UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

FACULTAD DE FARMACIA

Departamento de Farmacia y Tecnología Farmacéutica

TESIS DOCTORAL

Nanocápsulas de poliaminoácidos para la liberación selectiva de fármacos antitumorales

Giovanna Lollo

Santiago de Compostela, 2012

DOÑA MARÍA JOSÉ ALONSO FERNÁNDEZ, DOÑA DOLORES TORRES LÓPEZ Y DON MARCOS GARCÍA FUENTES CATEDRÁTICA, PROFESORA TITULAR E INVESTIGADOR POSTDOCTORAL RESPECTIVAMENTE, DEL DEPARTAMENTO DE FARMACIA Y TECNOLOGÍA FARMACÉUTICA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA.

INFORMAN:

Que la presente Memoria Experimental titulada: "Nanocápsulas de poliaminoácidos para la liberación selectiva de fármacos antitumorales", elaborada por Giovanna Lollo, 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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Ai miei genitori

“Per correr miglior acque alza le vele
omai la navicella del mio ingegno”
Dante Alighieri

TABLA DE CONTENIDO

RESUMEN-ABSTRACT

Resumen

El objetivo principal de esta tesis ha sido el diseño y desarrollo de nanocápsulas de poliaminoácidos para la liberación de fármacos antitumorales. Estos sistemas compuestos por un núcleo oleoso y una cubierta de ácido poliglutámico (PGA), y su modificación con polietilenglicol (PEG), emergen como una nueva plataforma de vehiculización selectiva para moléculas hidrofóbicas antitumorales como la plitidepsin y el docetaxel. Se ha prestado especial atención al efecto del PEG, evaluando diferentes grados de PEGilación (24 y 57% p/p de PEG), con la intención de conseguir sistemas con mayor permanencia en el torrente circulatorio y modificar así la cinética de eliminación en plasma de las moléculas asociadas. Las nanocápsulas de PGA-PEG con un elevado grado de PEGilación incrementaron al doble la vida media y el MRT de la plitidepsin, mientras el AUC resultó ser 5 veces mayor en comparación con la solución de plitidepsina en Cremophor® EL, formulación utilizada como referencia. Los experimentos *in vivo* mostraron que los sistemas altamente pegilados presentan una menor toxicidad, son mejor tolerados respecto a la formulación de referencia, y presentan una fuerte inhibición del crecimiento tumoral en el modelo xenograft de tumor renal (MRI-H-121). Adicionalmente, las nanocápsulas de PGA-PEG con elevado grado de PEGilación cargadas docetaxel mostraron una elevada actividad antitumoral en el modelo de glioma U87, reduciendo en un 60% el crecimiento del tumor y mostrando un aumento significativo de la supervivencia con respecto a los animales tradatos con el Taxotere®.

En una segunda etapa, se evaluó el potencial de otro tipo de nanocápsulas, las nanocápsulas de poliarginina (PARG), como nuevos vehículos para la administración oral de fármacos antitumorales. Los estudios llevados a cabo en cultivos celulares Caco-2 demostraron que las

nanocápsulas pueden a promover el transporte paracelular de fármacos modificando las uniones entre células epiteliales, internalizándose en la monocapa celular de manera eficiente. Tras su administración oral, mediante estudios de imagen *in vivo*, se confirmó la permanencia de las nanocápsulas en el tracto gastrointestinal.

Todos estos resultados ponen de manifiesto el potencial de las nanocápsulas de poliaminoácidos como sistemas de vehiculización para mejorar la absorción parenteral y oral de fármacos antitumorales.

Abstract

The main objective of this thesis has been the design and the development of polyaminoacids nanocapsules for the delivery of the hydrophobic anticancer compounds. These systems, composed of an oily core and a shell made of polyglutamic acid (PGA), and their modification with polyethylenglycol (PEG), were selected due to their capacity to efficiently encapsulate the hydrophobic anticancer compounds, plitidepsin and docetaxel. We have intensely investigated the effect of PEG, using two different pegylation degrees (24 and 57% w/w of PEG), to improve the circulation time and modify the pharmacokinetic behavior of the encapsulated plitidepsin. Plitidepsin-loaded PGA-PEG nanocapsules with a high PEG content increased 2 fold the t1/2 and the MRT compared with plitidepsin dissolved in Cremophor® EL, used as a reference formulation. Besides, the AUC of loaded nanocapsules was 5 fold higher than value obtained for the reference formulation. *in vivo* experiments showed that high pegylated systems combining a reduction of toxicity of plitidepsin, were well tolerate than the reference formulation, with a strong inhibition of tumor growth in a xenograft model of human renal cancer (MRI-H-121). Further, PGA-PEG nanocapsules loaded with docetaxel display a high antitumor activity in a glioma U87 mice model, inhibiting tumor growth in a 60% compared to the control (saline serum) and showing a significantly improvement over the survival rates compared with Taxotere®.

In a second stage, we evaluate the potential of another type of nanocapsules, polyarginine (PARG) nanocapsules as a new carrier for oral administration of an antitumor drug. The study performed in Caco-2 cells revealed that PARG nanocapsules can open the tight-junctions between epithelial cells, suggesting their capacity to enhance the paracellular transport of drugs. Moreover, PARG nanocapsules were internalized by

the monolayer in a very efficient manner. Their permanece in the gastrointestinal tract was confirmed by *in vivo* imaging studies using fluorescence technology.

All these results highlight the potential of polyaminoacids nanocapsules for improving parenteral and oral absorption of antitumor drugs.

Listado de abreviaturas

ANOVA	Analysis of variance	Análisis de varianza
AUC	Area under the curve	Area bajo la curva
BCK	Benzalkonium chloride	Cloruro de Benzalconio
BSA	Bovine serum albumine	Albumina sérica bovina
C max	Peak concentration	Concentración máxima
CLp	Plasmatic cleareance	Aclaramiento plasmático
DCX	Docetaxel	Docetaxel
HP	High pegylated	Alta PEGilación
HPLC	High prefomarce liquid cromatography	Cromatografía líquida de alta eficacia
IV	Intravenus injection	Inyección intravenosa
IVIS	*in vivo* imaging system	Sistema de imágenes *in vivo*
LDA	Laser doppler anemometry	Anemometría láser doppler
LNC	Lipidic nanocapsules	Nanocapsulas lipídicas
MDR	Multi Drug Resistance	Múltiple resistencia 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	Nanoemulsions	Nanoelmulsiones

NMR	Nuclear magnetic resonance	Resonancia magnetica nuclear
ODN	Oligodeoxynuceotide	Oligodesoxinucleotido
PACA	Poly(alquilcyaoacrylate)	Poli(alquilcianoacrilato)
PARG	Polyarginine	Poliarginina
PBCA	Poly(isobutylcyanoacrylate)	Poli(isobutilcianoacrilato)
PCL	Poly-epsylon-caprolactone	Poli-epsiolon-caprolactona
PCS	Photon correlation spectroscopy	Espectroscopia de correlacion fotonica
PEG	Polyethileglicol	Polietilenglicol
PGA	Poly glutamic acid	Ácido poli-l-glutámico
PLA	Poly lactic acid	Ácido Polilactico
PLGA	Poly lactic glyclic acid	Ácido Polilactico Glicolico
PTL	Plitidepsin	Plitidepsina
RES	Reticular endothelial system	Sistema retículo endotelial
SEM	Scanning electron microscopy	Microscopia electronica de barrido
siRNA	Small interfering RNA	RNA de interferencia
siRNA – AS	siRNA antisense	siRNA antisentido
t1/2	Half life	Semivida de absorción
t1/2α	Distribution half live	Semivida de distribucion
t1/2β	Elimination half live	Semivida de eliminación

Vd_B	Distribution volume	Volumen de distribución
Vdss	Distribution volumen (steady state)	Volumen de distribución (estado de equilibrio)
W/O	Water in oil simple emulsion	Emulsión simple agua en aceite
O/W	Oil in Water simple emulsion	Emulsión simple aceite en agua

CAPÍTULO 1. Introducción

Introducción

La aplicación de la nanotecnología en el ámbito del diagnóstico y tratamiento del cáncer ha dado lugar a importantes logros, que han contribuido a la mejora de la eficacia y seguridad de fármacos utilizados en la quimioterapia tradicional[1].

El gran inconveniente de la mayoría de los fármacos antitumorales actualmente comercializados sigue siendo la baja permanencia plasmática y distribución indiscriminada en el organismo, que hace que las células sanas resulten también afectadas, lo que da lugar a una inevitable toxicidad sistémica. Esta falta de especificidad, junto con los fenómenos de resistencia a fármacos, representa el principal obstáculo en la eficacia clínica de los medicamentos oncológicos[2].

Dentro de la nanomedicina podemos encontrar un gran número de plataformas cuyo objetivo es que el fármaco alcance de la manera más eficaz la diana tumoral, tales como nanopartículas, nanocápsulas, liposomas, micelas, conjugados, etc. Una descripción pormenorizada sobre algunos de los sistemas más avanzados en investigación se recoge en el capítulo 2 de esta memoria, por lo tanto, en esta breve introducción nos centraremos en la descripción de los sistemas conocidos como nanocápsulas.

Las nanocápsulas

Son sistemas nanométricos vesiculares formados por un reservorio interno, que puede estar constituido por agua o aceite, rodeado de una cubierta polimérica[3] (Figura 1). El núcleo tiene la función de

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1 **Blanco, E., A. Hsiao, et al.** (2011). "Nanomedicine in cancer therapy: Innovative trends and prospects." Cancer Sci. **102**(7): 1247-1252.

2 **Wang, A. Z., R. S. Langer, et al.** (2011). "Nanoparticle Delivery of Cancer Drugs." Annual review of medicine **63**(1).

3 **Mora-Huertas, C. E., H. Fessi, et al.** (2010). "Polymer-based nanocapsules for drug delivery." International Journal of Pharmaceutics **385**(1-2): 113-142.

favorecer la encapsulación del fármaco y protegerlo del entorno fisiológico. La cubierta polimérica, además de aportar estabilidad al sistema dentro y fuera del organismo, puede tener distintas funciones: interaccionar con membranas celulares y favorecer el transporte del fármaco, su acceso a las células diana, y/o controlar su liberación del fármaco[4].

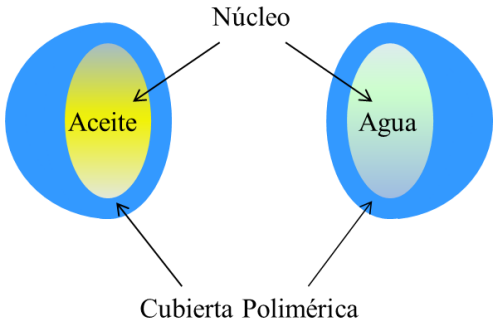

Figura 1: Representación de la estructura de nanocápsulas de núcleo oleoso y acuoso

Han sido desarrollados muchos métodos para la producción de las nanocápsulas y están basados en diferentes principios físico químicos, estos son: (i) polimerización interfacial[5] (ii) desplazamiento del solvente[6], (iii) inversión de fase por temperatura[7] y (iv) la adsorción de un polímero a una nanoemulsión preformada[8]. Moléculas de diferente naturaleza han sido eficientemente encapsuladas en las nanocápsulas, incluyendo tanto

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

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

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

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

[8] **Prego, C., M. Fabre, et al.** (2006). "Efficacy and Mechanism of Action of Chitosan Nanocapsules for Oral Peptide Delivery." Ibid. **23**(3): 549-556.

fármacos hidrofóbicos, los antitumorales[9,10], como moléculas altamente hidrofílicas, como el siRNA[11].

Las nanocápsulas pueden dividirse en dos grandes grupos dependiendo de los materiales empleados: nanocápsulas constituidas por materiales sintéticos y por biomateriales.

Nanocápsulas constituidas por polímeros sintéticos

La primera generación de nanocápsulas pertenecientes a esta clase fueron desarrolladas por el grupo de Couvreur[12] en los años 70 utilizando el poli(alquilcianoacrilato) (PACA) como material de recubrimiento. Estos nanosistemas obtenidos mediante polimerización interfacial[13], se han aplicado fundamentalmente a la encapsulación de moléculas hidrofóbicas. Aunque también, moléculas hidrosolubles como la calcitonina o la insulina, han sido encapsuladas previa formación de una suspensión en la fase oleosa y posterior formación instantánea de la cubierta alrededor de las gotículas de aceite[14].

En el primer caso, las nanocápsulas se forman por una rápida polimerización de los monómeros de alquilcianoacrilato en la interfaz de emulsiones aceite en agua (O/W) o agua en aceite (W/O) obteniéndose nanocápsulas con núcleos oleosos o acuosos, respectivamente. El diámetro de estas nanocápsulas ha resultado ser de 200-350 nm, aunque recientemente se ha descrito la posibilidad de reducir el tamaño de las

[9] **Renoir, J.-M., B. Stella, et al.** (2006). "Improved anti-tumoral capacity of mixed and pure anti-oestrogens in breast cancer cell xenografts after their administration by entrapment in colloidal nanosystems." The Journal of Steroid Biochemistry and Molecular Biology **102**(1–5): 114-127.

[10] **Lozano M. V., Lollo G**., et al. (Submitted). "Polyarginine nanocapsules: a new platform for intracellular drug delivery."

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

[12] **Couvreur, P., P. Tulkens, et al.** (1977). "Nanocapsules: a new type of lysosomotropic carrier." FEBS letters **84**(2): 323-326.

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

[14] **Aboubakar, M., F. Puisieux, et al.** (1999). "Study of the mechanism of insulin encapsulation in poly(isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization." Journal of Biomedical Materials Research **47**(4): 568-576.

nanocápsulas hasta 100 nm modificando adecuadamente la proporción de tensoactivos[15].

Como se mencionó anteriormente, además del método de polimerización interfacial, las nanocápsulas de PACA pueden ser obtenidas por desplazamiento del disolvente. Esta técnica fue descrita en un principio por Fessi[16] y está basada en la emulsificación espontánea del aceite debido a la difusión de un solvente orgánico (en el cual el polímero y el aceite se encuentran dispersos) en agua. Las nanocápsulas se forman debido a la precipitación del polímero pre-formado en la superficie de la nanoemulsión. Los sistemas obtenidos mediante esta técnica tienen un tamaño de entre 150 y 300 nm.

