapsulado en contacto con cultivos de células tumorales de cáncer de pulmón de la línea NCI–H460. Las variables incluidas en este estudio fueron el tipo de tensoactivo utilizado en la preparación de los sistemas, CTAB y cloruro de benzalconio, la concentración de docetaxel y el tiempo de contacto entre los sistemas y los cultivos celulares, 2 y 48 horas.

Para el tiempo de contacto de 2 horas, los resultados indicaron que el efecto citotóxico del docetaxel encapsulado fue marcadamente superior al del docetaxel en solución, tanto para las nanocápsulas preparadas con CTAB (Figura 13) como con cloruro de benzalconio (Cap. 2; Fig 6; pág 68). Ello nos lleva a concluir que las nanocápsulas favorecen la penetración celular del fármaco, siendo la citotoxicidad del fármaco encapsulado significativamente superior para todas las concentraciones de fármaco ensayadas. Es resaltable también el hecho de que no se han detectado diferencias atribuibles al tipo de tensoactivo catiónico utilizado en la formación de las nanocápsulas, y que las nanocápsulas blancas no dan lugar a toxicidad alguna.

Los resultados de viabilidad tras un contacto de 48 h, indicaron también un claro aumento del efecto antitumoral del docetaxel, tras la inclusión en los sistemas (Cap. 2; Fig 7, pág 69). Así, los valores de IC50 (concentración que causa una inhibición del crecimiento celular del 50%) del docetaxel encapsulado, tanto para las nanocápsulas de poliasparagina

preparadas con CTAB, como con cloruro de benzalconio (Tabla 8), alcanzaron valores que disminuyeron en un factor de 3 los obtenidos para el docetaxel en solución. De hecho, se pudo constatar la significatividad de este efecto para los dos tiempos de exposición y para los dos tipos de tensoactivos utilizados. Cabe destacar, sin embargo, que en las nanocápsulas preparadas con cloruro de benzalconio se detectó una cierta toxicidad al cabo de 24 h, para las concentraciones más elevadas de nanocápsulas blancas que sin embargo no aparece con el CTAB.

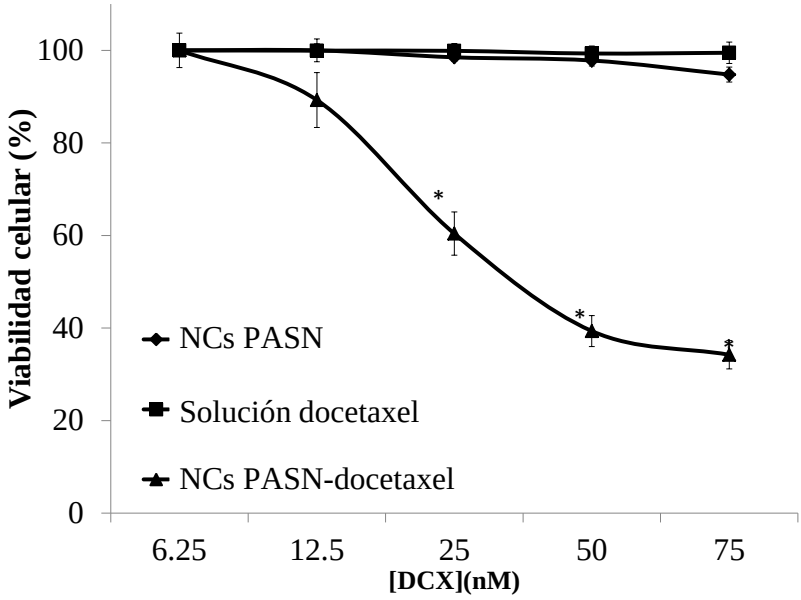

Figura 12.- Efecto de las nanocápsulas de poliasparagina (NCs PASN) blancas , docetaxel en solución y nanocápsulas de poliasparagina conteniendo docetaxel (NCs PASN-docetaxel) sobre la viabilidad de células NCI-H460 tras dos horas de exposición (tensoactivo utilizado: CTAB)

Diferencias estadísticamente entre Nanocápsulas de poliasparagina conteniendo docetaxel vs docetaxel en solución ($p<0.005$)

Tabla 8.- Valores de IC_{50} (nM; n = 4), obtenidos tras el contacto de las formulaciones indicadas con células NCI-H460, para distintos períodos de incubación.

Formulación	IC50 (nM)	
	2 h Exposición	48 h Exposición
Docetaxel	105 ± 9	36.4 ± 4
NCs PASN-docetaxel (CTAB)	30.9 ± 6.4*	10.51 ± 1.3*
NCs PASN-docetaxel (CBC)	26.2 ± 9.3*	13.25 ± 0.6*

* $P<0.01$ con respecto a la IC_{50} de docetaxel en solución (ANOVA, Tukey's test).

CTAB (bromuro de hexadeciltrimetilamonio);
CBC (cloruro de benzalconio)

La significativa mejora de la eficacia antitumoral del docetaxel encapsulado en nanocápsulas de poliasparagina, es concordante con lo previamente publicado para otros sistemas de este tipo en los que se han encapsulado taxanos, como es el caso de las nanocápsulas recubiertas con PEG [32], quitosano [33], poliarginina[34] o ácido hialurónico [35].

Parece fundamental, para explicar este efecto, la estructura nanocapsular, que favorece la internalización de los sistemas y la liberación intracelular del docetaxel. En segundo lugar, la hidrofilía de la

[32] **Garcion, E., et al.**, *A new generation of anticancer, drug-loaded, colloidal vectors reverses multidrug resistance in glioma and reduces tumor progression in rats.* Molecular Cancer Therapeutics, 2006. **5**(7): p. 1710-1722.
[33] **Lozano M.V., et al** (2008)
[34] **Lozano M.V., et al,** submitted.
[35] **Oyarzun-Ampuero, F., et al.**, *Hyaluronan nanocapsules: a new safe and effective nanocarrier for the intracellular delivery of anticancer drugs.* Submitted.

cubierta de estos sistemas, permitiría de manera análoga al PEG, evitar mecanismos de resistencia celular [36].

Teniendo en cuenta los resultados de citotoxicidad obtenidos, se planteó un estudio para profundizar sobre la interacción de las nanocápsulas con la línea celular en estudio. El objetivo consistió en determinar la existencia de la internalización de los nanosistemas. Para ello, en primer lugar, se prepararon nanocápsulas de poliasparagina conteniendo la sonda fluorescente DiD, El marcador se incorporó a la fase orgánica de cómo solución etanólica. La adición de esta molécula ocasionó una ligera variación tanto en el tamaño como en la carga superficial de las nanocápsulas, sin llegar a ser esta significativa (**Tabla 7**).

Los estudios de internalización celular, realizados mediante citometría de flujo, demostraron que la fluorescencia aparecía asociada a la totalidad de las células NCI-H460 tras una corta incubación con las nanocápsulas de 2 h, Esto claramente se debe relacionar con la entrada masiva de los nanosistemas en las células. De hecho, los resultados fueron muy diferentes tras el contacto de las células con la nanoemulsión fluorescente, o únicamente con el marcador, que indicaron que la fluorescencia no permanecía en las células. Todo ello ratifica por lo tanto, la hipótesis anteriormente expuesta sobre la facilidad de internalización de los nanosistemas.

[36] **Romberg, B., et al.**, *Pharmacokinetics of poly(hydroxyethyl-l-asparagine)-coated liposomes is superior over that of PEG-coated liposomes at low lipid dose and upon repeated administration.* Biochimica et Biophysica Acta - Biomembranes, 2007. **1768**(3): p. 737-743.

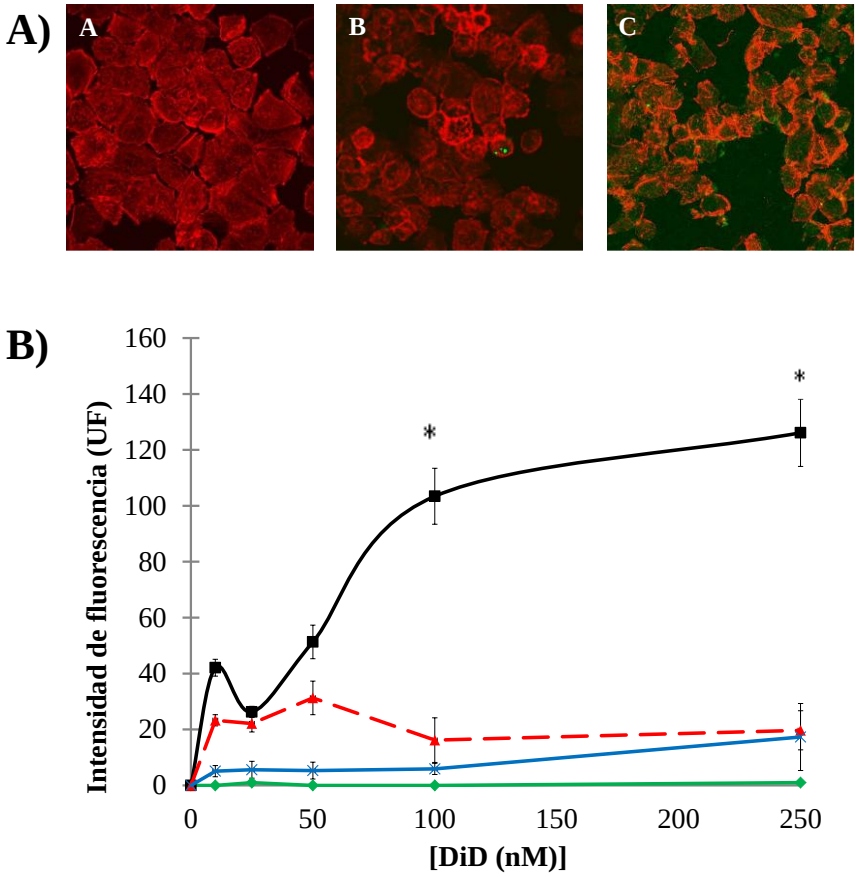

Figura 13. Estudios de captura celular de las nanocápsulas de poliasparagina preparadas con CTAB. **A)** imagen de microscopia de fluorescencia de las células NCI-H460 incubadas con las nanocápsulas de poliasparagina fluorescentes: a células teñidas con el marcador Bodipy® sin tratamiento; b. Células teñidas con Bodipy® incubadas con una dispersión de DiD y c. Células teñidas con Bodipy® incubadas con las nanocápsulas fluorescentes. **B)** Intensidad de fluorescencia de las células incubadas con diferentes tratamientos. Verde: Nanocápsulas blancas, Negro: Nanocápsulas conteniendo DiD; Rojo: Nanoemulsión conteniendo DiD; Azul: Dispersión de DiD.