Otros polímeros acrílicos que se han empleado en la formulación de nanocápsulas son los Eudragit®, compuestos a base de polimetacrilatos, que responden fisicoquímicamente a cambios de pH, lo que puede ser empleado en la mejora de la biodisponibilidad de fármacos tras su administración por vía oral[17].

Los poliésteres como la poli-ε-caprolactona (PCL), el ácido poliláctico (PLA) y su copolímero el ácido poli(láctico-glicólico) (PLGA) han sido empleados también para la preparación de nanocápsulas por medio de la técnica del desplazamiento del disolvente[18,19,20]. Esta técnica ha permitido obtener sistemas con una tamaño entre 100 y 350 nm con un

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15 **Vauthier, C., D. Labarre, et al.** (2007). "Design aspects of poly(alkylcyanoacrylate) nanoparticles for drug delivery." J Drug Targeting **15**(10): 641-663.

16 **Fessi, H., F. Puisieux, et al.** (1989). "Nanocapsule formation by interfacial polymer deposition following solvent deplacement." Int. J. Pharm. **55**: 25-28.

17 **Nassar, T., A. Rom, et al.** (2008). "A novel nanocapsule delivery system to overcome intestinal degradation and drug transport limited absorption of P-glycoprotein substrate drugs." Pharm. Res **25**(9): 2019-2029.

18 **Fessi, H., F. Puisieux, et al.** (1989). "Nanocapsule formation by interfacial polymer deposition following solvent displacement." International Journal of Pharmaceutics **55**(1): R1-R4.

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

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

espesor de la capa polimérica de 1 a 20 nm[21]. Además, las propiedades de la superficie de estas nanocápsulas pueden ser modificadas dependiendo de los propósitos terapéuticos. Es posible de hecho obtener nanocápsulas pegiladas utilizando copolímeros como el PEG-PCL[22], el PEG-PLA[23] o el PEG-PLGA[24]. La técnica empleada para la obtención de estos sistemas permite que el polímero oriente las parte hidrofóbicas hacia el núcleo oleoso, mientras que las cadenas de PEG se disponen en la superficie.

Nanocápsulas constituidas por biopolímeros

Polímeros naturales como los polisacáridos han sido ampliamente empleados para la formación de nanocápsulas. Nuestro grupo ha descrito por primera vez la preparación de nanocápsulas de quitosano mediante una modificación de la técnica de la deposición interfacial. El quitosano es incorporado en la fase acuosa y su deposición en la interfaz aceite/agua (O/W) tiene lugar debido a una interacción electrostática con la lecitina[25]. También hemos propuesto un método alternativo que prevé la formación de una nanoemulsión y la incubación de la misma en una solución de quitosano[26]. Esta técnica ha permitido obtener nanocápsulas de PEG-quitosano[27]. En este caso el PEG está orientado hacia la fase externa mientras que el quitosano debido a su naturaleza catiónica tiende a

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21 **Guinebretière, S., S. Briançon, et al.** (2002). "Nanocapsules of biodegradable polymers: preparation and characterization by direct high resolution electron microscopy." Materials Science and Engineering: C **21**(1–2): 137-142.

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

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

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

25 **Prego, C., D. Torres, et al.** (2006). "Chitosan-PEG nanocapsules as new carriers for oral peptide delivery: Effect of chitosan pegylation degree." J Control Release **111**(3): 299-308.

26 **Prego, C., D. Torres, et al.** (2006). "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 **6**(9-10): 2921-2928.

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

asociarse las gotículas cargadas negativamente disponiéndose en el la parte más interna. Asimismo, se han desarrollado nanocápsulas constituidas por ácido hialurónico para la administración IV de fármacos antitumorales. En este caso al tratarse de un polímero aniónico hemos adaptado el método mediante la incorporación de un tensoactivo catiónico para producir una interacción en la interface[28].

Nanocápsulas lipídicas

Una nueva generación de nanocápsulas denominadas nanocápsulas lipídicas (LNC), han sido preparadas por el grupo de Benoit[29]. Estos sistemas consisten un núcleo oleoso rodeado por un ligero recubrimiento a base de PEG-hidroxiestearato. Dichas nanocápsulas pueden ser preparadas por medio de un novedoso método de inversión de fase, libre de solventes. En este proceso todos los componentes del sistemas se mezclan dentro de la fase acuosa, posteriormente una serie de ciclos de temperatura es aplicada a la mezcla, alternando temperaturas altas y bajas durante el proceso[30]. El tamaño y la polidispersión de las nanocápsulas disminuyen en función de los números y de la temperatura de los ciclos. Una pequeña interfaz es creada sobre las nanogotículas de aceite debido a estos cambios de temperatura y el tensioactivo se concentra sobre dicha interfaz[31]. Finalmente el proceso termina en una temperatura inferior a la de inversión de fase (de la emulsión aceite/agua) seguido de la adición de agua fría. Este proceso rápido de enfriamiento-

[28] **Oyarzun-Ampuero, F., G. Rivera-Rodriguez, et al**. (Submitted). "Hyaluronan nanocapsules: a new safe and effective nanocarrier for the intracellular delivery of anticancer drugs."

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

[30] **Huynh, N. T., C. Passirani, et al.** (2009). "Lipid nanocapsules: A new platform for nanomedicine." International Journal of Pharmaceutics **379**(2): 201-209.

[31] **Anton, N., P. Gayet, et al.** (2007). "Nano-emulsions and nanocapsules by the PIT method: An investigation on the role of the temperature cycling on the emulsion phase inversion." Ibid. **344**(1-2): 44-52.

dilución permite la formación de las LNC con tamaño de partícula promedio de entre 20 y 100 nm.

Aplicaciones terapéuticas:

Nanocápsulas en la terapia del cáncer

Como se ha mencionado previamente, las limitaciones más importantes en la terapia del cáncer están relacionadas con la falta de especificidad de los fármacos tradicionales y los fenómenos de resistencia (MDR, *Multi drug resistance*) que suelen ocurrir tras repetidas administraciones[32,33]. Desde el punto de vista de la formulación, la mayoría de estos fármacos tienen una muy baja solubilidad en agua. Por lo tanto se necesitan cosolventes que muchas veces son los principales causantes de las reacciones adversas[34].

En este contexto las nanocápsulas representan una alternativa válida para la vehiculización de fármacos antitumorales. La encapsulación de dichos fármacos en el núcleo oleoso del nanosistema, ofrece una mayor solubilización y protección frente a la degradación en los fluidos biológicos[35]. El tamaño y las características de superficie del nanosistema puede facilitar su acumulación en el tejido tumoral mediante el efecto de incremento de la permeabilidad y retención (*Enhanced Permeability and Retention effect*)[36]. Además muchos estudios demuestran la inhibición del

[32] **Ehdaie, B.** (2008). "Application of Nanotechnology in Cancer Research: Review of Progress in the National Cancer Institute's Alliance for nanotechnology."

[33] **Gottesman, M. M., T. Fojo, et al.** (2002). "Multidrug resistance in cancer: Role of ATP-dependent transporters." Nat. Rev. Cancer **2**(1): 48-58, **Ehdaie, B.** (2008). "Application of Nanotechnology in Cancer Research: Review of Progress in the National Cancer Institute's Alliance for nanotechnology."

[34] **Gelderblom, H., J. Verweij, et al.** (2001). "Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation." European Journal of Cancer **37**(13): 1590-1598.

[35] **Brigger, I., C. Dubernet, et al.** (2002). "Nanoparticles in cancer therapy and diagnosis." Advanced Drug Delivery Reviews **54**(5): 631-651.

[36] Maeda, H., G. Y. Bharate, et al. (2009). "Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect." European Journal of Pharmaceutics and Biopharmaceutics 71(3): 409-419.

sistema MDR mediada por las nanocápsulas[37,38].

Existen numerosos trabajos relacionados con el uso de nanocápsulas en la formulación de fármacos antitumorales. En la decada de los 90, Lenaerts y colaboradores[39] encapsularon la ftalocianina, un importante agente en la terapia fotodinámica del cáncer, en nanocápsulas de PACA modificadas con poloxamer. Evidenciaron que el recubrimiento con algunos tipos de poloxámeros puede disminuir significativamente la captura de las nanocápsulas por parte de los órganos ricos en células fagocíticas favoreciendo su acumulación en el tumor. La concentración de este agente fotosensibilizante resultó ser, después de 12 horas de su administración, 200 veces más alta en el tumor que en otros órganos. Asimismo, las nanocápsulas lipídicas han demostrado ser vehículos adecuados para la encapsulación de taxanos. Específicamente la encapsulación de paclitaxel en estos sistemas aumentó su concentración en el sitio tumoral, llevando a una mayor reducción del volumen tumoral en comparación con el Taxol®[40]. El docetaxel, otro fármaco perteneciente a las clase de lo taxanos, fue eficazmente encapsulado en las nanocápsulas lipídicas y aumentó 5 veces el AUC del fármaco encapsulado en comparación con la formulación comercial Taxotere®[41].

Las nanocápsulas de quitosano modificadas con PEG, desarrolladas en nuestro grupo, han sido utilizadas para la liberación intracelular de docetaxel. Estas nanocápsulas resultaron ser vehículos adecuados para la encapsulación del fármaco en el núcleo oleoso y mostraron una elevada internalización del mismo dentro de las células,

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37 **Brigger, I., C. Dubernet, et al.** (2002). "Nanoparticles in cancer therapy and diagnosis." Advanced Drug Delivery Reviews **54**(5): 631-651.

38 **Shapira, A., Y. D. Livney, et al.** (2011). "Nanomedicine for targeted cancer therapy: Towards the overcoming of drug resistance." Drug Resistance Updates **14**(3): 150-163.

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

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

41 **Khalid, M., P. Simard, et al.** (2006). "Long Circulating Poly(Ethylene Glycol)-Decorated Lipid Nanocapsules Deliver Docetaxel to Solid Tumors." Pharm. Res. **23**(4): 752-758.

llevando a un aumento considerable del efecto citotóxico[42]. Asimismo, el docetaxel fue cargado en las nanocápsulas de poliarginina modificadas con PEG. Los resultados demostraron la validez de este sistema como vehículo para la administración parenteral de fármacos antitumorales, puesto que también potenciaron la acción del docetaxel favoreciendo su internalización celular[43].

El conjunto de los resultados recogidos en la literatura en relación a la utilización de nanocápsulas poliméricas en la terapia del cáncer indican que el éxito de las mismas está directamente relacionado con su capacidad protectora del fármaco encapsulado así como con su capacidad de vehiculizar el fármaco en el organismo favoreciendo su acumulación en el tumor.

Los poliaminoácidos como nuevos materiales de recubrimiento de nanoestructuras

La cubierta polimérica de las nanocápsulas puede conferir una vehiculización selectiva de los nanotransportadores hacia una diana específica. Un ejemplo de ello es el recubrimiento con el PEG de dichos sistemas, estrategia ampliamente utilizada para conferir propiedades furtivas, mejorar la biodistribución y farmacocinética de los vehículos una vez administrados por vía parenteral[44]. Otros polímeros empleados con la misma finalidad son los poliaminoácidos. Los poliaminoácidos son materiales biodegradables constituidos por largas cadenas de aminoácidos que pueden ser obtenidos por vía natural o por vía sintética y que han sido ampliamente utilizados como componentes de muchos nanosistemas

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

43 **Lozano M. V., Lollo G., et al.** (Submitted). "Polyarginine nanocapsules: a new platform for intracellular drug delivery."

44 **Huynh, N. T., E. Roger, et al.** (2010). "The rise and rise of stealth nanocarriers for cancer therapy: passive versus active targeting." Nanomedicine **5**(9): 1415-1433.

farmacéuticos[45]. Dentro de la gran cantidad de este tipo de polímeros que se han empleado con este fin destaca el poliaminoácido aniónico, el ácido poliglutámico, utilizado para incrementar la hidrofilia de la superficie de las partículas reduciendo así su rápida eliminación del torrente circulatorio[46].

Por otro lado el poliaminoácido catiónico, la poliarginina, ha demostrado su utilidad como promotor de la penetración celular, favoreciendo el paso de los fármacos asociados a través de la membrana plasmática[47].

Ácido Poliglutámico

El ácido poliglutámico (PGA) es un homopéptido aniónico, hidrofílico, biodegradable y no tóxico. Existen dos isoformas de este poliaminoácido, la α y la γ. La forma γ, obtenida por fermentación bacteriana está constituida por unidades de L-glutámico conectadas a través de enlaces entre el grupo α-amino y los γ-carboxilos del monómero adyacente. Debido a su reducida disponibilidad, esta isoforma y es poco utilizada en el ámbito tecnológico farmacéutico. La forma α, estructuralmente diferente y obtenida por síntesis, está constituida por unidades de L-glutámico unidas a través de enlaces amídicos (Figura 2). Los grupos γ carboxílicos libres están cargados negativamente y favorecen su solubilidad en agua. Además, los grupos α carboxilos pueden ser utilizados para enlazar otros polímeros, como el PEG, con la finalidad de obtener estructuras dibloque o grafted. La isoforma α es altamente

45 **Nair, L. S. and C. T. Laurencin** (2007). "Biodegradable polymers as biomaterials." Progress in Polymer Science **32**(8–9): 762-798.

46 **Romberg, B., J. M. Metselaar, et al.** (2007). "Poly(amino acid)s: Promising enzymatically degradable stealth coatings for liposomes." International Journal of Pharmaceutics **331**(2): 186-189.

47 **Takechi, Y., H. Tanaka, et al.** (2012). "Comparative study on the interaction of cell-penetrating polycationic polymers with lipid membranes." Chemistry and Physics of Lipids **165**(1): 51-58.

susceptible a los lisosomas así que puede ser fácilmente degradada en el organismo[48].

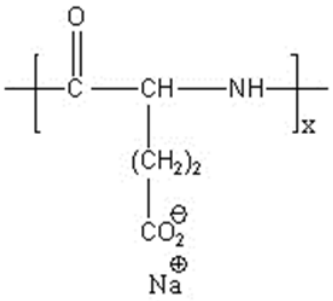

Figura 2: Estructura del ácido poli-L-glutámico

Debido a sus favorables propiedades físicoquímicas, el PGA ha sido ampliamente utilizado para el diseño y desarrollo de nuevas plataformas tecnológicas. Un ejemplo de ello es el Xyotax®, conjugado de PGA-docetaxel que se encuentra en fase clínica III[49]. Asimismo, el CT-2106, conjugado de ácido poliglutámico-glicina-camptotecina, actualmente en estudios de fase clínica II, ha demostrado favorecer el transporte y la acumulación de grandes cantidades de camptotecina en el tejido tumoral con respecto al fármaco solo[50].

Se han obtenido también micelas a partir del PGA y de PEG. El NC-6004 está constituido por micelas formadas a través de un complejo polímero-metal entre el copolímero PGA-PEG y el cisplatino. Actualmente se están evaluando protocolos para empezar la experimentación clínica de fase I[51],[52].

Poliarginina

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48 **Chun, L.** (2002). "Poly(l-glutamic acid)–anticancer drug conjugates." Advanced Drug Delivery Reviews **54**(5): 695-713.

49 **Jack W, S.** (2005). "Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane." J Control Release **109**(1–3): 120-126.

50 **Homsi, J., G. R. Simon, et al.** (2007). "Phase I Trial of Poly-l-Glutamate Camptothecin (CT-2106) Administered Weekly in Patients with Advanced Solid Malignancies." Clinical Cancer Research **13**(19): 5855-5861.

51 **Matsumura, Y.** (2008). "Polymeric Micellar Delivery Systems in Oncology." Japanese Journal of Clinical Oncology **38**(12): 793-802.

52 http://clinicaltrials.gov/ct2/show/NCT00910741?term=NC+6004&rank=1

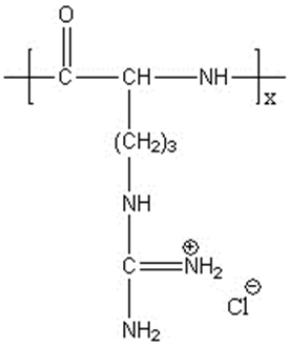

Figura 3: Estructura de la poliarginina.

La poliarginina es un poliaminoácido hidrofílico catiónico perteneciente a una clase de péptidos llamados péptidos promotores de la penetración celular[53] y se presenta como una interesante alternativa para favorecer la internalización de principios activos atreves de las membranas celulares[54] (Figura 3). Esta característica supone una ventaja a la hora de incrementar la baja permeabilidad de muchos fármacos actualmente administrados por vías sistémicas[55]. Ha sido demostrado como sistemas a base de poliarginina pueden incrementar el paso de macromoléculas hidrofílicas, a través del epitelio nasal[56]. Asimismo, estudios llevados a cabo en cultivo celulares han demostrado la capacidad de este polímero para abrir las uniones estrechas de las células favoreciendo el paso por vía paracelular de los fármacos asociados.

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53 **Takechi, Y., H. Tanaka, et al.** (2012). "Comparative study on the interaction of cell-penetrating polycationic polymers with lipid membranes." Chemistry and Physics of Lipids **165**(1): 51-58.

54 **Morishita, M., N. Kamei, et al.** (2007). "A novel approach using functional peptides for efficient intestinal absorption of insulin." Journal of Controlled Release **118**(2): 177-184.

55 **Takechi, Y., H. Yoshii, et al.** (2011). "Physicochemical Mechanism for the Enhanced Ability of Lipid Membrane Penetration of Polyarginine." Langmuir **27**(11): 7099-7107.

56 **Ohtake, K., T. Maeno, et al.** (2003). "Poly-l-Arginine Enhances Paracellular Permeability via Serine/Threonine Phosphorylation of ZO-1 and Tyrosine Dephosphorylation of Occludin in Rabbit Nasal Epithelium." Pharmaceutical Research **20**(11): 1838-1845.

CAPÍTULO 2.

Cáncer y Nanomedicamentos:
El auge de las nanoterapias oncológicas

Cáncer y Nanomedicamentos: El auge de las nanoterapias oncológicas

El término cáncer se utiliza para identificar y agrupar a un conjunto de más de cien enfermedades, todas ellas caracterizadas por un crecimiento celular acelerado e indiscriminado, que con el tiempo provocan la invasión y el daño a tejidos y órganos mediante la diseminación de éstas células a través del sistema sanguíneo y/o el sistema linfático. Dado que el cáncer es una enfermedad multifactorial que involucra múltiples mecanismos biológicos celulares, tales como la señalización y la apoptosis, las enfermedades enmarcadas con este nombre difieren significativamente unas de otras [1]. A pesar de los avances logrados en los últimos años, ésta enfermedad sigue siendo una de las causas de muerte más devastadoras a nivel mundial, apareciendo más de 10 millones de nuevos casos por año y produciendo la muerte de alrededor de 86 millones de personas a nivel mundial en el mismo intervalo de tiempo, principalmente, debido a la falta de un tratamiento eficaz y lo suficientemente accesible para combatir la enfermedad [2].

En la actualidad, la terapia contra el cáncer se encuentra mayoritariamente limitada a la radiación y quimioterapia, técnicas altamente invasivas e incomodas para el paciente y que en muchos casos conducen a la alteración de su salud integral. Los obstáculos más importantes frente a la consecución de una terapia oncológica eficaz se cifran en: (a) la distribución no especifica dentro del organismo de los fármacos antitumorales administrados, b) la incapacidad de alcanzar concentraciones lo suficientemente elevadas en el sitio del tumor y c) la resistencia desarrollada por las células cancerosas a diferentes tipos de quimioterapia. En este sentido, una de las herramientas principales con las que cuenta la medicina hoy en día es el uso de nanomedicamentos, entendiendo como tales a aquéllos sistemas terapéuticos que presentan un tamaño nanométrico (entre 1 y 1000 nm) que llevan asociado un principio activo en su estructura [3]. Se espera que en los próximos años los

avances en nanociencia y nanotecnología permitan desarrollar medicamentos, multifuncionales, y con una orientación selectiva a tejidos enfermos, capaces de atravesar barreras biológicas para liberar uno o múltiples agentes terapéuticos a nivel local, permitiendo se alcancen altas concentraciones de los mismos en tiempos apropiados y en condiciones fisiológicas específicas del área tumoral.

El objetivo de este artículo de revisión es el de presentar las diferentes estrategias terapéuticas desarrolladas hasta el momento basadas en la biodistribución selectiva también llamada orientación selectiva o "targeting". Estas estrategias se presentarán desde la perspectiva conceptual y del análisis crítico de los avances clínicos logrados hasta el momento.

La orientación selectiva o targeting de los nanomedicamentos

El "targeting" pasivo

Las estrategias adoptadas hasta el momento para conseguir la orientación y acumulación de los nanomedicamentos en las células tumorales se han basado en dos mecanismos diferenciados: el denominado "targeting" (direccionamiento o vehiculización) pasivo y el "targeting" activo.

El targeting pasivo consiste en el transporte de nanosistemas por simple convección a través de espacios intracelulares hacia el intersticio tumoral y su posterior acumulación en estos tejidos. El llamado efecto de permeabilidad y retención incrementados (Enhanced Permeability and Rentention (EPR) en inglés) explica este fenómeno (Figura 1). Este efecto, descrito inicialmente por Maeda [4], se fundamenta en la fisiología característica del endotelio de los capilares del tumor, cuyas células se encuentran frecuentemente separadas por espacios de entre 200 y 600 nm, permitiendo así el paso de nanoestructuras a través de ellas. Además, la

acumulación de las mismas en el tejido tumoral se ve favorecido por la pobre circulación linfática en este ambiente y la capacidad endocítica de las células tumorales hacia las citadas nanoestructuras [5, 6].

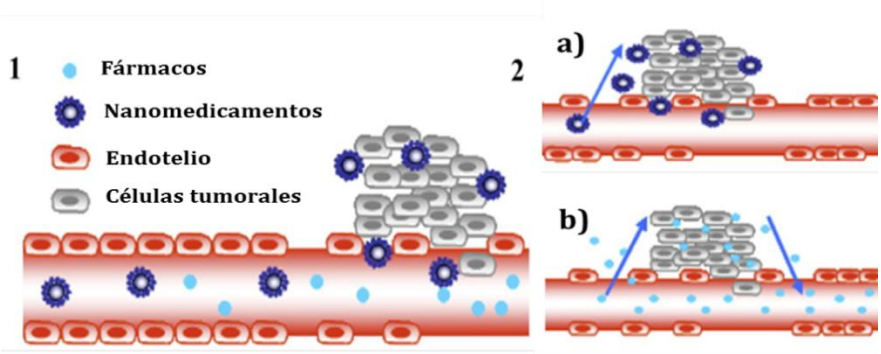

Figura 1.- Targeting pasivo. Representación esquemática del mecanismo de biodistribución selectiva por el efecto de permeabilidad y retención incrementado. En éste tipo de biodistribución selectiva los nanomedicamentos (nanosistema con fármaco asociado) y también los fármacos atraviesan fácilmente el endotelio de los vasos sanguíneso que irrigan el tumor debido a la existencia de grandes espacios en los mismos (1), los nanomedicamentos son retenidos debido a la pobre irrigación linfática (2a), mientras que los fármacos vuelven a circulación (2b), (Adaptado de [7], con permiso).

Además, se han identificado una serie de parámetros que influyen en el acceso de las nanoestructuras al tejido tumoral. Por ejemplo, se sabe que para que ocurra una extravasación eficiente a través de las fenestras del tejido tumoral los nanomedicamentos deben presentar un tamaño inferior a los 400 nm, no obstante, para evitar la filtración renal necesitan ser mayores a 10 nm y para que sean específicamente capturados por el hígado deben de presentar un tamaño menor a los 100 nm [7].

La carga superficial de las partículas, juega también un papel fundamental a la hora de conseguir nanomedicamentos de larga permanencia en el organismo después de su administración intravenosa o intramuscular. Dicha carga debe de ser preferentemente neutra o aniónica

para evitar la interacción de la nanoestructura con las opsoninas y, en general, con las células sanguínea [8]. La composición química y la hidrofilía de la superficie de los nanomedicamentos son otros dos factores de gran importancia a la hora de evitar el proceso de eliminación por el Sistema Fagocítico Mononuclear (Mononuclear Phagocitic System, MPS). Así, se sabe que las partículas con superficies hidrofilicas son generalmente "invisibles" para las células del MPS por lo que presentan un mayor tiempo de circulación, lo que aumenta las probabilidades de que accedan al tejido tumoral.

Para otorgar estas propiedades a los diferentes sistemas desarrollados, y por lo tanto mayores tiempos de permanencia en el organismo de los mismos, una de las herramientas más utilizadas es la modificación de la superficie de los nanovehículos mediante el uso de polímeros hidrofílicos [9]. La técnica más utilizada ha sido la denominada PEGilación [10], ya sea por el simple recubrimiento de los nanomedicamentos con PEG o modificando químicamente los componentes de los nanosistemas para que las cadenas del PEG queden expuestas en la superficie de los sistemas. Hasta la fecha se ha reportado la PEGilación de una gran variedad de nanosistemas con resultados bastante prometedores, en la mayoría de los casos aumentando considerablemente sus tiempos de vida media [11].

El "targeting" activo

El targeting activo hace referencia a la orientación activa del nanomedicamento, y no sólo una simple acumulación en los tejidos tumorales, motivada por su marcada especificidad hacia las células diana. Ésta especificidad se ha conseguido a través de procesos de reconocimiento celular aprovechando la sobreexpresión de varios tipos de receptores en la superficie de las células tumorales [12]. La acumulación de nanomedicamentos en el tumor ha demostrado incrementar

significativamente la efectividad terapéutica de los fármacos asociados, reduciendo a su vez la aparición de daños colaterales [13].

Varias son las técnicas empleadas en el desarrollo de nanomedicamentos dotados de una orientación específica, todas ellas relacionadas específicamente con características bioquímicas y fisiológicas particulares del tumor y con la sobreexpresión de receptores, condiciones del medio tumoral …etc. Todas ellas se han basado en la modificación de la superficie de los nanosistemas con diferentes tipos de moléculas o ligandos que van desde sencillas moléculas de bajo peso molecular a las más complejas macromoléculas. Un ejemplo de esto se muestra en la figura 2, donde se esquematiza el uso de nanomedicamentos funcionalizados con orientación a receptores superficiales en células tumorales [14].

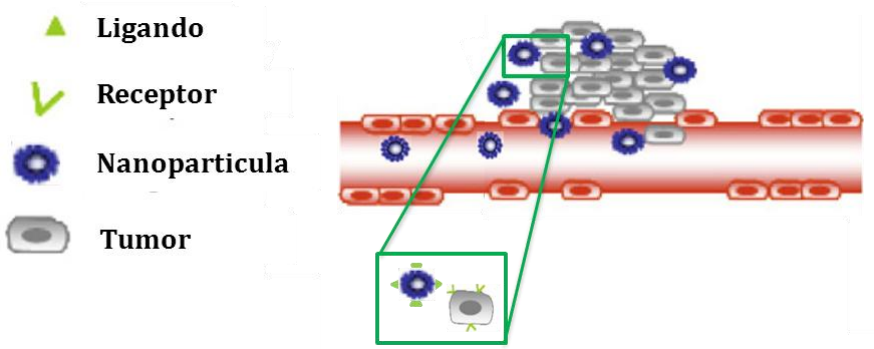

Figura 2.- Biodistribución activa. Representación esquemática del mecanismo de targeting activo, mediante el cual el nanomedicamento puede liberar el fármaco selectivamente en el tejido tumoral. La figura muestra la funcionalización del nanomedicamento con ligandos específicos a receptores sobreexpresados en las células tumorales (Adaptado de [7], con permiso)

El ejemplo más frecuente de moléculas de bajo peso molecular es el ácido fólico, sustrato principal del receptor folato, sobre-expresado en una gran cantidad de células tumorales como en el caso del cáncer ovárico

[15]. Asimismo, un ejemplo de macromolécula es el ácido hialurónico (AH), sustrato principal del receptor CD44 [16], sobre-expresado en una gran variedad de células tumorales, como en el ovárico, de estómago, de colon y varios tipos de leucemias [17]. Además de brindar propiedades de "targeting", el AH aporta propiedades escudo a los sistemas en que se ha empleado, lo que lo convierte en una interesante herramienta para conseguir los dos tipos de targeting en un mismo medicamento.