ESTUDIOS *IN VIVO:* BIODISTRIBUCIÓN Y EFICACIA ANTITUMORAL DE LAS NANOCÁPSULAS DE POLIASPARAGINA CONTENIENDO DOCETAXEL

Se llevaron a cabo dos estudios *in vivo* con las nanocápsulas de poliasparagina, utilizando el ratón como modelo animal. En primer lugar, un estudio de farmacocinética y biodistribución de las nanocápsulas fluorescentes en animales sanos tras su administración I.V. y, en segundo, actividad antitumoral de las nanocápsulas conteniendo docetaxel en el tratamiento de un modelo de glioma U87MG, siguiendo también administración I.V.

ESTUDIO DE HEMÓLISIS DE LAS NANOCÁPSULAS DE POLIASPARAGINA

Como estudio previo a la administración I.V., las nanocápsulas se sometieron a un estudio de hemolisis. Se considera la hemolisis como una respuesta al daño de la membrana celular ocasionado por los sistemas [37]. Para ello, se incubaron diferences concentraciones de nanocápsulas de poliaspagina, preparadas con CTAB con una suspensión de eritrocitos durante 15 minutos bajo agitación. Posteriormente, se determinó la cantidad de hemoglobina libre en dicha suspensión, considerándola como un producto de la lisis celular.

Los resultados obtenidos, indicaron que las nanocápsulas de poliasparagina mostraban para la concentración más alta ensayada, apenas un 7% de hemolisis, y para concentraciones inferiores valores no cuantificables. Las nanocásulas podían por lo tanto ser administradas por

[37] **Lu, J., et al.**, *Stability of Self-Assembled Polymeric Micelles in Serum.* Macromolecules, 2011. **44**(15): p. 6002-6008.

vía I.V. para ser sometidas a evaluación farmacocinética y de eficacia antitumoral.

ESTUDIOS DE CINÉTICA Y BIODISTRIBUCIÓN

Para estos estudios, se prepararon nanocápsulas de poliasparagina cargadas con la sonda fluorescente DiD de la misma manera en que se describió para los estudios de captura celular. Para estos estudios se ha utilizado la formulación conteniendo cloruro de benzalconio, debido a que al ser la sonda fluorescente de naturaleza anfifilica, comprometía fuertemente la estabilidad de las nanocápsulas cuando el tensoactivo era el CTAB. En estos estudios se utilizó como control una nanoemulsión aniónica, preparada de la misma forma que las nanoemulsiones catiónicas previamente descritas, pero sin la presencia del tensoactivo catiónico.

Las características físico-químicas de las nanocápsulas y de la nanoemulsión aniónica conteniendo DiD se muestran en la Tabla 9. Se aprecian valores similares a los presentados previamente para las nanocápsulas blancas o conteniendo el fármaco docetaxel. En este caso, se consiguió incrementar el contenido en docetaxel de la nanocápsulas hasta valores de 2 mg/ml, lo cual se hizo necesario para poder administrar la dosis in vivo de 2 mg/Kg. En esta formulación se incrementó la eficacia de encapsulación hasta valores del 90%, probablemente debido a la saturación de la fase acuosa con docetaxel, evitando una mayor difusión del fármaco hacia el exterior de las nanocápsulas.

Tabla 9.- Características físico-químicas de la nanoemulsión aniónica conteniendo DiD, y de nanocápsulas de poliasparagina (NCs PASN) blancas y conteniendo DiD o docetaxel (Resultados expresados como media ± D.E.; n ≥ 3)

Formulación	Tamaño (nm)	IP	Potencial ζ (mV)	E.E (%)
NCs PASN blancas (CTAB)	187 ± 7	0.1	-38 ± 3	-
Nanoemulsión -Did	192 ± 3	0.1	-20 ± 7	60 ± 5
NCs PASN-DiD (BKC)	163 ± 3	0.1	-29 ± 6	74 ± 4
NCs PASN- docetaxel (CTAB)	176 ± 3	0.1	-43 ± 6	90 ± 3

I.P.=Índice de polidispersión

Para la evaluación de la cinética en plasma de las nanocápsulas de poliasparagina cargadas con DiD, se inyectó una dosis de 1 mg/kg de peso de ratón por vía intravenosa en la cola de ratones Swiss, midiendo la intensidad de fluorescencia de las muestras de sangre extraídas a distintos tiempos.

La **Figura 14** muestra la cinética de eliminación en plasma de la sonda fluorescente DiD tras su administración I.V., que corresponde a un descenso biexponencial. En el caso de la nanoemulsión control, se observa una rápida disminución en la fluorescencia en plasma, alcanzando un valor del 15% del total de la fluorescencia en apenas 30 minutos. Por el contrario, tras la administración de las nanocápsulas, la fluorescencia asociada al DiD encapsulado permanece en un 40 % de la fluorescencia inicial, al cabo de 3 horas post-administración, alcanzando un 10% tras 24 h.

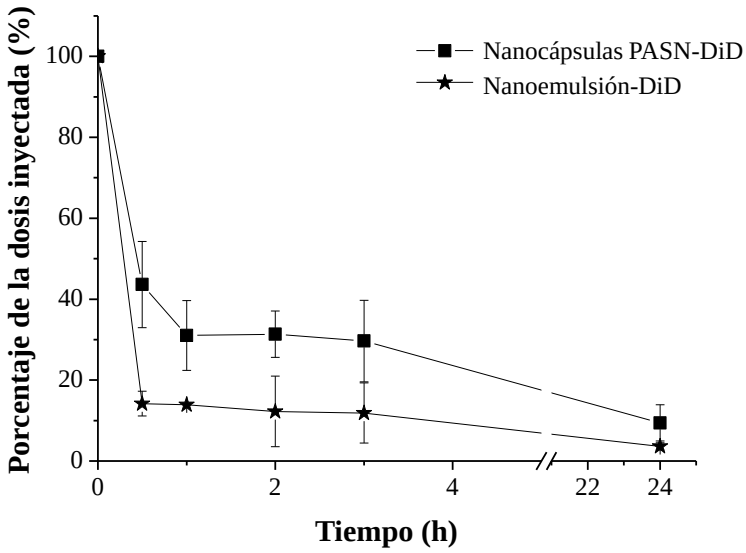

Figura 14.- Niveles plasmáticos de DiD, expresados como porcentaje de fluorescencia inicial, obtenidos tras la administración I.V. en ratones de las nanocápsulas de poliasparagina (PASN-DiD) y la nanoemulsión aniónica control conteniendo el marcador (media ± D.E.; n=3).

En la **Tabla 10**, se muestran los parámetros farmacocinéticos obtenidos tras el ajuste bicompartimental de los datos plasmáticos. Las nanocápsulas de poliasparagina logran un tiempo de vida media de eliminación que se incrementa de 8.17 h en la nanoemulsión a 12.64 h.

Por otro lado, los valores del tiempo medio de residencia (MRT) y del área bajo la curva (AUC) fueron apreciablemente superiores para las nanocápsulas que para la nanoemulsión control. Concretamente, los valores del MRT prácticamente se duplican, pasando de 6.59 h en el control a 12.75 h para las nanocápsulas. El AUC, por otra parte, experimentó un incremento del 138 % con respecto a la nanoemulsión aniónica. Estos parámetros confirman las propiedades de larga

circulación plasmática que parecen mostrar las nanocápsulas gracias a la cubierta hidrofílica de poliasparagina.

Tabla 10.- Parámetros farmacocinéticos obtenidos a partir de la cuantificación de la fluorescencia residual tras la inyección I.V. en ratones de las nanocápsulas de poliasparagina conteniendo DiD (NCs PASN-DiD) (Resultados expresados como Media ± D.E.; n ≥ 3)

Formulación	$t_{1/2}$ distribución [0-1h] (h)	$t_{1/2}$ eliminación [1-24h](h)	MRT [0-24h] (h)	AUC [0- 24h] (mg/ml*h)
NCs PASN-DiD	0.58 ± 0.1	12.64 ± 0.2	12.75±0.4	34.83 ± 2.3
Nanoemulsión-DiD	0.34 ± 0.3	8.17 ± 0.3	6.59±0.1	11.43 ± 2.1

Los resultados anteriores concuerdan con los descritos para otros nanosistemas con cubiertas de poliaminoácidos[38]. Así, la farmacocinética de liposomas recubiertos con un derivado de la poliasparagina se comparó con liposomas recubiertos con PEG, demostrándose que la cubierta de poliasparagina mejoraba los parámetros farmacocinéticos con respecto al PEG. Otros polímeros a base de poliaminoácidos han sido utilizados con el mismo fin, dando lugar a resultados similares [39].

Este aumento en la permanencia en plasma originado por la cubierta de poliasparagina, en comparación a la nanoemulsión sin recubrir, se

[38] **Romberg, B., et al.**, *Pharmacokinetics of poly(hydroxyethyl-l-asparagine)-coated liposomes is superior over that of PEG-coated liposomes at low lipid dose and upon repeated administration.* Biochimica et Biophysica Acta - Biomembranes, 2007. **1768**(3): p. 737-743.

[39] **Metselaar, J.M., et al.**, *A Novel Family of L-Amino Acid-Based Biodegradable Polymer-Lipid Conjugates for the Development of Long-Circulating Liposomes with Effective Drug-Targeting Capacity.* Bioconjugate Chemistry, 2003. **14**(6): p. 1156-1164.

justifica teniendo en cuenta la hidrofilía que la poliasparagina aporta a la superficie de las nanocápsulas, evitando la opsonización de los sistemas y su captura por parte del MPS [40], de un modo análogo al PEG.

En un estudio complementario, se ha evaluado la biodistribución de las nanocápsulas tras administración I.V. por medio de métodos de imagen, en particular utilizando fluorescencia en el infrarrojo cercano (NIR). Para ello, tras la inyección I.V. de las nanocápsulas fluorescentes se ha procedido a su seguimiento en tiempo real por medio de una cámara NIR, fotografiando al ratón anestesiado, para la posterior cuantificación de la fluorescencia.

Las imágenes obtenidas a las horas 1 y 24 post administración (Figura 15) muestran que de acuerdo a los resultados obtenidos en el estudio farmacocinético, muy posiblemente la nanoemulsión fue rápidamente eliminada de la circulación, además de localizarse rápidamente tanto en hígado como en riñón. En contraste, las nanocápsulas de poliasparagina consiguieron permanecer en circulación al menos 24 horas, sin observarse ninguna acumulación indeseada en órganos vitales. Esta larga permanencia en plasma es acorde a trabajos en los que previamente se ha descrito las características de larga permanencia que brinda la cubierta de poliasparagina [41, 42].

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[40] **Owens Iii, D.E. and Peppas, N.A.**, *Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles.* International Journal of Pharmaceutics, 2006. **307**(1): p. 93-102.

[41] **Romberg, B., et al.**, *Effect of liposome characteristics and dose on the pharmacokinetics of liposomes coated with poly(amino acid)s.* Pharm. Res., 2007. **24**(12): p. 2394-2401.