Otros receptores diana encontrados en células cancerosas son por ejemplo, el receptor del factor de crecimiento endotelial vascular (VEGFR por sus siglas en ingles) sobre-expresado en células cancerosas en procesos de angiogénesis, los receptores de transferrinas, los receptores de tirosin-quinasas, los receptores de crecimiento epidérmico humanos, y con mayor especificidad dependiendo del tipo de cáncer, diversos receptores de reciente descubrimiento como los CD44, HER-2, el receptor para la hormona liberadora de hormona luteinizante (LHRH) y los receptores de guanilil ciclasa C solo por mencionar algunos [13]. El acceso hacia estos receptores puede conseguirse mediante la funcionalización de los nanosistemas con los sustratos específicos.

Otra estrategia ampliamente difundida para conseguir una orientación activa es la funcionalización superficial de los nanosistemas con el uso de anticuerpos monoclonales [18]. El uso de este tipo de sistemas permite aumentar la especificidad del tratamiento a nivel celular. Actualmente, ésta estrategia se centra en algunas dianas como lo son las integrinas, annexinas, nucleolinas, VEGF, fosfatidilserinas, etc. [19].

Avances clinicos en el ámbito de los nanomedicamentos oncológicos

En la actualidad, el desarrollo de la nanomedicina ha llevado a que una gran variedad de nanomedicamentos se encuentren en un avanzado estado de desarrollo para su aplicación en la terapia del cáncer. Estos nanomedicamentos se presentan en diversas formas tales como

liposomas, conjugados poliméricos, micelas poliméricas y nanopartículas. A continuación se describen los nanomedicamentos ya comercializados o en avanzados estudios en fase clínica (Tabla 1).

Nanomedicamentos en forma de liposomas

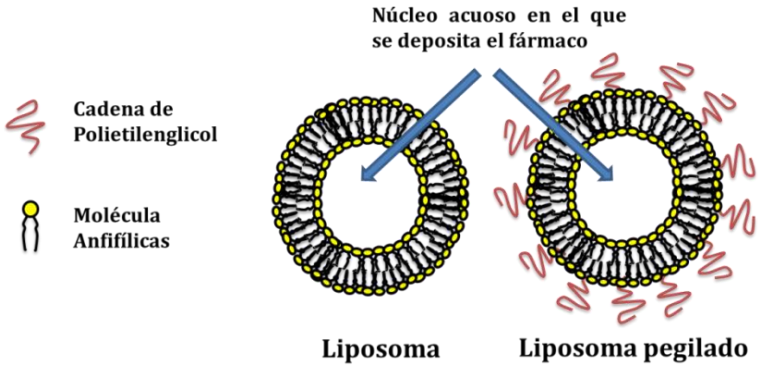

Figura 3.- Tipos de liposomas. Representación esquemática de dos diferentes presentaciones de liposomas, la forma más sencilla de los mismos a base de moléculas anfifílicas y a la derecha los liposomas modificados en su superficie con cadenas de PEG.

Los liposomas son vesículas artificiales constituidas, en su forma más simple, por una bicapa lipídica circundando una cavidad acuosa central (Figura 3) [20]. En una manera más compleja, los liposomas pueden contener una o múltiples bicapas alrededor de un núcleo y, dependiendo de la técnica de obtención empleada su tamaño puede comprender decenas o centenares de nanómetros. Su tamaño y características fisicoquímicas les permiten circular, penetrar y difundirse con resultados más óptimos a los obtenidos mediante un producto libre o una formulación farmacológica tradicional [21]. Las características que hacen de estos sistemas herramientas prometedoras en la vehiculización de fármacos son principalmente su carácter inerte, su elevada biocompatibilidad y sus aceptables perfiles de toxicidad y antigenicidad.

Las formulaciones liposomales son los primeros nanomedicamentos aprobados para su uso en humanos en el tratamiento de cáncer. Actualmente existen cuatro formulaciones diferentes comercializadas e indicadas para diferentes tipos de tumor (Tabla 1), dichos sistemas se diseñaron con el objetivo principal de encapsular fármacos antitumorales para aumentar su tiempo de vida media y disminuir los efectos adversos de los mismos.

Tabla 1.- Formulaciones liposomales actualmente comercializados o en fasse de evaluación clínica.

LIPOSOMAS			
Nombre comercial	**Fármaco**	**Indicación**	**Status (año)**
Doxil®	Doxorubicina	Cáncer de ovario, mama y sarcoma de Kaposi	Aprobado (2005)
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*

Dos de estos sistemas se han desarrollado con el interés particular de encapsular la antraciclina doxorubicina, el Doxil® y el Myocet®. El Doxil® se encuentra desde inicios del 2005 aprobado para su uso clínico en los Estados Unidos y el resto del mundo, y actualmente está indicado para el tratamiento del cáncer de ovario y en el sarcoma de Kaposi como monoterapia y, en asociación con el bortezomib, en el mieloma múltiple.

Por otra parte, el Myocet® se encuentra indicado en asociación con ciclofosfamida en el tratamiento de cáncer de mama metastático y en

el cáncer de ovario en Canadá y Europa, y en los Estados Unidos se encuentra en estudios clínicos avanzados. A pesar de que ambas formulaciones poseen características y naturaleza similares, la principal y gran diferencia entre Doxil® y Myocet® radica en la PEGilación de la superficie del primer sistema. Con esta estrategia, descrita previamente, se ha conseguido incrementar el tiempo de circulación plasmática de la doxorubicina en más de 40 h con respecto a lo obtenido por el sistema sin pegilar [22].

El Daunoxome®, aprobado desde 1996 por la FDA como medicamento de primera línea en el tratamiento del Sarcoma de Kaposi, es un sistema liposomal no pegilado que encapsula daunorobicina y que ha conseguido mejorar considerablemente la farmacocinética del fármaco y aumentar la esperanza de vida de los pacientes tratados. Finalmente, el Onco-TCS® (Marqibo®) es otra formulación liposomal no pegilada 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 [23].

Además de las formulaciones aprobadas y comercializadas, en la actualidad se encuentran en progreso 512 estudios clínicos en los Estados Unidos y 17 en Europa (http://www.clinicaltrials.gov, Octubre, 2011) que comprenden sistemas liposomales para aplicaciones en el tratamiento del cáncer, lo que representa un futuro más que prometedor para este tipo de medicamentos.

Los nanomedicamentos en forma de nanopartículas

Las nanopartículas son sistemas matriciales elaborados a partir de una gran variedad de materiales de origen natural, semisintético o sintético, en su mayoría polímeros. Dentro de los polímeros naturales investigados, encontramos algunas proteínas como la albumina,

polisacáridos como el quitosano o el ácido hialurónico o polipéptidos y poliaminoácidos. Por otra parte, los materiales de origen sintético más empleados para el desarrollo de nanopartículas son los poliésteres y poliacrilatos. El material empleado afecta de manera importante a las propiedades y estructura de las partículas y condiciona de manera determinante sus posibles aplicaciones clínicas, empezando por la vía de administración [24].

Tabla 2.- Formulaciones de nanopartículas actualmente en fase de evaluación clínica.

NANOPARTÍCULAS				
Nombre comercial	**Fármaco**	**Composición**	**Indicación**	**Status (año)**
Abraxane ®	Paclitaxel	Albumina	Cáncer de mama	Aprobado (2005)
Livatag® (Transdrug®)	Doxorubicina	Polialquil-cianoacrilatos	Hepatocarcinoma	Fase I/II
NBTXR3	-	Cristales de óxido de hafnio	Sarcoma de tejido blando	Fase I
Panzem®	Metoxi-estradiol	Dispersión nanocristalina de 2-metoxiestradiol	Cáncer ovárico y glioblastoma multiforme	Fase III

Existe una formulación de nanopartículas aprobada para su uso en humanos y otras en avanzados estudios de fase clínicos tanto en Estados Unidos, Europa y Asía (Tabla 2). La formulación comercializada Abraxane® es un sistema a base de nanopartículas de albumina, diseñado para la vehiculización del paclitaxel. Actualmente se encuentra aprobado por la FDA y la EMA para su uso en humanos y está indicado para el tratamiento del cáncer de mama metastático. Éste sistema ha demostrado una mayor eficacia comparado con el medicamento tradicional para esta

terapia, el Taxol®. Esta eficacia se asocia a la posibilidad de administrar mayores dosis de paclitaxel evitando los efectos secundarios causados por los excipientes de los tratamientos actuales, por ejemplo el Cremophor®, aceite de ricino pegilado, en el Taxol® [25]. Por otro lado, estudios han demostrado que la albumina también juega un papel agonista en la efectividad del paclitaxel, debido a su interacción con dos proteínas en circulación sanguínea. Una de las proteínas es la gp60, localizada en la superficie del endotelio vascular, la cual facilita la acumulación de las nanopartículas en el fluido intersticial del tumor [26]. La segunda es la osteonectina o SPARC (siglas en inglés de proteína secretada, acida y rica en cisteína) que se encuentra en la superficie de una gran variedad de células tumorales e interacciona con la albúmina provocando la acumulación de las nanopartículas en las células tumorales [27].

Otro de los grandes avances en la clínica de las nanopartículas lo representa el Livatag® (tecnología Transdrug®), un sistema nanopartículado a base de poli-isocianoacrilatos diseñado para la vehiculización de doxorubicina [7]. Éste sistema actualmente en ensayos clínicos fase II ha mostrado la capacidad de aumentar significativamente la supervivencia en pacientes con carcinoma hepatocelular, en comparación a la conseguida con el tratamiento clásico de quimioembolización [28].

Cabe aclarar que dentro del arsenal de sistemas conocidos como nanopartículas existen 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 nanoscristales 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.

Los nanomedicamentos en forma de conjugados poliméricos

El término conjugado se refiere a nanoestructuras híbridas consistentes en polímeros enlazados covalentemente a un agente terapéutico [29]. Dentro de los conjugados poliméricos se distinguen dos grupos: conjugados polímero-proteína y conjugados polímero-fármaco. La posible estructura de estos conjugados se describe en la figura 4. El objetivo perseguido con estos conjugados va desde mejorar la estabilidad del fármaco y reducir su inmunogenicidad hasta conseguir una biodistribución más adecuada [30].

A continuación se describen las formulaciones basadas en esta estrategia que se encuentran comercializadas o en fase de evaluación clínica, cuyo conjunto se recoge en la Tabla 3. Se omite la presentación de conjugados que se encuentran en forma de micelas por ser éstos abordados en otra sección.

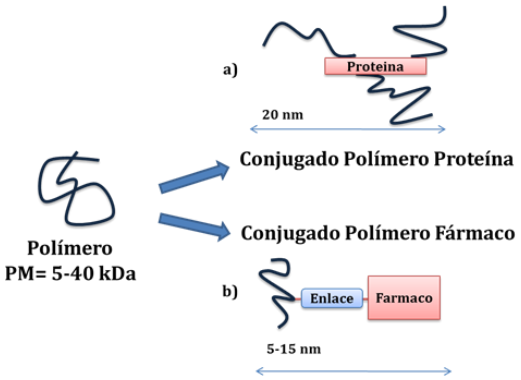

Figura 4.- Diferentes tipos de conjugados poliméricos. Se muestran los dos tipos de conjugados poliméricos que se encuentran en estudio clínico: aa) Conjugado polímero-proteína, en éste caso el ingrediente activo terapéutico es una proteína, pudiendo ser un enzima o un anticuerpo,b) Conjugado polímero-fármaco, en cuyo caso el ingrediente activo es una molécula terapéutica.

Conjugados polímero-proteína

En 1990, se comercializó el primer conjugado polimérico bajo el nombre de Zinostatin stimalamer®, sistema también conocido por las siglas SMANCS. Este sistema es un conjugado de estireno-anhídrido maléico (SAM) y la proteína con actividad antitumoral neocarzinostatina (NCS) indicado para el tratamiento de carcinoma hepatocelular. Este sistema consiguió aumentar considerablemente la lipofilia de la proteína y, de este modo, su asociación al agente de contraste Lipiodol®, permitiendo la visualización del tumor a la vez que un aumento del tiempo de vida media de la proteína.

Una de las estrategias de conjugación que merece ser destacada por su importancia es la PEGilación. El primer conjugado polímero-proteína, el Oncaspar®, comercializado en 1994, consiste en la unión covalente del enzima L-asparaginasa a una cadena de PEG. El Oncaspar® está indicado como tratamiento de primera línea en pacientes con leucemia linfoblástica. Mediante la conjugación del enzima se consiguió aumentar su tiempo de vida media pasando de horas a días, disminuyendo así la frecuencia de la administración. Además, la PEGilación permitió disminuir las reacciones de hipersensibilidad de la L-asparaginasa [31].

Otras dos formulaciones que se encuentran actualmente en estudios clínicos fase II para el tratamiento de melanoma y carcinoma renal son el PEG-Asys® y el PEG-Intron™, ambos consistentes en interferones alfa pegilados. El primero interferón alfa 2-a y el segundo alfa 2-b.

Conjugados polímero-fármaco

El Opaxio®, también conocido como Xyotax®, fue el primer conjugado polímero-fármaco en alcanzar la fase clínica III. Se trata de un conjugado del ácido poliglutámico, y el paclitaxel, que está siendo

estudiado para su indicación clínica en el tratamiento del cáncer de esófago, colorectal, mama, ovario y pulmón [32]. Esta formulación ha sido desarrollada para incrementar la solubilidad del fármaco y así evitar los efectos indeseable asociado al uso de disolventes lipídicos como el Cremophor®. Además, el paclitaxel así formulado ha conseguido aumentar su efectividad antitumoral.

El Prolindac® es otro conjugado polimérico construido a base de (hidroxipropil)meta-acrilamida (HPMA) para la vehiculización un análogo del platino, el oxaliplatino. De esta forma se ha conseguido aumentar la eficacia del fármaco, que actualmente se encuentra en estudios de fase clínica II para el tratamiento de cáncer de ovario [33, 34].

El polímero HPMA ha sido también utilizado para formar conjugados con la doxorubicina, estando dos formulaciones denominadas PK1 y PK2 en ensayos clínicos fase II. La primera de ellas, la PK1, se está ensayando para el tratamiento del cáncer de colon, mama y pulmón, habiendo conseguido una reducción considerable de la toxicidad sistémica de la doxorubicina [35]. La segunda de las formulaciones, la PK2, presenta la particularidad de poseer residuos de galactosamina que favorecen la acumulación hepática del complejo [30].

La PEGilación también ha dado buenos resultados en términos clínicos en la formación de conjugados polímero-fármaco, estando algunas formulaciones de éste tipo en avanzadas fases de estudios clínicos. Así, el sistema NKTR-102 [28], es un conjugado entre el fármaco irinotecan y el PEG que actualmente se encuentra en estudios de fase II con la indicación para el tratamiento de cáncer de colon, mama y ovario. Ésta formulación ha permitido aumentar su eficacia antitumoral en virtud de una mayor concentración de irinotecan en el tumor.

Otra formulación basada en la PEGilación, es la denominada Prothecan®, la cual a pesar de encontrarse actualmente descontinuada, sirve de base para explicar una interesante estrategia. Se trata de la

camptotecina pegilada con una doble finalidad: aumentar el tiempo de vida media del fármaco, y conservar la conformación de la lactona activa de la camptotecina. Los estudios clínicos en fase II demostraron una mejora considerable de la efectividad antitumoral del fármaco aunque similar a la conseguida con otros fármacos de la misma familia tales como el topotecan y el exatecan. Actualmente se está desarrollando una nueva formulación con un derivado de la campotecina, SN38, basada en esta estrategia [36]. 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, obteniéndose un aumento considerable de la residencia del fármaco dentro del tejido tumoral [37].

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

CONJUGADOS

Conjugados polímero-proteína				
Nombre comercial	**Proteína**	**Polímero**	**Indicación**	**Status (año)**
Zinostatin Stimalmer®	SMANCS	Estireno-anhídrido maléico	Carcinoma hepatocelular	Aprobado (1990)
Oncaspar®	L-asparaginasa	PEG	Leucemias	Aprobado (1994)
PEG-Asys®	Interferon α-2a	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	Hidroxipropil-metaacrilamida (HPMA)	Cáncer de ovario	Fase II
PK1	Doxorubicina	HPMA	Cáncer de mama, de pulmón y de colon.	Fase II
PK2	Doxorubicina	HPMA - Galactosamina	Carcinoma hepatocelular	Fase II
NKTR-102	Irinotecan	PEG	Cáncer de mama Cáncer colorectal, de pulmón y de ovario	Fase III Fase II
Prothecan®	Camptotecina	PEG	Cáncer gástrico y de esófago	Fase II
CRLX101	Camptotecina	Ciclodextrinas	Cáncer de pulmón no microcítico	Fase II

Los nanomedicamentos en forma de micelas

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 en el que comúnmente se deposita al fármaco y una superficie hidrofílica (Figura 5) [38]. Por su sencillez y versatilidad en cuanto a preparación y componentes empleados, las micelas son consideradas hoy en día como los nanomedicamentos con mayor potencial en clínica a corto plazo. En la tabla 4 se resumen los sistemas más avanzados hasta la fecha.

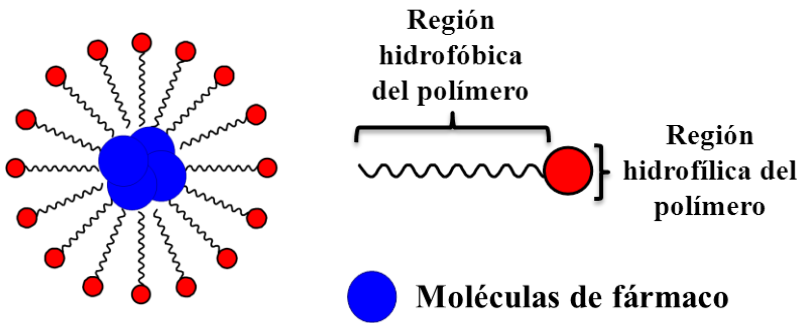

Figura 5.- Micelas. Esquema de una micela polimérica mostrando los componentes de la misma.

En la actualidad existen cinco formulaciones que se encuentran en avanzados estudios clínicos en la terapia de diferentes tipos de cáncer, todas ellas con resultados prometedores. Los taxanos, como el docetaxel y el paclitaxel, debido a su naturaleza hidrofóbica y a su muy baja solubilidad en agua son algunos de los candidatos ideales para ser formulados mediante ésta herramienta. Así, el Genexol® PM es una formulación consistente en micelas poliméricas, construidas por un polímero de tipo dibloque de ácido poliláctico-PEG (PLA-PEG)

encapsulando al paclitaxel. Los sistemas obtenidos presentan un rango de tamaños de los 20 a los 50 nm [39]. Estas micelas se encuentran en estudios clínicos de fase III en su indicación para cáncer de mama, de pulmón no microcítico y páncreas.

Otra formulación de paclitaxel, es el denominado NK105 [40], constituida por micelas de PEG-Poliaspartato con un tamaño medio de 80 nm. Esta formulación se encuentra en estudios clínicos de fase II en su indicación para el cáncer de estómago [41]. Finalmente, en lo referente a la vehiculización de paclitaxel en sistemas micelares, se encuentra el Paclical®, micelas a partir de vitamina A (en la plataforma denominada XR-17) que se encuentran en fase III para el tratamiento de carcinoma ovárico [30]. Este sistema presenta como principales ventajas la eliminación de la necesidad de pre-medicación y la eliminación de los efectos adversos causados por los principales excipientes de la formulación comercial de paclitaxel.

Tabla 4.- Formulaciones de micelas 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 estomago	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	Ácido poliglutámico-PEG	Cáncer pancreático	Fase I
SP1049C	Doxorubicina	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 gemtabicina

Otra formulación actualmente en evaluación clínica fase II para el tratamiento de cáncer de mama es el NK012, constituida por micelas de PEG-poliglutamato que contienen un análogo de camptotecina, metabolito del irinotecan, el SN38. 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 [42].

El cisplatino, incorporado en micelas de otro copolímero, el ácido poliglutámico-PEG, denominado NC-6004 (Nanoplatin™) [43] es otro sistema micelar indicado para el tratamiento del cáncer de páncreas en asociación con la gemcitabina, que se encuentra en fase II. Los resultados iniciales sugieren una reducción significativa de los efectos colaterales de neurotoxicidad y nefrotoxicidad asociados al cisplatino [44].

El docetaxel, un taxano, formulado en micelas de PEG-ácido poli-D-l-láctico[45], se encuentra en estudios clínicos fase I, en su indicación para el tratamiento del cáncer de mama bajo el nombre de Nanoxel-PM®. Ésta formulación ha conseguido una reducción significativa de los efectos adversos del Taxotere®.

Finalmente, el SP1049C, un sistema de micelas construidas a partir de una mezcla de copolímeros, Pluronic L61 y Pluronic F127, para la vehiculización de la doxorubicina se encuentra actualmente en fase clínica III, indicado para el tratamiento de adenocarcinomas y de cáncer de estómago [24]. Esta formulación presenta un perfil de toxicidad mucho más favorable que el del fármaco solo, además de tener actividad frente a tumores generalmente resistentes a la doxorubicina sola.

Áreas emergentes de la nanomedicina oncológica

Dentro de las áreas emergentes en nanomedicina oncológica destacan las terapias basadas en la aplicación de fuentes de energía externa (terapia fotodinámica), la terapia génica o el desarrollo de vacunas específicas contra la enfermedad.

Terapia fotodinámica basada en el uso de nanomedicamentos

La terapia fotodinámica es una técnica que se fundamenta en el uso de energía luminosa. El mecanismo de acción de ésta terapia conlleva el uso de compuestos denominados fotosensibles, los cuales al ser

irradiados por una fuente laser y en presencia de oxígeno, conducen a la formación de especies citotóxicas [58].

A pesar del éxito previsible de esta terapia, dentro de sus limitaciones cabe destacar la escasa estabilidad, el carácter hidrofóbico y la biodistribución indiscriminada de los agentes empleados. Por tanto, el uso de estrategias nanotecnológicas ofrece un panorama alentador al brindar la posibilidad de mejorar la estabilidad y solubilidad de los compuestos fotosensibles, a la vez que propiciar la orientación de dichos agentes en el organismo, consiguiendo una mayor especificidad de la terapia.

Al ser una estrategia relativamente nueva, no existen actualmente estudios clínicos de su aplicación en la terapia del cáncer, sin embargo, existen varios sistemas candidatos en desarrollo preclínico. Así por ejemplo, algunas formulaciones liposomales como el Foslip® o su análogo pegilado, FosPEG®, actualmente se encuentran en estudios preclínicos para el tratamiento del cáncer de mama encapsulando el agente fotosensible mTHPC [43, 44]. También han sido desarrollados con éste propósito nanopartículas de cerámica o metálicas modificadas en su superficie a fin de lograr su orientación especifica [39, 40]. Dentro de ellas destaca la formulación denominada Pc4SN consistente en nanopartículas de silicio que asocian el agente fotosensible PC4 y cuyo uso está previsto para el tratamiento de melanomas [42, 45].

Además del uso de la luz como fuente de energía para la excitación de compuestos en la terapia contra el cáncer, en la actualidad se están estudiando otro tipo de fuentes energéticas tales como energías térmicas, magnéticas o de captura de neutrones [59]. No obstante, y a pesar de la escasa toxicidad de estas terapias, una de las limitaciones asociadas al desarrollo clínico de las mismas reside en la incomodidad que representan para el paciente, por lo que su utilización que ha de venir compensada por un incremento significativo de su eficacia.

Terapia basada en el uso de nanopartículas magnéticas

El desarrollo de la ciencia de los materiales y la evolución de las técnicas para la consecución de nanopartículas a base de hierro, níquel o cobalto que exhiben propiedades magnéticas, llamadas nanopartículas magnéticas, ha permitido avanzar significativamente en cuanto a su potencial como terapias oncológicas. Una de las ventajas asociadas a esta terapia reside en su orientación selectiva mediada por el uso de fuerzas magnéticas [60].

El targeting por esta vía es llevado a cabo por medio del uso de un campo magnético externo, generalmente generado por magnetos de rara naturaleza o de campos y gradientes muy altos como aquellos compuestos por neodimio, hierro y Boro (Nd-Fe-B) [61]. El principio básico del targeting guiado mediante campos magnéticos es colocar un magneto dentro del tejido diana, por ejemplo dentro del tumor, para conseguir una acumulación de las nanopartículas orientadas sobre el mismo si es que éstas están asociadas a algún tipo de fármaco, o bien, para ocasionar mediante el mismo procedimiento la acumulación de las nanopartículas en los vasos sanguíneos circundantes al tumor con la finalidad de obstruirlos y aislar al tumor de los nutrientes necesarios, ésta última técnica ha sido diseñada y desarrollada desde inicios de 1970, sin embargo, problemas de biocompatibilidad e inestabilidad impidieron entonces mayores avances en esta área. Finalmente, otra estrategia ampliamente investigada en el uso de nanopartículas magnéticas es el de la hipertermia, ésta técnica se fundamenta en la producción de calor por parte de las nanopartículas magnéticas al ser expuestas a ciertos tipos de campos magnéticos por corriente alterna (AC), esto ocasiona el calentamiento de las mismas a más de 45ºC lo que produce daños considerables a las células cancerosas [62].

El uso de nanopartículas magnéticas en la terapia del cáncer no solo se limita a su aplicación terapéutica, algunos estudios han

demostrado que mediante el uso de estos sistemas es posible llevar a cabo el diagnóstico mediante técnicas de contraste. Actualmente, las nanopartículas magnéticas están siendo investigadas para la visualización de metástasis en los nodos linfáticos, algo que con las técnicas actuales es imposible de conseguir [63].

Terapias génicas

La posibilidad de inducir la expresión de una proteína terapéutica (insertando un gen funcional) o en el caso contrario de suprimir la expresión aberrante de una proteína (inhibiendo la expresión de un gen defectuoso) cuando ésta sea el origen de una determinada enfermedad, abre innumerables posibilidades para revolucionar la práctica clínica [64]. Como en otros casos, la nanotecnología ofrece interesantes oportunidades para proteger al material genético frente a su degradación y obre todo para conseguir su liberación selectiva a nivel intracelular.

Hasta el momento se han desarrollado dos tipos de sistemas para la transferencia de material genético, los virales y no virales o de naturaleza sintética [65]. Los sistemas sintéticos son los que aborda principalmente la nanotecnología en virtud de la combinación adecuada de biomateriales que pueden ser a su vez de origen natural o sintético. De la gran variedad de vehículos sintéticos desarrollados hasta el momento [66], cabe destacar la formulación liposomal denominada Allovectina 7®, la cual contiene una secuencia de ADN plasmídico que codifica la cadena pesada HLA-B7 y la microglobulina □2, ambos constituyentes del antígeno MHC-1[67]. El plásmido se combina con lípidos catiónicos y se inyecta intratumoralmente. Actualmente se encuentra en estudios clínicos en fase II indicado para el tratamiento del melanoma metastático y para el cáncer de cabeza y cuello [68].

Otra formulación liposomal de éste tipo, y que actualmente se encuentra iniciando estudios clínicos en fase I [69], es la Atu-027,

construido a partir de lípidos catiónicos pegilados, indicado para el tratamiento de tumores sólidos. La molécula de siRNA en este caso está diseñada para la inhibición de la proteína quinasa N3 (PKN-3), la inhibición del mecanismo de ésta proteína está asociado con varios mecanismos antiangiogénicos [70].

Por último, existe una formulación de nanopartículas, a base de un polímero linear de ciclodextrinas, funcionalizadas con transferrina, denominada CALAA-01, que se encuentra actualmente en estudios de fase clínica I para el tratamiento de tumores sólidos. El ingrediente activo es una molécula de ARN interferente pequeño (siRNA) capaz de reducir la expresión de la subunidad M2 de la ribonucleotido reductasa (R2), una enzima esencial requerida para la biogénesis del ADN [71] sobreexpresada en una gran variedad de cánceres gástricos asociada a la quimioresistencia, y cuya inhibición se ha descubierto como una interesante estrategia terapéutica, especialmente para las líneas tumorales MKN-1, MKN-7, and SNU-719 [72].