[42] **Romberg, B., et al.**, *Pharmacokinetics of poly(hydroxyethyl-l-asparagine)-coated liposomes is superior over that of PEG-coated liposomes at low lipid dose and upon repeated administration.* Biochimica et Biophysica Acta - Biomembranes, 2007. **1768**(3): p. 737-743.

Estas propiedades de larga circulación, abren la posibilidad a que las nanocápsulas de poliasparagina puedan alcanzar el tejido diana tumoral, y gracias a la ya descrita necesidad de asparagina por parte de este tipo de células, ésta cubierta puede otorgar propiedades de *targeting* a los sistemas.

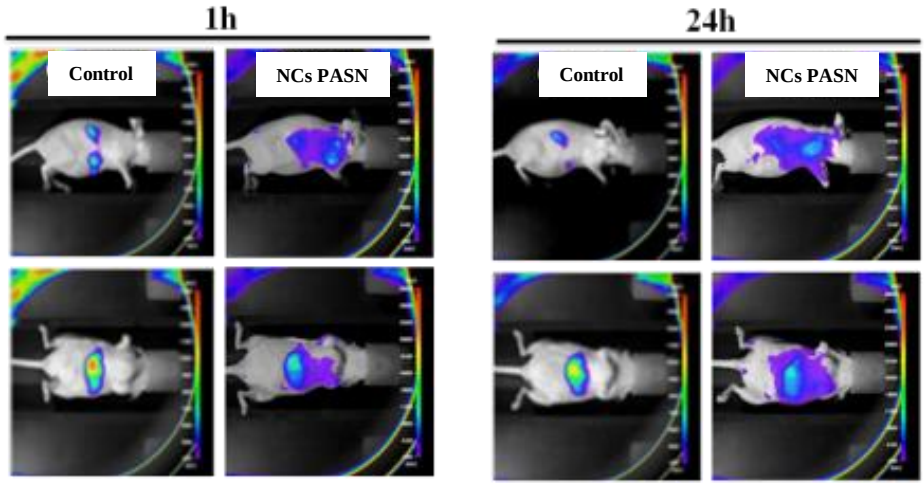

Figura 15.- Imagen de la fluorescencia *in vivo* de ratones atímicos inyectados con formulaciones fluorescentes. Control – nanoemulsión aniónica conteniendo DiD; NCs PASN-DiD– nanocápsulas de poliasparagina cargadas con DiD.

ESTUDIOS DE EFICACIA ANTITUMORAL DE LAS NANOCÁPSULAS DE POLIASPARAGINA CONTENIENDO DOCETAXEL

Finalmente, se ha evaluado la eficacia antitumoral *in vivo* del docetaxel incluido en las nanocápsulas de poliasparagina. El modelo tumoral utilizado se obtuvo tras la inyección subcutánea de células de glioma U87MG en ratones atímicos desnudos.

La formulación comercial, Taxotere ® se utilizó como control positivo, mientras que el control negativo fue suero salino fisiológico. La dosis I.V. de docetaxel por tratamiento fue de 2 mg de docetaxel por kg de peso de ratón, en 200 µl.

Los valores del volumen tumoral relativo (Figura 16) muestran, que tras una sola administración I.V., la eficacia del docetaxel, tanto incorporado en las nanocápsulas de poliasparagina, como en la formulación comercial, inhibe el crecimiento tumoral de manera significativa, en comparación al grupo control. ($p < 0.01$; días 18 y 21 post-tratamiento).

Concretamente, se ha observado una diferencia de tamaño de casi 60 % del volumen tumoral entre las formulaciones con docetaxel y los animales control. A pesar de que ambos tratamientos conducen a reducciones de tamaño similares, el volumen del tumor tratado con las nanocápsulas parece llegar a un punto de estancamiento, sin presentar cambios significativos en el volumen entre el día 18 y 21. Al contrario, en el caso del Taxotere® la curva de la evolución tumoral parece indicar que el tumor sigue creciendo de una manera exponencial. Ello nos induce a pensar en una liberación sostenida del docetaxel una vez que las nanocápsulas alcanzan el área tumoral.

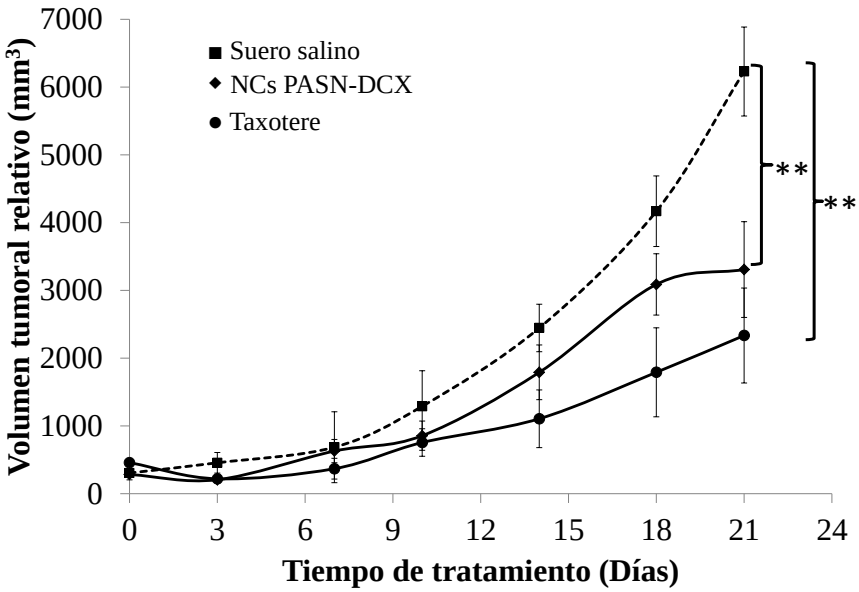

Figura 16.- Efecto antitumoral de las nanocápsulas de poliasparagina conteniendo docetaxel y del Taxotere tras una única administración endovenosatras en comparación a grupo control que recibió suero salino, en un modelo de glioma U87MG en ratones (Resultados expresados como media ± S.E.; n =3) ** diferencia significativa (p<0.05) del tratamiento respecto al suero salino.

Los perfiles de crecimiento tumoral obtenidos con los tratamientos utilizados, parecen acordes a aquellos estudios en los que se evalúa la eficacia antitumoral de taxanos por medio de nanosistemas. Así, nanocápsulas pegiladas han demostrado vehiculizar eficientemente el docetaxel además de mejorar sus parámetros farmacocinéticos y aumentar los tiempos de vida media, por lo tanto mejorando la actividad antitumoral del fármaco [43]. Otros nanosistemas de naturaleza oleosa han demostrado

[43] **Khalid, M.N., et al.**, *Long circulating poly(ethylene glycol)-decorated lipid nanocapsules deliver docetaxel to solid tumors.* Pharm. Res., 2006. **23**(4): p. 752-758.

también mejorar considerablemente la concentración alcanzada en el tumor del docetaxel [44], mecanismo que puede explicar la mejora proporcionada por nuestros sistemas.

En el mismo estudio, se evaluó también la supervivencia de los animales tratados, para obtener conclusiones sobre su toxicidad asociada a los tratamientos. Para el estudio de estos valores, se procedió a la determinación del "índice de incremento de la supervivencia" (IST) a partir de un estudio de Kaplan-Meier.

La evolución de la supervivencia entre grupos se representa en el siguiente grafico de Kaplan-Meier (**Figura 17**). Los valores del IST (**Tabla 11**) indicaron que el docetaxel incluido en las nanocápsulas de poliasparagina daba lugar a un aumento considerable de los tiempos de supervivencia, si se comparaban con los de la formulación comercial. Así, mientras el incremento del tiempo de supervivencia tras el tratamiento con las nanocápsulas fue del 67%, en comparación al control, el Taxotere® sólo consiguió un aumento del 50%.

[44] **Liu, D., et al.**, *Nanostructured lipid carriers as novel carrier for parenteral delivery of docetaxel.* Colloids and Surfaces B: Biointerfaces, 2011. **85**(2): p. 262-269.

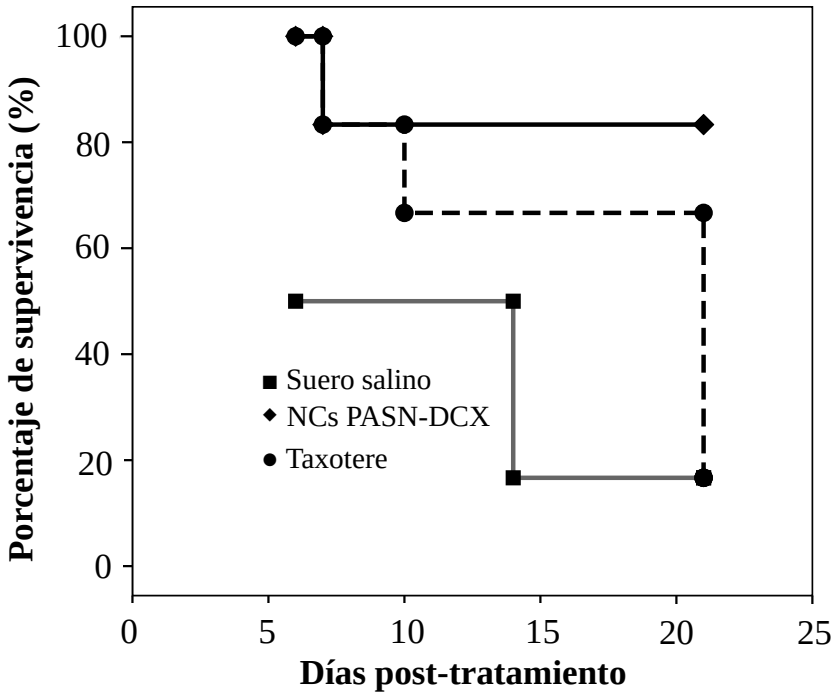

Figura 17.- Curvas de supervivencia de Kaplan-Meier de los ratones portadores del modelo de glioma U87MG tumor tras la administración I.V. de los siguientes tratamientos: (♦) nanocápsulas de poliasparagina conteniendo docetaxel (NCS PASN-DCX) (●)Taxotere® y (■) suero salino

Tabla 11.- Valores de tiempo de supervivencia de ratones portadores del modelo de glioma U87MG tras la administración I.V de docetaxel, en nanocápsulas de poliasparagina o en su formulación comercial Taxotere®. Se comparan con los valores obtenidos para el grupo control, suero salino.

Tratamiento	**Tiempo de supervivencia (días)**			**Incremento del tiempo de supervivencia (%)**		
	Rango	Mediana	Media SD	IST $_{median}$	IST$_{mean}$	*p*- vs serum
NCs PASN-DCX	14-21	0	18.7± 2	0	66.96	0.036
Taxotere ®	11-21	21	16.8± 3	50	50	0.056
Control (Suero)	6-14	14	11.2± 2	-	-	

% IST porcentaje en el tiempo de supervivencia en función del tiempo obtenido con el control.