Nano-vacunas contra el cáncer

La aplicación de la nanotecnología al desarrollo de vacunas ofrece interesantes posibilidades al permitir diseñar nanosistemas que promueven la captación del antígeno por las células presentadoras de antígeno (CPA) [73]. Además, los nanosistemas permiten la incorporación de agentes adyuvantes auxiliares que al liberarse conjuntamente con el antígeno permiten aumentar su potencia, o bien para modular la respuesta inmune y dar lugar a respuestas celulares [74]. Las vacunas contra el cáncer ofrecen múltiples ventajas con respecto a las terapias tradicionales, principalmente debido a una especificidad incrementada, toxicidad reducida y el efecto a largo plazo producido por la memoria inmunológica. Dichas vacunas pueden desarrollarse como una estrategia profiláctica o terapéutica siendo

en ambos casos el objetivo la biodistribución selectiva hacia las células dendríticas [75].

Algunas formulaciones de liposomas y nanopartículas se encuentran actualmente en investigación, resaltando la L-BLP25 (o Stimuvax®), una formulación desarrollada en 1998, que consiste en un sistema liposomal, a base de colesterol, dimiristoil fosfatidilglicerol y dipalmitoil fosfatidilcolina, que encapsula al péptido sintético BLP25 y al adyuvante MPLA. Esta se encuentra actualmente en fase clínica III para el tratamiento de cáncer de pulmón no microcítico en Asia y ha mostrado un aumento considerable en la supervivencia de los grupos tratados [76].

Tendencias futuras en nanoterapias oncológicas: el nanoteranóstico

El desarrollo exponencial experimentado por la biología y la medicina molecular en las últimas décadas ha permitido dilucidar numerosos mecanismos celulares y moleculares involucrados en la aparición y evolución del cáncer lo que puede ser aprovechado por la nanotecnología para el diseño de terapias más eficaces, seguras y cómodas para el paciente, además para la detección cada vez más temprana de la enfermedad y la monitorización de la misma a través del tratamiento.

En este sentido, en los últimos años se ha ido forjando una nueva herramienta dentro de la nanomedicina contra el cáncer denominada "teranóstico", la cual precisamente consiste en la suma de las estrategias de diagnóstico, tratamiento y evaluación de la enfermedad en un mismo dispositivo nanométrico aprovechando los avances en el targeting y las técnicas de contraste actuales (Figura 6) [77].

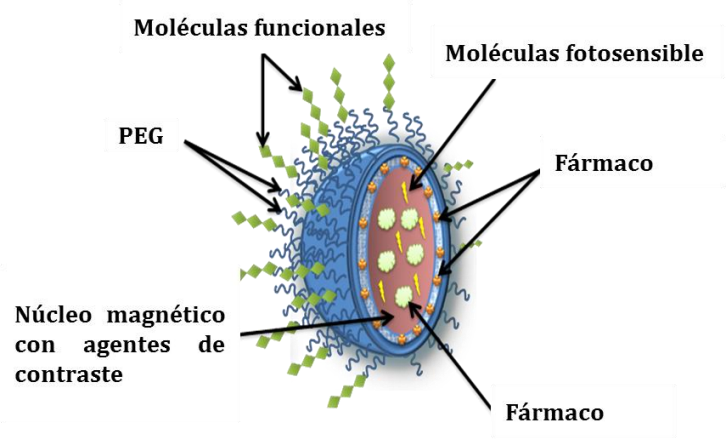

Figura 6.- Modelo de un sistema teranóstico. El nanosistema incluye además de un fármaco, un agente fotosensible, un agente magnético y agentes de contraste para permitir tanto la orientación selectiva como el diagnóstico y la monitorización de la terapia.

Dentro de las ventajas y soluciones que ofrece el teranóstico se encuentran entre otras, 1) la posibilidad de monitorizar en tiempo real la biodistribución ya sea del fármaco administrado o del nanomedicamento en su conjunto, esto último siempre de la mano de la asociación entre un agente terapéutico y un agente de contraste en el sistema. 2) Analizar la distribución y acumulación del fármaco o nanomedicamento en el sitio de acción a través de técnicas de imagen o de contraste, utilizando técnicas como el PET o RMN. 3) Monitorizar los diferentes mecanismos de liberación de fármacos desde los nanomedicamentos. 4) Provocar o controlar la liberación de los fármacos desde el nanomedicamento a través del uso de energías externas que provoquen cierta reacción en la estructura del nanomedicamento. 5) Ayudar a la elección de una terapia determinada en el tratamiento, o en su caso ayudar a predecir la respuesta terapéutica de un nanomedicamento, esto por medio de la monitorización del nanomedicamento en el organismo, sabiendo donde se distribuye y donde se localiza el mismo podemos saber si dicha terapia es efectiva para

determinado tipo de tumor, por ejemplo en los casos de tumores que presentan el efecto EPR [78].

Y finalmente, el objetivo principal del teranóstico radica en 8) combinar el diagnóstico y el tratamiento de la enfermedad en una misma terapia. Por medio del uso de agentes de contraste, moléculas funcionales, sistemas nanométricos y el fármaco adecuado, es posible que los sistemas empleados en el nanoteranóstico permitan el desarrollo de sistemas capaces de diagnosticar o identificar el cáncer desde sus estadios más tempranos, permitiendo la visualización de las células anormales y su consecuente tratamiento farmacológico [79].

Conclusiones y perspectivas

Muchas son las estrategias utilizadas en el desarrollo de nanomedicamentos oncológicos, brindando la gran mayoría resultados prometedores en el tratamiento de diferentes tipos de cáncer. Es previsible, por tanto, que cada vez más frecuencia nos encontremos con sistemas de éste tipo como tratamientos de primera línea en la terapia del cáncer.

Además, la nanomedicina podría aportar un cambio sin precedentes en los paradigmas actuales referentes a la comprensión de la interacción de los fármacos y dispositivos terapéuticos con las células tumorales, en tiempo real y en una escala celular e incluso molecular. Éste conocimiento podría resultar en nuevas estrategias para el diagnóstico y la prevención de la enfermedad, ya que previsiblemente los nanomedicamentos permitirán la detección y el seguimiento de la enfermedad desde los primeros indicios de su aparición.

El objetivo a largo plazo de lo que podríamos denominar nano-oncología consiste en consolidar una medicina personalizada que pueda tratar el cáncer aún antes de que este se manifieste como una amenaza a la vida del paciente, mediante técnicas de reconocimiento especifico de

células cancerosas o a parámetros genéticos que determinen la enfermedad. Además, los nanomedicamentos permitirán tratar la enfermedad a la vez que, por medio de técnicas de imagen podrá realizar una monitorización en tiempo real de la evolución del mismo.

Referencias

1. Diccionario del Cáncer. National Cancer Institute. 2011 15-06-2011 [cited 2011 24-11-2011]; Available from: http://www.cancer.gov/diccionario/.

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

3. Ferrari, M., Cancer nanotechnology: opportunities and challenges. Nat Rev Cancer, 2005. 5(3): p. 161-171.

4. Maeda, H., G.Y. Bharate, and J. Daruwalla, Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. European Journal of Pharmaceutics and Biopharmaceutics, 2009. 71(3): p. 409-419.

5. Fang, J., H. Nakamura, and H. Maeda, 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.

6. Torchilin, V., Tumor delivery of macromolecular drugs based on the EPR effect. Adv Drug Deliv Rev, 2011. 63(3): p. 131-5.

7. Danhier, F., O. Feron, and V. Préat, To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. Journal of Controlled Release, 2010. 148(2): p. 135-146.

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

9. Gabizon, A. and D. Papahadjopoulos, The role of surface charge and hydrophilic groups on liposome clearance in vivo. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1992. 1103(1): p. 94-100.

10. Howard, M.D., et al., PEGylation of nanocarrier drug delivery systems: State of the art. Journal of Biomedical Nanotechnology, 2008. 4(2): p. 133-148.

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

12. Vila-Jato, J.L., Nanotecnología Farmacéutica: Realidades y posibilidades farmacoterapéuticas. Monografías2009, Madrid, España: Instituto de España, Real Academia Nacional de Farmacia. 409.

13. Byrne, J.D., T. Betancourt, and L. Brannon-Peppas, Active targeting schemes for nanoparticle systems in cancer therapeutics. Advanced Drug Delivery Reviews, 2008. 60(15): p. 1615-1626.

14. Wang, M. and M. Thanou, Targeting nanoparticles to cancer. Pharmacological Research, 2010. 62(2): p. 90-99.

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. Oyarzun-Ampuero, F.A., et al., A new drug nanocarrier consisting of polyarginine and hyaluronic acid. European Journal of Pharmaceutics and Biopharmaceutics, 2011. 79(1): p. 54-57.

17. Mizrahy, S., et al., Hyaluronan-coated nanoparticles: The influence of the molecular weight on CD44-hyaluronan interactions and on the immune response. J Control Release, 2011(0).

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

19. Fay, F. and C.J. Scott, Antibody-targeted nanoparticles for cancer therapy. Immunotherapy, 2011. 3(3): p. 381-394.

20. Lian, T. and R.J.Y. Ho, Trends and developments in liposome drug delivery systems. Journal of Pharmaceutical Sciences, 2001. 90(6): p. 667-680.

21. Malam, Y., M. Loizidou, and A.M. Seifalian, Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. Trends in Pharmacological Sciences, 2009. 30(11): p. 592-599.

22. Martin, F. Comparison of Liposomal Doxorubicin Products: Myocet Vs. DOXIL. Apples to Apples 2011 01/25/2011 [cited 2011 13-10-2011]; Available from: http://www.fda.gov/ ohrms/dockets/ ac/01/ slides/ 3763s2_08_martin/sld001.htm.

23. 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.

24. Hervella, P., V. Lozano, and M. Garcia-Fuentes, Nanomedicine: New Challenges and Opportunities in Cancer Therapy. Journal of Biomedical Nanotechnology, 2008. 4(3): p. 276-292.

25. Gelderblom, H., et al., Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. European Journal of Cancer, 2001. 37(13): p. 1590-1598.

26. Alexis, F., et al., Nanoparticle Technologies for Cancer Therapy Drug Delivery, M. Schäfer-Korting, Editor 2010, Springer Berlin Heidelberg. p. 55-86.

27. Desai, N., et al., SPARC Expression Correlates with Tumor Response to Albumin-Bound Paclitaxel in Head and Neck Cancer Patients. Translational Oncology, 2009. 2(2): p. 59-64.

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

29. Duncan, R., Polymer conjugates as anticancer nanomedicines. Nature Reviews Cancer, 2006. 6(9): p. 688-701.

30. Vicent, M.J. and R. Duncan, Polymer conjugates: nanosized medicines for treating cancer. Trends in Biotechnology, 2006. 24(1): p. 39-47.

31. Graham, M.L., Pegaspargase: a review of clinical studies. Advanced Drug Delivery Reviews, 2003. 55(10): p. 1293-1302.

32. Canal, F., J. Sanchis, and M.J. Vicent, Polymer–drug conjugates as nano-sized medicines. Current Opinion in Biotechnology, (0).

33. 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.

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

35. Li, C. and S. Wallace, Polymer-drug conjugates: Recent development in clinical oncology. Advanced Drug Delivery Reviews, 2008. 60(8): p. 886-898.

36. Pasut, G. and F.M. Veronese, PEG conjugates in clinical development or use as anticancer agents: An overview. Advanced Drug Delivery Reviews, 2009. 61(13): p. 1177-1188.

37. 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.

38. Blanco, E., et al., Nanomedicine in cancer therapy: Innovative trends and prospects. Cancer Science, 2011. 102(7): p. 1247-1252.

39. Oerlemans, C., et al., Polymeric Micelles in Anticancer Therapy: Targeting, Imaging and Triggered Release. Pharmaceutical Research, 2010. 27(12): p. 2569-2589.

40. NK105 Paclitaxel Micelle. 2011 [cited 2011 17-10-2011]; Available from: http://www.nanocarrier.co.jp/en/research/pipeline/01.html.

41. 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.

42. 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.

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

44. Uchino, H., et al., Cisplatin-incorporating polymeric micelles (NC-6004) can reduce nephrotoxicity and neurotoxicity of cisplatin in rats. British Journal of Cancer, 2005. 93(6): p. 678-687.

45. Lee, S.-W., et al., Development of docetaxel-loaded intravenous formulation, Nanoxel-PM™ using polymer-based delivery system. Journal of Controlled Release, (0).

46. Garcia-Fuentes, M., D. Torres, and M.J. Alonso, Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules. Colloids and Surfaces B-Biointerfaces, 2003. 27(2-3): p. 159-168.

47. Pinto Reis, C., et al., Nanoencapsulation II. Biomedical applications and current status of peptide and protein nanoparticulate delivery systems. Nanomedicine: Nanotechnology, Biology and Medicine, 2006. 2(2): p. 53-65.

48. Plapied, L., et al., Fate of polymeric nanocarriers for oral drug delivery. Current Opinion in Colloid & Interface Science, 2011. 16(3): p. 228-237.

49. Di Colo, G., Y. Zambito, and C. Zaino, Polymeric enhancers of mucosal epithelia permeability: Synthesis, transepithelial penetration-enhancing properties, mechanism of action, safety issues. Journal of Pharmaceutical Sciences, 2008. 97(5): p. 1652-1680.

50. Dai, J., et al., pH-sensitive nanoparticles for improving the oral bioavailability of cyclosporine A. International Journal of Pharmaceutics, 2004. 280(1–2): p. 229-240.

51. Morishita, M., et al., A novel approach using functional peptides for efficient intestinal absorption of insulin. Journal of Controlled Release, 2007. 118(2): p. 177-184.

52. Morishita, M. and N.A. Peppas, Is the oral route possible for peptide and protein drug delivery. Drug Discovery Today, 2006. 11(19-20): p. 905-910.

53. Prego, C., et al., Efficacy and Mechanism of Action of Chitosan Nanocapsules for Oral Peptide Delivery. Pharmaceutical Research, 2006. 23(3): p. 549-556.

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

55. Lee, E., et al., In vivo antitumor effects of chitosan-conjugated docetaxel after oral administration. Journal of Controlled Release, 2009. 140(2): p. 79-85.

56. Gaucher, G., et al., Polymeric micelles for oral drug delivery. European Journal of Pharmaceutics and Biopharmaceutics, 2010. 76(2): p. 147-158.

57. Peltier, S., et al., Enhanced Oral Paclitaxel Bioavailability After Administration of Paclitaxel-Loaded Lipid Nanocapsules. Pharmaceutical Research, 2006. 23(6): p. 1243-1250.

58. Paszko, E., et al., Nanodrug applications in photodynamic therapy. Photodiagnosis and Photodynamic Therapy, 2011. 8(1): p. 14-29.

59. Sharma, R. and C.J. Chen, Newer nanoparticles in hyperthermia treatment and thermometry. Journal of Nanoparticle Research, 2009. 11(3): p. 671-689.

60. Rozanova, N. and J.Z. Zhang, Metal and Magnetic Nanostructures for Cancer Detection, Imaging, and Therapy. Journal of Biomedical Nanotechnology, 2008. 4(4): p. 377-399.

61. Prijic, S. and G. Sersa, Magnetic nanoparticles as targeted delivery systems in oncology. Radiology and Oncology, 2011. 45(1): p. 1-16.

62. Jordan, A., et al., Endocytosis of dextran and silan-coated magnetite nanoparticles and the effect of intracellular hyperthermia on human mammary carcinoma cells in vitro. Journal of Magnetism and Magnetic Materials, 1999. 194(1-3): p. 185-196.

63. Nune, S.K., et al., Advances in lymphatic imaging and drug delivery. Advanced Drug Delivery Reviews, 2011. 63(10-11): p. 876-885.

64. Hu, Y.-L., et al., Mesenchymal stem cells: A promising targeted-delivery vehicle in cancer gene therapy. Journal of Controlled Release, 2010. 147(2): p. 154-162.

65. Rochlitz, C.F., Gene therapy of cancer. SWISS MED WKLY, 2001. 131: p. 4-9.

66. Jeong, J.H., T.G. Park, and S.H. Kim, Self-Assembled and Nanostructured siRNA Delivery Systems. Pharmaceutical Research, 2011. 28(9): p. 2072-2085.

67. Bedikian, A.Y. and M. Del Vecchio, Allovectin-7 therapy in metastatic melanoma. Expert Opinion on Biological Therapy, 2008. 8(6): p. 839-844.

68. Bedikian, A.Y., et al., A phase 2 study of high-dose Allovectin-7 in patients with advanced metastatic melanoma. Melanoma Research, 2010. 20(3): p. 218-226.

69. Study With Atu027 in Patients With Advanced Solid Cancer. 2011 November 06, 2011 [cited 2011 November 06, 2011]; Available from: http://clinicaltrials.gov/ct2/show/NCT00938574?term=atu+027&rank=1.

70. Aleku, M., et al., Atu027, a Liposomal Small Interfering RNA Formulation Targeting Protein Kinase N3, Inhibits Cancer Progression. Cancer Research, 2008. 68(23): p. 9788-9798.

71. Davis, M.E., The First Targeted Delivery of siRNA in Humans via a Self-Assembling, Cyclodextrin Polymer-Based Nanoparticle: From Concept to Clinic. Molecular Pharmaceutics, 2009. 6(3): p. 659-668.

72. Morikawa, T., et al., Expression of ribonucleotide reductase M2 subunit in gastric cancer and effects of RRM2 inhibition in vitro. Human Pathology, 2010. 41(12): p. 1742-1748.

73. O'Hagan, D.T. and R. Rappuoli, Novel approaches to pediatric vaccine delivery. Adv. Drug Delivery Reviews, 2006. 58(1): p. 29-51.

74. Vicente, S., A. Sanchez, and M.J. Alonso, Nanovacunas, in Nanotecnología Farmacéutica: Realidades y posibilidades farmacoterapéuticas, J.L. Vila-Jato, Editor 2009, Instituto de España, Real Academia Nacional de Farmacia: Madrid, España. p. 320.

75. Shurin, M.R., et al., Regulatory dendritic cells New targets for cancer immunotherapy. Cancer Biology & Therapy, 2011. 11(11): p. 988-992.

76. Sangha, R. and C. Butts, L-BLP25: A Peptide Vaccine Strategy in Non–Small Cell Lung Cancer. Clinical Cancer Research, 2007. 13(15): p. 4652s-4654s.

77. Pene, F., et al., Toward theragnostics. Critical Care Medicine, 2009. 37(1): p. S50-S58 10.1097/CCM.0b013e3181921349.

78. Kievit, F.M. and M.Q. Zhang, Cancer Nanotheranostics: Improving Imaging and Therapy by Targeted Delivery Across Biological Barriers. Advanced Materials, 2011. 23(36): p. H217-H247.

79. Lammers, T., et al., Nanotheranostics and Image-Guided Drug Delivery: Current Concepts and Future Directions. Molecular Pharmaceutics, 2010. 7(6): p. 1899-1912.

ANTECEDENTES, HIPÓTESIS Y OBJETIVO

Antecedentes

1. La nanomedicina representa un campo innovador con un potencial inmenso para mejorar el tratamiento del cáncer proporcionando nuevas oportunidades para la liberación específica de fármacos en el tejido tumoral reduciendo así su distribución indiscriminada en el organismo[57].

2. El diseño y desarrollo de nanocápsulas para la vehiculización de fármacos antitumorales, constituidas por un núcleo oleoso y una cubierta polimérica, permiten por un lado mejorar la formulación, evitando el uso de excipientes que pudieran ocasionar graves efectos adversos[58], y por otro favorecer la internalización de los principios activos que trasportan[59,60].

3. La modificación de la superficie de las nanocápsulas con polímeros de distinta naturaleza permite el diseño de sistemas aptos tanto para su administración por vía parenteral[61] como por vía oral[62]. Específicamente, nanoestructuras de ácido poliglutámico (PGA)[63] y sus derivados pegilados (PGA-PEG) han sido elaboradas con la finalidad de obtener sistemas estéricamente estabilizados que no sean reconocidos por los macrófagos del RES, lo cual permite que su aclaramiento del torrente circulatorio

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57 **Blanco, E., A. Hsiao, et al. (2011).** "Nanomedicine in cancer therapy: Innovative trends and prospects." Cancer Sci. 102(7): 1247-1252.

58 **Engels, F. K., R. A. A. Mathot, et al.** (2007). "Alternative drug formulations of docetaxel: a review." Anti-Cancer Drugs **18**(2): 95-103.

59 **Huynh, N. T., C. Passirani, et al.** (2009). "Lipid nanocapsules: A new platform for nanomedicine." Int. J. Pharm. **379**(2): 201-209.

60 **Lozano M. V., Lollo G., et al.** (Submitted). "Polyarginine nanocapsules: a new platform for intracellular drug delivery."

61 **Danhier, F., O. Feron, et al.** (2010). "To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery." Journal of Controlled Release **148**(2): 135-146.

62 **Prego, C., M. García, et al.** (2005). "Transmucosal macromolecular drug delivery." Ibid. **101**(1-3): 151-162.

63 **Chun, L.** (2002). "Poly(l-glutamic acid)–anticancer drug conjugates." Advanced Drug Delivery Reviews **54**(5): 695-713.

sea lento y su permanencia en el organismo aumente notablemente[64,65].

4. Es ampliamente reconocido el potencial del poliaminoácido poliarginina para promover la absorción de fármacos por vía oral[66]. Los nanosistemas constituidos por este poliaminoácido han demostrado ser capaces de atravesar membranas favoreciendo la internalización de los fármacos asociados[67]

[64] **Matsumura, Y.** (2008). "Polymeric Micellar Delivery Systems in Oncology." Japanese Journal of Clinical Oncology **38**(12): 793-802.

[65] **Jack W, S.** (2005). "Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane." Journal of Controlled Release **109**(1-3): 120-126.

[66] **Morishita, M., N. Kamei, et al.** (2007). "A novel approach using functional peptides for efficient intestinal absorption of insulin." Ibid. **118**(2): 177-184.

[67] **Takechi, Y., H. Yoshii, et al.** (2011). "Physicochemical Mechanism for the Enhanced Ability of Lipid Membrane Penetration of Polyarginine." Langmuir **27**(11): 7099-7107.

Hipótesis

1. El desarrollo de nanocápsulas constituidas por un núcleo oleoso y una cubierta a base de PGA, elaboradas a partir de la técnica de desplazamiento del solvente, pueden ser una estrategia adecuada para la vehiculización de fármacos hidrofóbicos antitumorales. La cubierta de PGA incrementa la hidrofilia de los sistemas, reduciendo la adsorción de opsoninas y por lo tanto, aumentando su permanencia en el organismo.

2. El uso de un copolímero pegilado de ácido poli-L-glutámico (PGA-PEG) en la superficie de las nanocápsulas otorga propiedades furtivas a los nanosistemas permitiendo su larga circulación en el torrente circulatorio. Asimismo esta modificación mejora los parámetros farmacocinéticos, incrementando la posibilidad de alcanzar una vehiculización pasiva vía EPR del fármaco asociado en el tumor.

3. Los nuevos sistemas nanocapsulares constituidos por un núcleo oleoso y una cubierta a base de poliarginina pueden comportarse como vehículos trasportadores de fármacos hidrofóbicos antitumorales tras su administración oral. Es importante destacar para ello no solo su capacidad para proteger la molécula encapsulada sino también para favorecer la internalización a través del epitelio intestinal.

Objetivo

Teniendo en cuenta los antecedentes expuestos y las hipótesis planteadas, el objetivo global de la presente memoria se ha dirigido al diseño de nanosistemas innovadores para la vehiculización de fármacos antitumorales partiendo de la plataforma de nanocápsulas. Asimismo, con este estudio se ha pretendido profundizar en los mecanismos de acción y en la valoración del potencial de las nanoestructuras desarrolladas como nanoterapias en el cáncer. Para lograr este objetivo se han planteado las siguientes etapas:

Desarrollo de nanocápsulas de PGA y de PGA-PEG como nuevas plataformas en la terapia del cáncer:

Esta etapa se ha dirigido al diseño y caracterización de sistemas nanocapsulares constituidos por el poliaminoácido PGA y su derivado pegilado PGA-PEG con distintos grados de PEGilación, para la vehiculización de fármacos antitumorales. Por ello se ha explotado el potencial de dichos vehículos con el fin de mejorar la biodisponibilidad y la eficacia antitumoral del fármaco asociado.

Se han llevado a cabo estudios *in vivo* en dos modelos tumorales: un modelo xenograft de tumor renal (MRI-H121) y un modelo de glioma (U87).

Los resultados de este apartado se recogen los capítulos 3, 4 y 5:

Capítulo 3: Polyglutamic acid and polyglutamic acid-polyethyleneglycol nanocapsules: a new nanocarrier for parenteral delivery of anticancer drugs.

Capítulo 4. Long circulating PEG-polyglutamic acid nanocapsules for an improved antitumor efficacy: *in vivo* evaluation in a murine solid tumour model.

Capítulo 5. Pegylated polyglutamic acid nanocapsules as carriers for anticancer drug delivery: *in vivo* proof-of-principle

Desarrollo de nanocápsulas de PARG y evaluación de su potencial en la administración oral de fármacos antitumorales.

El objetivo de esta etapa ha sido la investigación del potencial del recubrimiento con poliarginina de las nanocápsulas para la vehiculización oral de fármacos antitumorales. Para ello se ha evaluado el mecanismo de interacción del nanosistema con la línea celular Caco-2 y finalmente la biodistribución *in vivo* de los sistemas después de su administración oral.

Los resultados de este apartado se recogen en el capítulo 6.

Capítulo 6. A novel approach for oral delivery of peptides: polyarginine nanocapsules.

CAPÍTULO 3.

Polyglutamic acid and polyglutamic acid-polyethyleneglycol nanocapsules: a new nanocarrier for parenteral delivery of anticancer drug

Abstract

Designing nanocarriers that combine the capacity to encapsulate hydrophobic drugs with long-circulating properties is a critical objective for cancer nanomedicine. In this work, we disclose a new biodegradable nanocarrier for anticancer drug delivery based on nanocapsules coated with the polyaminoacid poly-L-glutamic acid (PGA) or with a polyethyleneglycol-grafted PGA copolymer (PGA-PEG). PGA and PGA-PEG nanocapsules were successfully prepared using a modified solvent displacement technique where the polyanionic polymer was electrostatically adsorbed onto a polycationic lipid core. The results showed that PGA and PGA-PEG nanocapsules have an average size in the range of 200 nm and negative zeta potential that changed depending on the presence of PEG on the surface of the nanocarriers. These nanocarriers could accommodate a significant amount of the lipophilic anticancer drug with encapsulation efficiencies above 90%. PGA and PGA-PEG nanocapsules showed very high stability in shelf-life studies performed at different temperatures and could be optionally freeze-dried for more prolonged storage times. *in vivo* studies in mice showed that plitidepsin-loaded PGA and PGA-PEG nanocapsules presented significantly higher maximum tolerated doses as compared to the reference formulation, a solution of plitidepsin in ethanol and Cremophor® EL. This indicates a significant reduction in formulation toxicity with the nanocapsules as compared to the conventional formulations. Finally, pharmacokinetic studies performed in mice showed a slow elimination of plitidepsin when encapsulated in PGA and PGA-PEG nanocapsules, which we attribute to the stealth properties derived from the polymeric coating of the nanocarriers.

Introduction

The clinical use of most anticancer drugs is associated to severe side effects and decreased quality of life for the patients. These effects are related to the biodistribution of large fractions of the drugs to non-target tissues, where they result in toxic effects to normal cells [1]. Interestingly, the side effects of many anticancer treatments are not only related to the active ingredient, but also to the excipients used for formulating them for parenteral administration. This is due to the extreme water insolubility of many anticancer drugs that requires the use of organic solvents to prepare conventional parenteral formulations. Typical organic solvents used in these formulations are Cremophor® EL/ethanol mixtures that have been deemed responsible of serious adverse effects including peripheral neuropathy and acute hypersensitivity reactions characterised by dyspnoea, flushing, rash, chest pain, tachycardia, hypotension, angio-oedema, generalised urticarial [2].