Considerando conjuntamente los resultados del crecimiento tumoral y supervivencia, con los de cinética plasmática e imagen, se podría apuntar como hipótesis que es una biodistribución más adecuada de los nanosistemas lo que conduce a una mayor acumulación de las nanocápsulas en el área tumoral y un menor acceso a otros tejidos, en comparación a la formulación comercial. Esta capacidad de *targeting* tumoral estaría asociada con la larga permanencia plasmática de los nanosistemas recubiertos de poliasparagina, como se ha demostrado para otros nanosistemas pegilados [45] y otros con superficies hidrofilicamente

[45] **Khalid, M.N., et al.**, *Long circulating poly(ethylene glycol)-decorated lipid nanocapsules deliver docetaxel to solid tumors.* Pharm. Res., 2006. **23**(4): p. 752-758.

modificadas [46], aunque en el caso de la poliasparagina, se mantiene la posibilidad de una cierta capacidad de *targeting* debido a la avidez de las células tumorales por la asparagina [47].

PARTE II. NANOCÁPSULAS DE CASEÍNA PARA LA VEHICULIZACIÓN DE FÁRMACOS HIDROFÓBICOS POR VÍA ORAL.

En esta segunda etapa, nos planteamos el diseño y el desarrollo de un nuevo sistema de nanocápsulas poliméricas, en la que la cubierta está constituida por la proteína caseína. Resulta especialmente novedosa la inclusión de la caseína como elemento de cubierta, porque hasta la fecha no se ha descrito ningún sistema similar. El interés del uso de las caseínas como componente de las nanocápsulas radica en dos puntos fundamentales. En primer lugar su aceptable perfil de seguridad como lo demuestra el formar parte de la lista de excipientes considerados GRAS (reconocidos en general como seguros) por la FDA para uso en humanos [48], por su biodegradabilidad y ausencia de toxicidad [49]. En segundo lugar, sus especiales características fisicoquímicas hacen que esta proteína pueda comportarse tanto como polímero hidrofílico, como

[46] **Romberg, B., et al.**, *Pharmacokinetics of poly(hydroxyethyl-l-asparagine)-coated liposomes is superior over that of PEG-coated liposomes at low lipid dose and upon repeated administration.* Biochimica et Biophysica Acta - Biomembranes, 2007. **1768**(3): p. 737-743.

[47] **Allan, J.D., et al.**, *L-asparagine L-asparaginase and cancer.* Lancet, 1967. **1**(7491): p. 682-&.

[48] **FDA**. *Inactive Ingredient Search for Approved Drug Products*. 2012 [cited 2012 24-01-2012]; Available from: http://www.accessdata.fda.gov/.

[49] **Elzoghby, A.O., et al.**, *Casein-based formulations as promising controlled release drug delivery systems.* Journal of controlled release : official journal of the Controlled Release Society, 2011. **153**(3): p. 206-16.

tensoactivo [50]. Además esta proteína presenta una interesante respuesta a cambios de pH, permitiendo la formación de cubiertas tipo gel con respuesta a pH [51].

El procedimiento de obtención utilizado para la formación de nanoestructuras fue análogo al previamente descrito para las nanocápsulas de poliasparagina, pero sin la inclusión del tensoactivo catiónico en la fase orgánica y estando constituida la fase acuosa únicamente por la solución de caseína.

De los cuatro tipos de caseína existentes, hemos seleccionado dos, la β- y la κ-caseína, para evaluar la importancia de la estructura molecular en las características finales de las nanocápsulas. La β-caseína, posee un punto isoeléctrico de 4.83-5.07, y es la variedad más lipofílica, por lo tanto es la que tiene más posibilidades se adsorberse sobre superficies hidrofóbicas como por ej. gotículas oleosas [52]. La κ-caseína con un punto isoeléctrico de 5.45– 5.7, fue elegida por ser la responsable natural de la estabilidad de las micelas de caseína en la leche [53], y haber sido ya utilizada como estabilizante en la administración oral de nutracéuticos.

En un estudio preliminar, nos hemos planteado la identificación de los parámetros críticos en la formación de las nanocápsulas. Así, se ha evaluado la influencia del volumen de aceite, concentración de lecitina en el núcleo oleoso, y de la concentración de caseína en la fase acuosa, sobre el tamaño y la carga superficial de los nanosistemas.

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[50] **Dalgleish, D.G.**, *Casein Micelles as Colloids: Surface Structures and Stabilities.* Journal of Dairy Science, 1998. **81**(11): p. 3013-3018.

[51] **Farias, M.E., et al.**, *Casein glycomacropeptide pH-dependent self-assembly and cold gelation.* International Dairy Journal, 2010. **20**(2): p. 79-88.

[52] **Maher, P.G., et al.**, *Optimization of beta-Casein Stabilized Nanoemulsions Using Experimental Mixture Design.* Journal of Food Science, 2011. **76**(8): p. C1108-C1117.

[53] **Tuinier, R. and de Kruif, C.G.**, *Stability of casein micelles in milk.* J. Chem. Phys., 2002. **117**(3): p. 1290-1295.

En relación al tamaño de las nanocápsulas (**Figura 19**), se observaron diferentes comportamientos dependiendo del tipo de proteína utilizada. Concretamente, para cualquiera de las dos variedades de caseína (se muestran en esta discusión solo los datos de la β-caseína a modo de ejemplo) el tamaño de partícula no se vio significativamente afectado ($p < 0.05$) por la concentración de proteína en la fase acuosa, obteniendo sistemas en torno a 180 nm en el rango menor de concentración de lecitina y de 250 nm en el rango superior.

Al contrario, la concentración tanto de Miglyol como de lecitina afectó considerablemente al tamaño de las nanocápsulas obtenidas, especialmente el porcentaje de lecitina, al dar lugar a cambios en el tamaño en torno a 100 nm, entre el porcentaje mínimo y el máximo. Un fenómeno particular ocurre con el Miglyol ya que, mientras que en los sistemas con β-caseína el volumen de Miglyol no aparece como un factor que afecte significativamente al tamaño, con la κ–caseína se observa una influencia significativa ($p < 0.05$) sobre el tamaño cuando el porcentaje de lecitina es máximo.

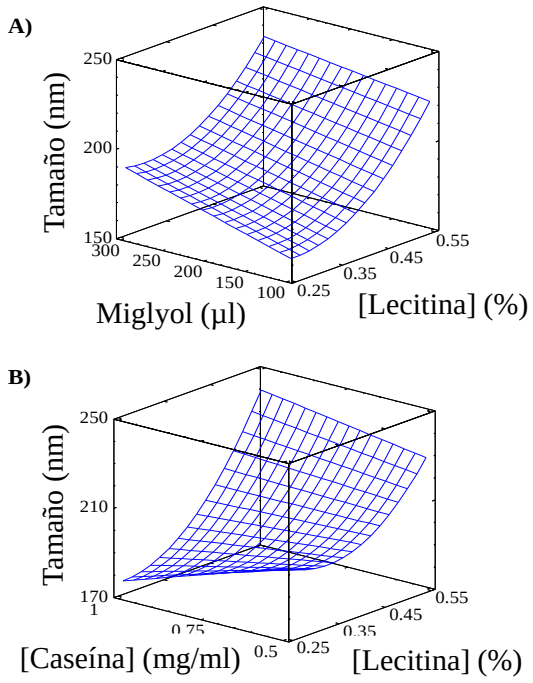

Figura 18.- Superficie de respuesta mostrando el efecto de diferentes parámetros sobre el tamaño de las nanocápsulas de β-caseína. A) Influencia del volumen de aceite y de la concentración de lecitina (concentración de caseína= 1mg/ml) y B) Influencia de la concentración de proteína y de lecitina (volumen de Miglyol= 150 µl)

En la **Figura 19** se representa la influencia de las mismas variables sobre el potencial zeta de las partículas. Se observa que la carga superficial se modificó significativamente en función de la concentración de lecitina ($p < 0.05$). Estos resultados pueden ser atribuidos a la carga negativa de los residuos hidrofílicos de la lecitina expuestos en la superficie de los sistemas. Al contrario, ni la concentración de caseína ni la cantidad de Miglyol afectaron a dicho parámetro.

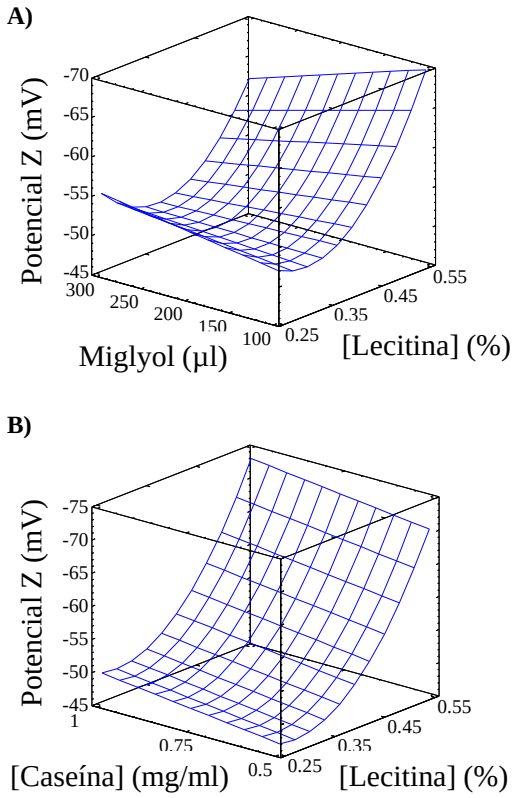

Figura 19.- Superficie de respuesta mostrando el efecto de diferentes parámetros sobre la carga superficial de las nanocápsulas de β-caseína: A) Influencia del volumen de aceite y de la concentración de lecitina (concentración de caseína= 1mg/ml) y B) Influencia de la concentración de proteína y de lecitina (volumen de Miglyol= 150 µl)

De acuerdo con la información anterior, los valores elegidos para el desarrollo de las nanocápsulas de caseína fueron:

- concentración de caseína: 1 mg/ml
- volumen de Miglyol: 150 µl
- concentración de lecitina: equivalente al 0.3 % p/v de formulación.

La Tabla 12 recoge la caracterización fisicoquímica de la nanoemulsión sin recubrimiento, y de las nanocápsulas blancas elaboradas en las citadas condiciones. Se observa que las nanocápsulas formaron poblaciones monodispersas, con tamaño medio entre 165-178 nm, dependiendo del tipo de lecitina. La carga superficial muestra valores negativos bastante elevados, debidos fundamentalmente al pH de la formulación (7.4) en el que la lecitina, cuyo punto isoeléctrico se sitúa en torno a 6.7, está fuertemente cargada.

Tabla 12.- Características físico-químicas de la nanoemulsión control y de las nanocápsulas de β- y κ-caseína (NCs β-CN y NCs κ-CN) blancas y conteniendo docetaxel (DCX) (Resultados expresados como media ± D.E.; n ≥ 3)

	Tamaño (nm)	**I.P.**	**Potencial z (mV)**	**E.E. (%)**
Nanoemulsión control	198 ± 16	0.1	-30 ± 6	-
NCs β-CN blancas	165 ± 11	0.1	-51 ± 3	-
NCs κ-CN blancas	178 ± 11	0.1	-39 ± 1	-
NCs β-CN DCX	168 ± 7	0.1	-49 ± 6	70 ± 6
NCs κ-CN DCX	181 ± 16	0.1	-38 ± 5	84 ± 3

Las imágenes de microscopía electrónica de transmisión (Figura 20) nos indican que las nanocápsulas, tanto de β-caseína como de κ-caseína constituyeron una población homogénea con tamaño de partícula menor a los 200 nm. Además, es de destacar la diferencia en la apariencia de ambos tipos de nanocápsulas, principalmente en la configuración de la cubierta.

En función de dicha diferencia, las pequeñas variaciones en el tamaño de partícula entre las nanocápsulas de β-caseína y las de κ-caseína y la información previa relativa a otros sistemas a base de caseínas [54], podemos especular sobre una diferencia en la conformación de la superficie de las nanocápsulas en función del tipo de caseína utilizada.

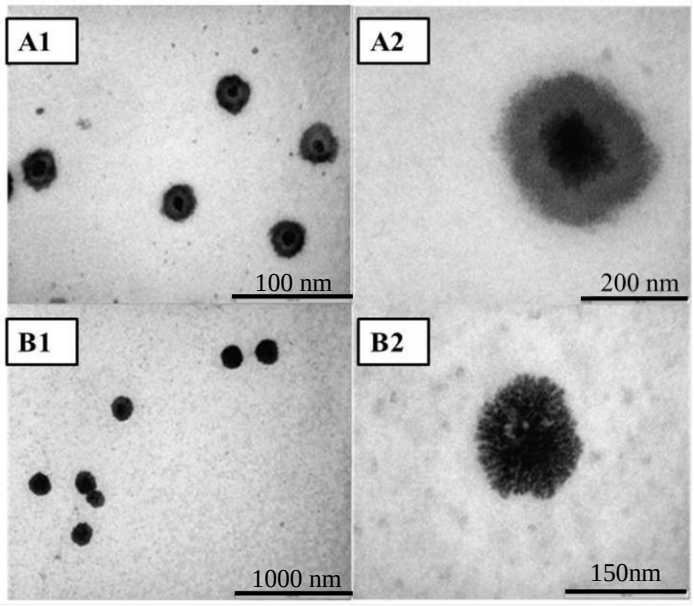

Figura 20.- Imágenes de microscopia electrónica de transmisión de las nanocápsulas de A) β-caseína y de B) κ-caseína.

A partir de estos datos e imágenes, proponemos la formación de dos tipos diferentes de cubierta en las nanocápsulas dependiendo del tipo de caseína utilizada: una cubierta compacta o de tipo "*mushroom"* para el caso de la β-caseína y una conformación extendida o de "cepillo" o

[54] **de Kruif, C.G. and Zhulina, E.B.**, *κ-casein as a polyelectrolyte brush on the surface of casein micelles.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1996. **117**(1-2): p. 151-159.

"*brush*" para el caso de la κ-caseína (Figura 21). En este sentido, ha sido ampliamente descrito el comportamiento de la κ-caseína al estabilizar las micelas de caseína en la leche y, sobre todo, su particular forma de disponerse en la superficie de las mismas [55]. Así, esta variedad de caseína se dispone en forma de microvellosidades sobre la superficie de los sistemas[56] otorgando estabilidad a los mismos por medio de repulsión estérica. Por otro lado, en el caso de la β-caseína se ha descrito, con menor frecuencia, su capacidad para cubiertas de tipo compacto y más lisas, en las que el principal mecanismo de estabilización transcurre por interacciones electrostáticas [57].

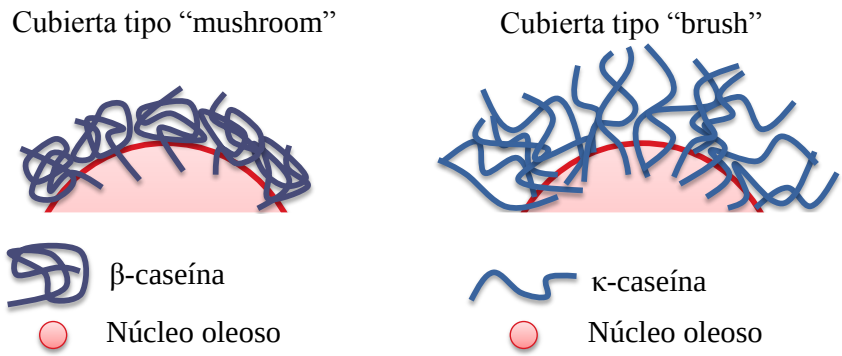

Figura 21.- Representación esquemática de los tipos de conformación propuestos para la cubierta de las nanocápsulas de caseína.

Nuestra hipótesis es que la diferencia en la conformación de la cubierta entre tipos de caseína puede estar condicionada por la

[55] **Tuinier, R. and de Kruif, C.G.**, *Stability of casein micelles in milk.* J. Chem. Phys., 2002. **117**(3): p. 1290-1295.
[56] **Dalgleish, D.G.**, *Casein Micelles as Colloids: Surface Structures and Stabilities.* Journal of Dairy Science, 1998. **81**(11): p. 3013-3018.
[57] **Leclerc, E. and Calmettes, P.**, *Interactions in Micellar Solutions of β-Casein.* Physical Review Letters, 1997. **78**(1): p. 150-153.

conformación molecular que presentan al incluirse en el nanosistema. A pesar de que la estructura tridimensional de las caseínas no está aun claramente identificada, en base a aproximaciones teóricas podemos asimilar algunos aspectos de ella [58, 59]. Dicha conformación está determinada principalmente por el número de residuos de aminoácidos reactivos y en su disposición espacial dentro de la cadena de las proteínas. Así, la mayor compactación en las cadenas de β-caseína, se debe a la existencia de cinco residuos de fosfoserina, un grupo altamente reactivo y a que, a las condiciones de pH de la formulación, pueden dar lugar a interacciones electrostáticas con otros grupos cargados cercanos, o del tipo disulfuro entre si (Capítulo 4, Figura 7, pág 129). Estas interacciones provocan la compactación de la cubierta y que la conformación de los residuos hidrofílicos de la proteína sea más bien plegada.

En la κ-caseína, sólo existe un residuo de fosfoserina, provocando menos interacciones del tipo disulfuro y por tanto un menor plegamiento de las cadenas hidrofílicas de la proteína. Además, la alta densidad de carga en las cadenas, por la presencia de aminoácidos altamente polares, provoca la repulsión electrostática entre las cadenas hidrofílicas provocando que se extiendan formando las "cerdas" en la superficie.

ENCAPSULACIÓN Y LIBERACIÓN DE DOCETAXEL A PARTIR DE LAS NANOCÁPSULAS DE CASEÍNA

[58] **Kumosinski, T.F., et al.**, *Three-Dimensional Molecular Modeling of Bovine Caseins: A Refined, Energy-Minimized κ-Casein Structure.* Journal of Dairy Science, 1993. **76**(9): p. 2507-2520.

[59] **Kumosinski, T.F., et al.**, *Three-Dimensional Molecular Modeling of Bovine Caseins: An Energy-Minimized β-Casein Structure.* Journal of Dairy Science, 1993. **76**(4): p. 931-945.

La encapsulación del fármaco docetaxel en las nanocápsulas de β y κ-caseína se realizó por el mismo procedimiento anteriormente descrito para las nanocápsulas de poliasparagina. Al ser sistemas de núcleo oleoso, las nanocápsulas de caseína consiguieron eficacias de encapsulación de docetaxel similares a las de poliasparagina, alcanzando valores entre 71-84 % (Tabla 12). El tamaño y carga superficial de las nanocápsulas apenas experimentaron modificaciones tras la incorporación del fármaco.

El tipo de caseína de la cubierta no influyó sobre el perfil de liberación de docetaxel en tampón fosfato pH 7.4 . El proceso de cesión de docetaxel al medio transcurrió de un modo similar al descrito para la poliasparagina, mostrando dos fases principales, una primera fase de liberación rápida relacionada con la dilución del sistema y el reparto del fármaco. Sin embargo, en este caso, en una segunda etapa se produjo una liberación más lenta del fármaco, alcanzando un el 60 % del total al cabo de 4 horas de incubación (Figura 9, Capítulo 4, pág 132).

ESTUDIO DE LA ESTABILIDAD DE LAS NANOCÁPSULAS DE CASEÍNA EN MEDIOS GASTROINTESTINALES SIMULADOS.

Un estudio fundamental en el desarrollo de estos nuevos sistemas, al estar considerados para la administración oral de fármacos, es el dedicado a evaluar su estabilidad en las condiciones del tracto gastrointestinal.

Los resultados presentados en la Figura 22 muestran que la estabilidad de las nanocápsulas de caseínas bajo condiciones de pH gástrico está dadas principalmente por la presencia de la cubierta de proteínas en la superficie del nanosistema. No obstante, al cabo de 3 horas, las

nanocápsulas de κ-caseína mostraron un aumento considerable de tamaño. Dicho aumento puede estar relacionado con el conocido proceso de gelificación ácida de la κ-caseína, causado principalmente por un colapso de las cadenas hidrofílicas de la superficie, al haber un cambio de conformación de las mismas [60]. Este proceso impide la estabilización estérica de los sistemas, hecho que no ocurre sin embargo la β-caseína, cuya conformación en superficie, no da lugar a este fenómeno desestabilización por pH.

La nanoemulsión aniónica, utilizada como control, al carecer de cubierta polimérica, vio incrementado su tamaño en 200 nm apenas 1 hora después de su incubación. Este resultado nos indica la importancia de la existencia de una cubierta polimérica en la mejora de la estabilidad de los nanosistemas, a nivel gástrico.

En la **Figura 23** se muestra la evolución del tamaño de partícula de las nanocápsulas de caseína y de la nanoemulsion control, tras su incubación en medio intestinal simulado sin enzimas. Como podemos apreciar, la nanoemulsión sin recubrir ve incrementado su tamaño en casi 100 nm, durante el período de estudio. Este incremento se relaciona se relaciona con el efecto desestabilizante que los electrolitos catiónicos tiene sobre la superficie de los nanosistemas cargados negativamente.

[60] **Horne, D.S.**, *Formation and structure of acidified milk gels.* International Dairy Journal, 1999. **9**(3–6): p. 261-268.

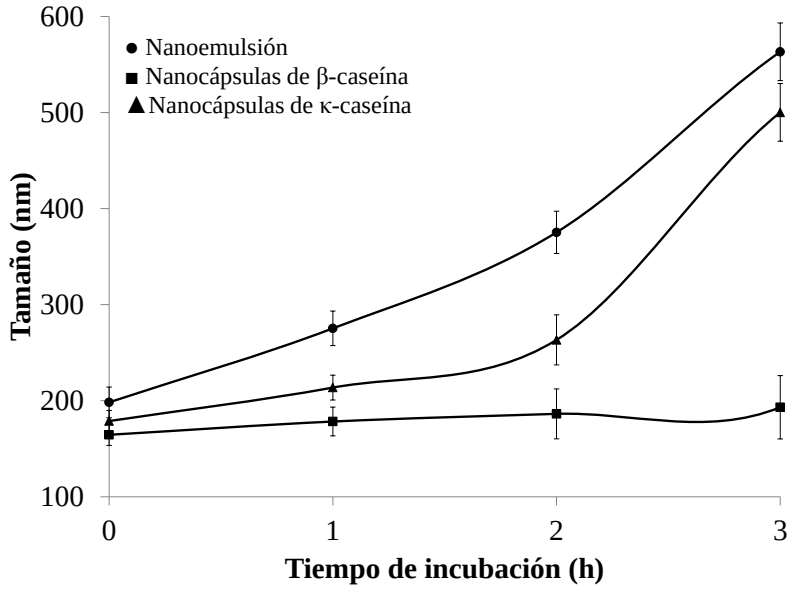

Figura 22.- Evolución del tamaño de partícula de las nanocápsulas blancas de β- y κ-caseína tras su incubación en medio gástrico simulado sin enzimas, pH = 1.2 y 37 ºC.

En este caso, sin embargo, tanto las nanocápsulas de β como de κ-caseína son totalmente estables durante el período de incubación. Dado el punto isoeléctrico de ambas caseínas (4.8-5.07 para la β- y 5.45-5.77 para la κ-), a pH intestinal presentan una alta carga negativa, lo que da lugar a que se desencadene un mecanismo de estabilización electrostática, además del esperado por estabilización estérica.

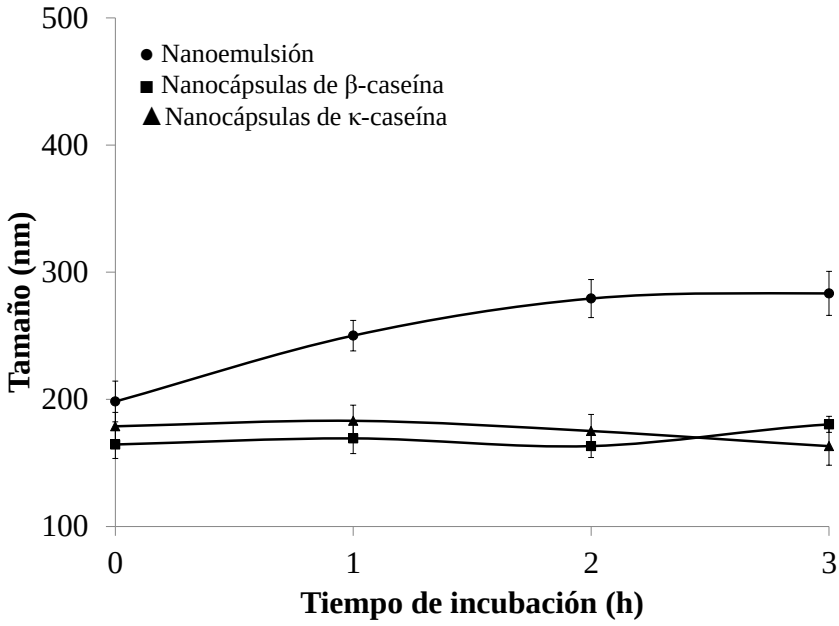

Figura 23.- Evolución del tamaño de partícula de las nanocápsulas blancas de β- y κ-caseína tras su incubación en medio intestinal simulado sin enzimas, pH = 6.8 y 37 ºC

En conjunto, la buena estabilidad presentada por las nanocápsulas en fluidos fisiológicos simulados convierten a esta plataforma en una interesante alternativa para la administración oral de fármacos.

CONCLUSIONES

CONCLUSIONES

El trabajo experimental recogido en la presente memoria se ha dirigido al desarrollo de nuevos nanotransportadores constituidos por poliaminoácidos como la poliasparagina o proteínas como la caseína. Los resultados obtenidos nos han permitido extraer las siguientes conclusiones.

1. Parte I. Nanocápsulas de poliasparagina para la vehiculización de fármacos antitumorales.

1.- Se han obtenido nuevas estructuras nanocapsulares constituidas por un núcleo oleoso rodeado de una cubierta de poliasparagina. La formación de las nanocápsulas fue posible gracias a la incorporación en el núcleo oleoso de un tensoactivo catiónico, que facilitó el depósito del poliaminoácido en superficie. Dichos sistemas presentaron un tamaño de rango nanométrico, siempre inferior a 200 nm y carga superficial negativa en torno a -20 -34 mV.

2.- Las nanocápsulas de poliasparagina incorporaron el antitumoral docetaxel con una eficacia próxima al 80%. Tras el contacto con la línea celular de cáncer de pulmón NCI-H460, las nanocápsulas dieron lugar a un incremento considerable en la citotoxicidad del docetaxel. Así, en comparación al fármaco libre, el valor de la IC50 se redujo significativamente en un factor de 3, hecho atribuible a una mejor internalización de los nanosistemas, y liberación intracelular del docetaxel.

3.- El seguimiento en plasma de los nanotransportadores de poliasparagina fluorescentes permitió comprobar su prolongada permanencia plasmática,

aumentando el tiempo de vida media en un 55 % con respecto a la nanoemulsión sin recubrir.

4.- Tras su administración I.V. en ratones portadores del modelo de glioma U87MG, las nanocápsulas de poliasparagina han demostrado mejorar considerablemente el tiempo de supervivencia de los animales tratados en comparación a la formulación comercial Taxotere®, consiguiendo una reducción similar del crecimiento tumoral.

5.- Las nanocapsulas se mostraron estables durante un período de almacenamiento de 2 meses a 4º y 37ºC, y se han podido liofilizar facilmente tras la incorporación de pequeñas porporciones de trealosa.

2. Parte II. Nanocápsulas de caseína para la vehiculización de fármacos hidrofóbicos por vía oral.

1.-Se han obtenido nuevas estructuras nanocapsulares con una cubierta a base de β- o κ-caseína. Los sistemas mostraron un rango de tamaño nanométrico, ligeramente inferior en el caso de la β-caseína y significativamente dependiente de la concentración de lecitina utilizada. La carga superficial, de elevado valor negativo, mostró igualmente esta dependencia, para las dos variedades de proteína.

2- Los sistemas se mantuvieron en general estables en fluídos gastrointestinales simulados, sin enzimas. En medio gástrico, sin embargo, los sistemas elaborados con β-caseína experimentaron un incremento de tamaño, tras dos horas de contacto. Estas diferencias se atribuyen a las diferentes conformaciones que adoptan las variedades de

caseína en la superficie de los nanosistemas, que determinan el tipo de estabilización que le otorgan

3. Independientemente del tipo de cubierta, las nanocápsulas de caseína consiguieron una elevada eficacia de encapsulación (entre 70 y 80%) para fármacos hidrofóbicos como el docetaxel.

4. Las nanocápsulas de caseína se mantuvieron estables en el almacenamiento a 4º y 37º, durante un período de 6 meses. Además, mediante el uso de trealosa ha sido posible la obtención de un producto liofilizado.

Anexos

Anexo 1. Hyaluronan nanocapsules: a new safe and effective nanocarrier for the intracellular delivery of anticancer drugs.

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Summary

Aim: The development of a new drug nanocarrier for the intracellular delivery of hydrophobic anticancer drugs. The nanocarrier -named as nanocapsules- is composed of a lipid core surrounded by a shell made of hyaluronic acid (HA). This polymer was chosen because of its potential ability to prolong the circulation time of the nanocarrier and its specific interaction with certain cancer tumors. **Results:** HA nanocapsules were produced by a modified solvent displacement technique, which allows the formation of the polymer shell around the oily core as a consequence of the interaction between a cationic surfactant and the anionic polysaccharide. The resulting nanocapsules have a size of ~200 nm and a negative zeta potential and showed a capacity to efficiently encapsulate the model hydrophobic drug docetaxel. As expected, the nanoencapsulated docetaxel exhibited an enhanced cytotoxicity (3-fold increase as compared to a control solution) upon *in vitro* incubation with the cancer cell line NCI-H460. Moreover, the nanocapsules suspension could be freeze-dried and remained stable during storage. **Conclusion:** these novel nanostructures hold promise as intracellular drug delivery systems.

Keywords: Nanocapsules, hyaluronic acid, cancer, hydrophobic drugs, intracellular delivery.

Introduction

Currently, it is known that the use of nanoscale drug delivery vehicles represents a very promising strategy for improving the biodistribution and intracellular delivery of anticancer drugs. Taxanes (paclitaxel, docetaxel) are good examples of potent chemotherapeutic agents, which could greatly benefit from these delivery carriers. Indeed, despite their efficacy, these drugs display significant draw-backs related to their indiscriminate biodistribution and the necessity to use toxic solubilizers for their intravenous administration [1]. Besides the marketed formulation of albumin nanoparticles (Abraxane), a number of nanocarriers have been disclosed in the literature for the delivery of these specific compounds. Particularly attractive for this purpose are the nanocapsules, which can easily accommodate hydrophobic drugs [2-6]. For example, the group of Benoit and co-workers has reported the potential of PEG-coated lipid nanocapsules loaded with paclitaxel in different cancer animal models. Overall, the authors observed that PEGylated nanocapsules have long circulating properties as well as the ability to improve the intracellular accumulation of drugs in the tumor cells [7-8]. An alternative nanocapsule-type carrier was recently reported by our group for the delivery of docetaxel (DCX) [5-6]. These nanocapsules are made of chitosan and polyarginine and display a number of properties, among which it is important to highlight: i) their capacity to be internalized by different cell lines, such as the breast cancer (MCF-7) and lung cancer (A-549) cell lines, ii) the improved efficacy of DCX-loaded nanocapsules in MCF-7, A-549 and NCI-H460 (non small cell lung cancer) cell lines compared to DCX alone; and iii) the possibility to functionalize them with ligands such as anti-TMEFF-2 in order to target them to TMEFF-2, a transmembrane protein, that is overexpressed in non microcytic tumors [9].

Following this experience, we found it important to modify the polymer corona of the nanocapsules using hydrophilic negatively charged polymers, such as hyaluronic acid (HA). This polysaccharide attracted our attention because of its reported ability to prolong the plasma circulation time of liposomes and nanoparticles [10-14]. Additionally, HA offers the possibility of targeting the drug-loaded nanocarrier to the cancer cells that overexpress the endogenous receptor for this polymer (called CD-44 receptors). As a consequence of these properties, HA-based nanocarriers have resulted in an improved tumor growth inhibition and a decreased systemic toxicity, when compared to the free drug [10,11,15-21].

Taking this background information into account our aim in this work has been to develop a new and alternative HA-based anticancer drug delivery nanocarrier exhibiting unique pharmaceutical properties such as being: i) acceptable from the safety point of view, ii) able to load important amounts of lipophilic anticancer drugs, iii) having the ability to target and enter cancer cells providing intracellular drug delivery and, finally, iv) are easy to produce and scale-up and are stable under storage. The results presented in this report clearly show that HA nanocapsules fulfill such an interesting profile.

Materials & methods

Chemicals: Docetaxel (DCX, from Fluka), poloxamer (Pluronic F-68®), benzalkonium chloride (BKC) and hexadeciltrymethylammonium bromide (CTAB) were purchased from Sigma-Aldrich (Spain). Miglyol 812®, which is a neutral oil formed by esters of caprylic and capryc fatty acids and glycerol, was donated by Sasol Germany GmbH (Germany). The surfactant Epikuron 145V, which is a phosphatidylcholine-enriched fraction of soybean Lecithin, was donated by Cargill (Spain). Hyaluronic

acid of 29 kDa was purchased from Imquiaroma (Barcelona, Spain) and that of 160 kDa was kindly donated by Bioiberica (Barcelona, Spain).

Preparation of HA nanocapsules:

HA nanocapsules were prepared following two different procedures that have been previously described by our group [5,22]. The first, called "two-stage procedure" consists of adding 125 μL of Miglyol 812 to an organic phase comprising 30 mg of lecithin, and 4 mg of BKC or 1.8 mg of CTAB, dissolved in 0.5 ml of ethanol and 9.5 ml of acetone. This organic phase was added to an aqueous phase (20 mL). The formation of the cationic nanoemulsions was instantaneous, which was evident due to the milky appearance of the mixture (interestingly, the cationic nanoemulsion was also obtained after the addition of the organic phase to a homogenizer/sonicator, data not shown). The above solution was rotaevaporated until a volume of 10 mL, and then incubated with an aqueous solution of HA (0.1-50 mg) in a volume ratio 4:1.5 (cationic nanoemulsion:HA solution); when the anionic HA interacts with the cationic nanoemulsion, it forms a polymer corona at the oil/aqueous interface thus originating HA nanocapsules. The second, called "one-stage procedure" involves adding 125 μL of Miglyol 812 to an organic phase comprising 30 mg of lecithin, and 4 mg of BKC or 1.8 mg of CTAB, dissolved in 0.5 ml of ethanol and 9.5 ml of acetone. This organic phase was added to an aqueous phase (20 mL) that contains the polymer HA (0.1-50 mg). The above solution was rotaevaporated until a volume of 10 mL, here the formation of HA nanocapsules occurs concomitantly to the evaporation of the solvent due to the adsorption of the polymer onto the nanocarrier-surface. In both cases, the incorporation of DCX, required the previous dissolution of this molecule in ethanol to obtain a final

concentration of 1 mg/mL. Next, an aliquot of the stock solution was added to the organic phase and the same procedure was followed. The final DCX concentration obtained in HA nanocapsules carriers was 7.27 μM.

Physicochemical Characterization of HA nanocapsules

The size and zeta potential of the colloidal systems were determined by photon correlation spectroscopy and laser Doppler anemometry, with a Zetasizer Nano-ZS (Malvern Instruments, United Kingdom). Each batch was analyzed in triplicate.

The morphological examination of the systems was performed by transmission electron microscopy (TEM) (CM12 Phillips, Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid and immobilized on copper grids with Formvar® for viewing by TEM.

Encapsulation Efficiency of DCX-Loaded HA nanocapsules

The encapsulation efficiency of DCX in the nanocarriers was determined indirectly by the difference between the total amount of DCX and the free drug recovered in the continuous phase. The total amount of drug was estimated by dissolving an aliquot of non isolated DCX-loaded HA nanocapsules with acetonitrile. This sample was centrifuged during 20 min at 4000 × *g* and the supernatant was measured with a high performance liquid chromatography (HPLC) system. The non encapsulated drug was determined by the same method following separation of the nanocapsules from the aqueous medium by ultracentrifugation (27500 *g*, 60 min). DCX was assayed by a slightly

modified version of the method proposed by Lee *et al.* (1999) [23]. The HPLC system consisted of an Agilent 1100 series instrument equipped with a UV detector set at 227 nm and a reverse phase Zorbax Eclipse XDB- C8 column (4.6 × 150 mm i.d., pore size 5 μm Agilent, U.S.A.). The mobile phase consisted of a mixture of acetonitrile and 0.1% v/v orthophosphoric acid (55:45, v/v) and the flow rate was 1 mL/min. The standard calibration curves of DCX were linear ($r2 > 0.999$) in the range of concentrations between 0.3-2 μg/mL.

In vitro Release Studies

The release studies of DCX from HA carriers were performed by incubating a sample of the formulation with milli-Q water at an appropriate concentration to ensure sink conditions. The vials were placed in an incubator at 37 °C for horizontal shaking. A total of 3 mL of the suspension were collected and ultracentrifuged (27500 *g*, 60 min) using Herolab high speed centrifuge labware tubes (Herolab GmbH, Germany) at different time intervals. The DCX released was calculated indirectly by determining how much was left in the system by processing the isolated HA nanocapsules with acetonitrile before HPLC analysis.

Cellular assays

Cell viability assay and IC_{50} estimation: Human non-small cell lung cancer cell line NCI-H460 was cultured in RPMI 1640 medium (ATCC), supplemented with 10% (v/v) fetal bovine serum (FBS) at 37° C in a humidified atmosphere containing 5% carbon dioxide. Tretazolium salt 3-(4,5-dimewthylthiazol-2-yl)2,5 diphenyltetrazolium bromide (MTT, Acros Organics) was used for mytocondrial activity evaluation. Briefly,

cells were plated onto 96-well plates, with a seeding density of $15x10^3$ cells/well in 100 µL of culture medium. After 24 h, the medium was renewed but contained the following three treatments: DCX, DCX- loaded HA nanocapsules and blank HA nanocapsules. Finally, after 48 h cell survival was measured by the MTT assay [24]. In brief, medium was removed and the cells were washed twice with 100 µL Hank`s Balanced Salt Serum (HBSS). Then 20 µL of a MTT solution (5 mg/mL in PBS) and 100 µL HBSS were added to the wells and maintained at 37°C in an atmosphere with 5% CO_2 for 4h. Afterwards, buffers were replaced by 100 µL DMSO per well and maintained at 37° C in an atmosphere with 5% CO_2 overnight. Absorbance (λ= 515 nm) was measured in a spectrophotometer (Tecan, Ultra evolution) removing any background absorbance (λ= 630 nm). Moreover, short incubation times of 2 h were assayed in order to determine the efficacy of HA nanocapsules to quickly interact with the cells and deliver the drug intracellularly. Thus, after a 2 h incubation with the three treatments, the medium was replaced by a fresh one and cells were grown for 48 h. Finally, cell viability was measured as described.

The percentage of cell viability was calculated by the absorbance measurements of control growth in the presence of the formulations at various concentration levels. IC_{50} values were obtained by fitting the data with non linear regression, with Prism 2.1 software (GraphPad, San Diego, CA).

Stability study at storage conditions

The suspension stability of HA nanocapsules prepared with the surfactants BKC and CTAB were evaluated according to terms of time and temperature of storage. Therefore, aliquots of the nanocapsules

suspension without dilution were placed in sealed tubes at 4 and 37°C for storage. Size and polydispersity index of the nanocarriers were measured for a period of three months, meanwhile zeta potential values were controlled at the end of the study. Each sample corresponds to a different HA nanocapsules batch.

Freeze-dried studies of HA nanocapsules

Concentrations of HA nanocapsules (0.5 and 1% w/v) and of the cryoprotectant trealose (5 and 10%) were considered the variables for the lyophilization study. Therefore, 2 mL dilutions of HA NCs were transferred into 5 mL volume glass vials and were frozen at -20°C. The lyophilization procedure consisted of an initial drying step for 60 h at -35°C, followed by a secondary drying for 24 h in a high vacuum atmosphere. Finally, the temperature was slowly increased up to 20°C until the end of the process (Labconco Corp., USA). HA nanocapsules were recovered by adding 2 mL of ultrapure water to the freeze-dried powders followed by manual resuspension and were characterized as explained above.

Statistical analysis

Cell culture results were evaluated in order to determine the statistical significance between the different formulations studied. The statistical evaluation of the cell viability results was performed by an ANOVA test followed by the post hoc Tukey test comparison analysis (SigmaStat Program, Jandel Scientific, version 3.5).

Results and discussion

The main aim of this work was the development of a new anticancer drug nanocarrier consisting of a lipid core surrounded by a shell made of HA. The conceptual bases of the system were: simplicity (minimum amount of components and easy to produce), acceptability (from the regulatory stand point) and efficacy (improvement of citoxicity in cancer cells). Thus, DCX was chosen as a model compound and its efficacy was evaluated in lung cancer cell line NCI-H460. As indicated in the introduction, the biopolymer HA was chosen because of its interesting biopharmaceutical properties [13-14,25].

Development and characterization of HA nanocapsules

HA nanocapsules were prepared by a number of simple techniques, which involved the formation of a cationic nanoemulsion and the attachment of the outer HA corona. One of the techniques was the solvent displacement technique, which allowed the emulsification process to occur simultaneously to the attachment of the polymer corona. Other techniques involved two steps, i.e. an emulsification process either by the solvent displacement technique, homogenization or sonication, followed by coating with the polymer. In all cases, the formation of the polymer capsule is driven by the ionic interaction of the positively charged surfactant with the negatively charged HA. Following an initial screening of different experimental approaches, we chose the two-step solvent diffusion technique previously used for the formation of chitosan nanocapsules [22], and investigated the influence of different formulation parameters on the physicochemical properties of the resulting systems. The formulation parameters were the type and concentration of cationic surfactant, and the concentration of HA. The cationic surfactants, selected

on the basis of their acceptable toxicological profile, were benzalkonium chloride (BKC) and hexadecyltrimethylammonium bromide (CTAB) [26]. On the other hand, the quantity of the surfactants used was the minimum that allowed the formation of stable systems. In the case of CTAB this amount was 1.8 mg, and in the case of BKC 4.0 mg.

Tables 1a and b show the characteristics of nanocapsules prepared by the two-stage procedure using the surfactants BKC or CTAB, respectively. As can been noted, the use of adequate concentrations of HA with the cationic nanoemulsions results in the formation of homogenous populations of nanocapsules of around 250 nm. The results also show a shielding of the original positive zeta potential of the nanoemulsion, which leads to an inversion to negative values, as the concentration of HA increases. This dependency of the zeta potential with the amount of HA evidences the surface localization of HA molecules and indicates the necessity of using a minimum amount of HA in order to obtain stable nanocapsules. The formation of the HA corona results in a minor increase in the size of the nanocapsules with respect to the nanoemulsions, a result that supports the idea that HA is tightly attached to the oily droplets.

Tables 1a and b also indicate that the viability of the system is compromised under certain conditions. More specifically, we observed a precipitation of the system due to the neutralization of the positive surface charge caused by the addition of increasing amounts of HA. After the inflection point, the addition of subsequent amounts of HA leads to the inversion of the zeta potential and the subsequent stabilization of the system. Finally, there is another precipitation point that is related to the excess of the HA in the formulation. Here, the precipitation phenomenon could be attributed to a combination of factors such as the high ionic strength in the medium [27] (due to the high concentration of sodium ions added together with the hyaluronate), combined with the increase in the

viscosity, which might modify the kinetic of adsorption of the polymer onto the cationic nanoemulsion.

Tables 2a and 2b display the results of size and zeta potential of the HA nanocapsules prepared according to a single stage procedure. Overall, the conclusion is that the incorporation of HA either during or after the emulsification process does not affect the formation of the HA corona (both show similar zeta potential values and precipitation conditions). However, the size of the nanocapsules elaborated by a single stage procedure was smaller than that of nanocapsules obtained by a two-stage procedure (~170nm and ~250nm, respectively). This difference in size has also been observed for chitosan nanocapsules prepared following a single [5] or two-stage procedure [22] and could be attributed to the stabilizing role of the polymer during the emulsification process.

The size and appearance of the nanocapsules has also been observed by transmission electron microscopy (TEM). As an example, figure 1 illustrates the size and structure of those prepared with the BKC surfactant (figure 1). Overall, the nanocapsules exhibited a round shape and the core-corona structure typically observed for polymeric nanocapsules [5,22].

In a second part of the nanocapsules development process, we evaluated the influence of the HA MW (160 kDa vs. 29 kDa used in the original study) in the characteristics of the nanocapsules prepared by the two-step solvent diffusion technique. Table 3 shows the characteristics of the formulations prepared by incubation of the preformed emulsion containing BKC with high MW HA. Overall, for the range of mass of 6.2-25 mg, the size of the nanocapsules was larger and their zeta potential higher than the corresponding ones obtained with HA of 29 kDa (table 1a). This behavior correlates very well with that previously observed for chitosan nanocapsules [27] and is attributed to the different thickness of

the HA coating around the oily nanodroplets depending on the HA MW. Namely, the use of high MW led to an increase in the thickness of the coating and, thus, to an increase of the size of the nanocapsules. In addition, the increase in the zeta potential values observed for the high MW HA nanocapsules could be a consequence of the greater number of carboxylic groups of AH at the shell surface. These minor differences were not, however, observed for the nanocapsules containing the surfactant CTAB. This could be attributed to a different entanglement of the HA molecules depending on the surfactant used for their formation.

Encapsulation and release of docetaxel (DCX) from HA nanocapsules

The high efficacy of DCX in the treatment of a wide range of solid tumors [28], together with its hydrophobic character makes this molecule an attractive candidate for inclusion in the developed nanocapsules. Among the tested blank nanocapsules formulations, we selected the one containing 12.5 mg of 29 KDa HA (see tables 1a and 1b) for the following studies as it contained the greatest amount of HA without causing precipitation. As shown in table 4, DCX could be efficiently encapsulated in the HA nanocapsules prepared with the surfactants BKC or CTAB, hardly affecting the characteristics of the original blank formulations. These data of encapsulation correlate very well with previously published works where DCX was also encapsulated in lipid nanocapsules, and is attributed to the affinity of the drug for the core components [5,29].

In an additional experiment, we evaluated the release pattern of the encapsulated DCX upon incubation of highly diluted nanocapsules prepared with the surfactants BKC or CTAB in aqueous medium. The results showed similar drug release profiles for nanocapsules containing BKC or CTAB with only critical differences in the initial time-release point (figure 2). The release follows a biphasic profile, characterized by a

rapid initial release, followed by a second stage in which no further changes were observed. The initial release stage, which is typically observed in oily systems [5,6,30], has been attributed to the dilution of the nanocapsules in the incubation medium and the subsequent partition of the drug between the oily core and the external aqueous phase. The absence of release in the second stage confirms the high affinity of the DCX by the oil core. While these data provide us with information about mechanistic details, in their interpretation it is important to point out that the release behavior observed *in vitro* was not expected to correlate with the *in vivo* behavior. In the *in vivo* situation, the presence of physiological occurring macromolecules and ions could significantly influence the release profile as reported Ahmed *et al.* (2011) [31].

In vitro efficacy of the DCX- loaded HA nanocapsules in human lung cancer cell line

Cell viability studies were performed in order to assess the efficacy of the HA nanocapsules in the non-small cell lung cancer NCI-H460 cell line. Figures 3a and b show the cellular viability profiles of DCX-loaded HA nanocapsules (formulation containing 1.8 mg of CTAB and 12.5 mg of HA 29 KDa) upon cell exposure for up to 2 and 48 h respectively. The results indicate that the encapsulation of DCX in HA nanocapsules lead to a significant increase of its cytotoxicity. The toxicity values were multiplied by a factor of 2 or 3 depending upon the incubation time. The results also show that blank nanocapsules did not cause any noticeable damage to the cells. These results are summarized in table 4, which illustrates the cytotoxicity values of DCX-loaded nanocapsules measured by their IC_{50}. Overall, the potency of DCX was increased up to more than 3 times upon its encapsulation into HA nanocapsules.

Figures 4a and b show the cytotoxicity profiles of cell exposed to HA nanocapsules prepared with the surfactant BKC for up to 2 and 48 h, respectively. The cellular behavior displayed in figure 4a is similar to that observed for nanocapsules prepared with the surfactant CTAB. Accordingly, the results in table 4 show that DCX-loaded nanocapsules are 3.6 times more efficient than the free drug in terms of the IC_{50} values. This improved efficacy was also observed after 48 h incubation (figure 4b), leading to a 3-fold reduction in the IC_{50} value (table 4). In this case, a certain reduction of cell viability was also observed at high concentrations of the blank nanocapsules (figure 4b), a result that was attributed to the higher toxicity of BKC over CTAB [26], and the necessity of using a greater amount of BKC *vs*. CTAB for the formation of stable nanocapsules.

The significant improvement in cytotoxicity observed for HA nanocapsules loaded with DCX is in agreement with the results observed for other nanocapsules coated with polyethyleneglycol [7], chitosan [5] or polyarginine [6] tested in a variety of cell lines, including: glyoma cells (9L and F98 cell lines), human breast carcinoma (MCF-7), and human lung cancer (A-549, and NCI-H460). The improvement in cytotoxicity has been attributed to the internalization and intracellular drug delivery capacity of the nanocapsules in association with a potential reversal of the multidrug resistance effect.

It is important to note here that the above indicated nanocapsules and those functionalized with a monoclonal antibody (anti TMEFF-2) have also been tested in a variety of animal models of cancer, such as: glyoma [7], hepatocellular carcinoma [8], colon adenocarcinoma [29], and lung cancer [9]. The results obtained so far have shown an improved or similar efficacy of the encapsulated drug compared to that obtained for the free drug. These are promising preliminary data as the nanocapsules are

expected to have a more acceptable toxicity profile (they avoid the use of toxic solubilizers) than the commercial drugs.

Stability studies of HA nanocapsules during storage

Stability is a critical issue in the development of a nanocarrier formulation. Variations on temperature are known to significantly compromise the stability of colloidal systems, and show evident importance during storage [32]. The size increase of the formulations could be attributed to the weakening/breaking-down of linkers between the molecules that form the nanocarriers, and may alter their drug release and induce destabilization of the formulations. A decrease in size could also occur due to the detachment of components from the nanocarriers or to an increase on the interaction strengths between linkers, potentially affecting the desired efficacy of the nanocarrier. In the present study we evaluated the stability of the DCX-loaded nanocapsules prepared with the surfactant CTAB under storage at 4° and 37°C, for a period of 3 months. Figure 5 shows that nanocapsules were generally stable during storage irrespective of the temperature conditions. The overall trend of prolonged stability is justified by the electrostatic repulsive effect due to the high zeta potential values of nanocapsules (around -40 mV). Similar results were obtained when the surfactant BKC was used for the elaboration of the nanocapsules (data not shown). This acceptable stability profile has been previously observed for other polymer nanocapsules [6,33], and attributed to the polymer corona surrounding the oily nanodroplets.

Formation of a lyophilized product of HA nanocapsules

Lyophilization is one of the most frequent and efficient methods of preserving the properties of nanoparticulate systems during storage. Nevertheless, this process becomes quite complex in the case of nanocapsules, due to the fluidity of the polymer shell and also to the presence of the oil core, which is susceptible of leakage [34]. In order to facilitate the lyophilization of the nanocarriers and avoid their collapse, the use of cryoprotectant agents is necessary [35]. The first evidence that polymeric nanocapsules were effectively protected, by isotonic concentrations of cryoprotectants derived from sugar, during a lyophilization process was carried out by Calvo *et al.* (1997) [36]. Subsequent works [6,37] have indicated that trehalose is an adequate agent for preserving the stability of fluid nanosystems during lyophilization. The main arguments, that support the use of this agent over other cryoprotectants, is its less hygroscopicity together with its higher glass transition temperature.

Figure 6 shows the size of HA nanocapsules containing BKC upon reconstitution of the freeze-dried product. Overall the results indicate that at relatively high concentrations of nanocapsules (1% w/v) it is possible to achieve an adequate resuspension of the dried product without altering the size of the nanocapsules.

Conclusions

In this paper we report the formation and characterization of a new type of drug nanocarriers named as HA nanocapsules. These nanocarriers are able to successfully encapsulate the hydrophobic drug docetaxel because of their hydrophobic core. In addition, thanks to their HA

hydrophilic coating, these nanocarriers have the capacity to interact with NCI-H460 cancer cells and improve the pharmacological effect of the model drug docetaxel. Finally, pharmaceutical parameters such as drug release, stability during storage and reconstitution of freeze-dried HA nanocapsules render these novel systems promising platforms for the intracellular delivery of anticancer drugs.

Future perspective

Cancer is a chronic illness whose prevalence has been strongly augmented during last decades. This is specially attributed to an increase in life expectancy together with changes in lifestyle. The high cellular toxicity and indiscriminate spreading of a number of antineoplasic drugs after its administration following conventional therapies strongly stimulate the development of new/improved formulations. Within this sense, the arguments in favour of the potential of nanosystems are forceful; for example, the development of antineoplasic colloidal systems with the capacity of avoiding the use of toxic drug-solubilizers together with a better cellular target have emerged and become a challenging approach for systemically and topical applications. However, significant formulations improvements are still required to achieve a better clinical impact. In the case of hidrofobic drugs, the release control (temporal and spatial) still is a barrier that is strongly desired by oncologist and other clinicians. The developments of new anticancer formulations as those proposed here represent great opportunities to solve these problems. This, in combination with traditional or new antineoplasic drugs represents for the near future a big deal that can originate easier, safer and cheaper treatments.

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