The limitations of conventional therapies have resulted in a great interest for the use of nanocarriers for anticancer drug delivery. From a technological perspective, the ideal nanocarrier should be well tolerated and capable of encapsulating highly hydrophobic drugs. Among the different delivery strategies, polymeric nanocapsules present many positive features for this application [3, 4, 5]. The oily core of polymeric nanocapsules is an ideal environment to encapsulate most antitumor drugs at high payloads, with good encapsulation efficacies and in a stable environment. On the other hand, the polymeric shell can be chosen to improve the biodistribution and the pharmacokinetic profile of the nanocarrier. Prolonged plasmatic circulation times of anticancer drugs have been linked to passive tumour targeting through the enhanced permeability and retention effect (EPR) [6,7]. This improved biodistribution combined with the safer formulation profile ultimately results in delivery systems with enhanced efficacy/toxicity ratios [8, 9].

Polyglutamic acid (PGA) is a highly anionic polyaminoacid composed of naturally occurring L-glutamic acid linked by peptide bonds. PGA is highly biocompatible and readily biodegradable by digestion with lysosomal enzymes. It is a suitable material for drug delivery design not only owing to its safety profile but also because it renders the surface of the nanocarriers more hydrophilic avoiding the uptake by the mononuclear phagocytic system [10]. This enhanced circulation times are important to achieve enhanced accumulation of anticancer drugs at the tumor site by the EPR effect, as it has been observed for PGA-paclitaxel® conjugates in phase III clinical trials [11, 12]. As a strategy to further increase the long circulation properties of PGA nanocapsules, we investigated the use of a PEG-grafted PGA (PGA-PEG) derivative as an alternative coating polymer [13, 14].

In this work, PGA and PGA-PEG nanocapsules were designed for the delivery of a new anticancer drug, plitidepsin. Plitidepsinis a highly hydrophobic cytostatic active ingredient originally isolated from the marine tunicate Aplidium Albicans, and now manufactured synthetically by PharmaMar S.A. as a potential treatment for a variety of cancers [15]. Unloaded and plitidepsin-loaded PGA and PGA-PEG nanocapsules were prepared and studied physicochemically, regarding their capacity to encapsulate plitidepsin and *in vivo* for their capacity to improve the maximum tolerated dose and the pharmacokinetic parameters and of the drug.

Materials and methods

Chemicals

Plitidepsinwas kindly provided by PharmaMar S.A. (Spain). Poloxamer (Pluronic F-68®), benzalkonium chloride (BKC) and poly-L-

glutamic acid (PGA) (Mw 15-50 KDa) were purchased from Sigma-Aldrich (Spain). Miglyol®812, which is neutral oil formed by formed by esters of caprylic and capric fatty acids and glicerol, was donated by Sasol Germany GmbH (Germany). The surfactant Epikuron 170, which is a phosphatidylcholine-enriched fraction of soybean Lecithin, was donated by Cargill (Spain).

Synthesis of PGA-PEG

PGA (100 mg, 0.662 mmol of repetition unit, Mn 10900 by multi angle laser light scattering, degree of polymerization 72) and MeO-PEG-NH2 (43.6 mg, 8.4 µmol, Mn 5219, Mw 5242 by MALDI-TOF) were dissolved in H2O (2 mL). l-Hydroxy-benzotriazole (11 mg, 84 µmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (13 mg 84 µmol) were added and the reaction was allowed to stir overnight. The resulting product was purified by ultrafiltration (Amicon YM30, 15 x 50 mL H2O) to afford 123 mg of PGA-PEG (degree of PEGylation 1.17 % by 1H NMR, 87 % yield, 24% w/w of PEG). 1H NMR (500 MHz, D2O): δ 4.51-4.16 (m, 72 H), 3.89-3.57 (m, 523 H), 3.43 (s, 3.5H), 2.65-1.84 (m, 288H). Preparation of PGA and PGA-PEG nanocapsules

Preparation of PGA and PGA-PEG nanocapsules

PGA nanocapsules were prepared by the solvent displacement technique [16, 17]. Briefly, the organic phase composed of plitidepsin (1.2 mg), 0.125 ml Miglyol® 812, 7 mg of the cationic surfactant BKC, 30 mg Epikuron 170 in 0.5 ml of ethanol and 9 ml acetone was added onto an aqueous phase composed of the non-ionic surfactant Pluronic 188 (0.25% w/v) and the polymer PGA or PGA-PEG. Nanocapsules were formed immediately upon the mixture of both phases. The organic solvents were

evaporated under vacuum. Unloaded nanocapsules were prepared by the same method, in absence of plitidepsin in the organic phase.

Nanoemulsions and PEG-coated nanoemulsions were used as controls to study the properties of the polymeric coating. They were obtained by the same technique as described above, but without adding PGA or PGA-PEG to the external water phase. Instead, PEG-surface modified nanoemusions were formed by including 6.42 mg of PEG-stearate to the organic phase, the calculated amount of modified lipid required for having the same amount of PEG than with the PGA-PEG coating.

Physicochemical Characterization of PGA and PGA-PEG nanocapsules

PGA and PGA-PEG nanocapsules were characterized with regard to size, zeta potential and morphology. Particle size and polydispersion index were determined by photon correlation spectroscopy (PCS) after dilution with bidistilled water. Analyses were carried out at 25ºC with an angle detection of 173º. The zeta potential values were calculated from the mean electrophoretic mobility values, as determined by laser Doppler anemometry (LDA). PCS and LDA analysis were performed in triplicate using a NanoZS® (Malvern Instruments, Malvern, UK).

The morphology of nanocapsules was studied by Transmission Electron Microscopy (TEM) using a Philips CM-12 (FEI Company, Eindhoven, The Netherlands), following negative staining with a phosphotungstic acid solution (2%, w/v) and immobilization on copper grids with Formvar®.

Plitidepsin encapsulation and release studies

The encapsulation efficiency of plitidepsin in the nanocapsules was determined by the difference between the amount of plitidepsin in the

supernatant and the total amount in the nanocapsules. Plitidepsin content in the supernatant was established upon isolation of the drug from the nanocapsules by ultrafiltration in Amicon columns (Amicon Ultra-4, 100000MWCO, Millipore, Spain). Then, samples of the supernatants or the nanocapsule suspension (for the total plitidepsin content) were dissolved in acetonitrile and analyzed by HPLC.

The *in vitro* drug release from the nanocapsules was performed in PBS (0,01 M) with 4% bovine serum albumin (BSA). Samples were incubated at 37°C and withdrawn at appropriate time intervals (15 min, 1 h, 3 h, 6 h and 24 h). Total plitidepsin content was determined by HPLC after dissolving a portion of each sample in acetonitrile, followed by mild centrifugation (3 min, 4000 g) to precipitate suspended proteins. Released plitidepsin was calculated upon isolation of the free drug by ultracentifugation (27400 g, 1 h, 15ºC). The supernatant was then analyzed by HPLC following the same treatment described above for total plitidepsin content.

The HPLC system consisted of an Agilent 1100 series instrument equipped with UV detector set at 225 nm. The analytic method for plitidepsin quantification has been previously reported by PharmaMar (Spain) [15].

Stability of plitidepsin-loaded PGA and PGA-PEG nanocapsules during storage

The stability of plitidepsin-loaded nanocapsules was evaluated under storage conditions for 8 weeks at 4ºC, room temperature and 37ºC. Three parameters were assessed at different time points: (i) macroscopic aspect (presence of aggregated, cream formation, changes in color, etc.); (ii) particle size, polydispersity and zeta potential; (iii) plitidepsin concentration in the preparation and encapsulation efficiency. All these characteristics were determined as described above.

Freeze-drying studies of plitidepsin loaded PGA nanocapsules

Blank and plitidepsin-loaded PGA nanocapsules at different concentrations between 1 and 0.5% w/v were freeze-dried by immersion in liquid nitrogen in the presence of trehalose (10% w/v). The freeze-drying programme consisted in an initial drying step at -35°C, and secondary drying were temperature was finally equilibrated at 20ºC over a period of 60 h (Labconco Corp., USA). PGA nanocapsules were resuspended by adding 1 mL of ultrapure water to the freeze-dried cake followed by gentle agitation. The size and polydispersity of the resuspended nanocapsules was evaluated by PCS.

in vivo studies

Animals

Studies were performed with CD-1 male mice (Harlan Interfauna Iberica S.L., Barcelona, Spain), housed Makrolon cages (10 animals/cage). Animals were subjected to preliminary observation and to an acclimatisation period. The animal house was maintained at 21-23ºC, with 35-55% relative humidity. Illumination was controlled to allow for 12 hours of light and 12 hours of darkness. All animals were observed for morbidity/mortality in the whole assay duration.

Toxicological evaluation

The Maximun Tolerated Dose (MTD) of different formulations was evaluated after intravenous administration. The formulations tested were: plitidepsin-loaded PGA nanocapsules, PGA-PEG nanocapsules,

nanoemulsions, PEG-coated nanoemulsions and the reference formulation (Cremophor®EL/Ethanol/Water 15/15/70 w/w/w) solution. The formulations at different plitidepsin doses (0.2-1 mg/kg) were administered as a single intravenous bolus (IV) in the lateral vein of the tail. Groups of 8 animals were used per each dose level. A control group consisting of 8 animals was administered with non-loaded nanocapsules, to evaluate potential toxicity. The animals were weighed at the start of the study, twice a week, and before being sacrificed. Mortality checks were performed at least once a day during the whole assay. Any mouse showing signs of extreme weakness, toxicity or in a moribund state was sacrificed. The animals were monitored at least once a day during the whole assay and any clinical responses were carefully noted. The observations included changes in weight, skin and fur, eyes and mucous membranes, respiratory, circulatory, central nervous and autonomic nervous systems, somatomotor activity and behavior.

Pharmacokinetic evaluation

Pharmacokinetic studies of plitidepsin were performed upon IV administration of different formulations to CD-1 mice. The formulations tested were: PGA nanocapsules, PGA-PEG nanocapsules, nanoemulsions and PEG-coated nanoemulsions. Mice with 20-25 g of weight were selected for these studies. A volume of 250 µl of the different plitidepsin formulations were injected in the lateral vein of the tail. The injected plitidepsin dose was 0.1 mg/kg for NE and PEG NE and 0.4 mg/kg for PGA s and PGA-PEG nanocapsule. Blood samples were collected in EDTA microtubes at the following times postinfusion: 5 min, 15 min, 30 min, 1 h, 3 h, 6 h, 24 h and 48 h. The samples were centrifuged at 4000 g for 15 minutes at approximately 5ºC. The resulting plasma was frozen at -20ºC until analysis by HPLC-MS/MS.

Plitidepsin concentrations were quantified by HPLC-MS/MS after solid-liquid extraction with a mixture of tert-butyl methyl ether (TBME): hexane (1:1, v/v). The pharmacokinetic parameters of plitidepsin were performed using a non-compartmental pharmacokinetic method with a WinNonlin™ Professional Version 4.01 (Pharsight Corporation, Mountain View, CA, USA). AUC values given are normalized to the dose given (0.1mg/kg).

Results and Discussion

This article describes for the first time the design of a novel drug nanocarrier platform based on polyglutamic acid nanocapsules (PGA nanocapsules). The rationale for selecting PGA as the coating polymer was its biocompatibility and its potential ability to provide stealth properties to the carrier [10, 18]. As an alternative, a PEG-grafted PGA (PGA-PEG) copolymer was also investigated as the coating material. Herein, we discuss the preparation of these nanocarriers, their physicochemical characterization, their capacity to load and release the anticancer drug plitidepsin, and their capacity to modify the toxicity and pharmacokinetics of this drug.

Preparation and characterization of plitidepsin-loaded PGA and PGA-PEG nanocapsules

Nanocapsules were obtained according to a modified solvent displacement technique where the coating polymer is deposited onto the oily core by electrostatic interaction. A similar approach had successfully been used before by our group to prepare chitosan and polyarginine nanocapsules [19-21]. However, in this case, the inclusion of a cationic surfactant, benzalkonium chloride, was required to drive the interaction between PGA and the nanoemulsion (Figure 1).

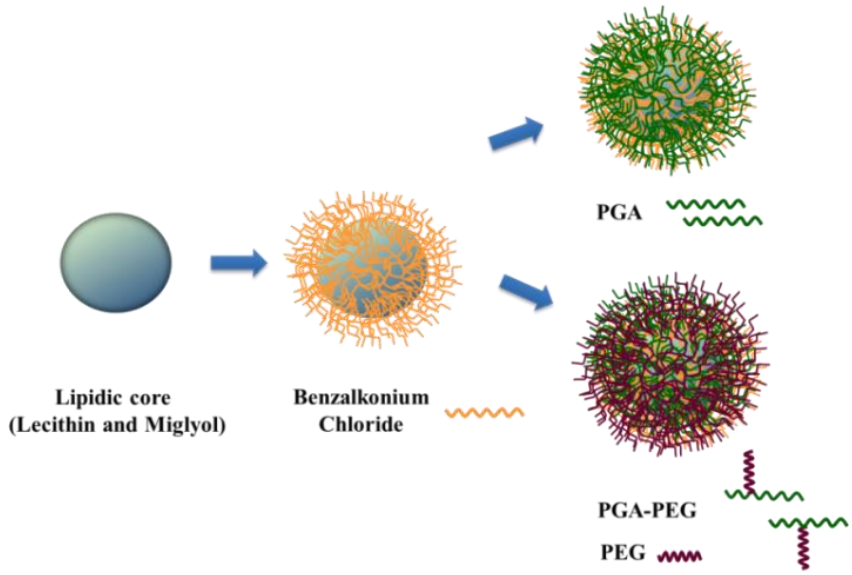


Figure 1: Illustration of the structure and preparation method of PGA and PGA-PEG nanocapsules.

The physicochemical characterization of blank and plitidepsin-loaded PGA and PGA-PEG nanocapsules is reported on Table 1. Also, data showing the physicochemical properties of two nanoemulsions is presented. The nanoemulsion was prepared as a control system made with the same composition as PGA nanocapsules, but without the PGA coating. The PEGylated nanoemulsion (PEG NE) is the same system as the NE, but adding enough PEG-stearate to have similar amounts of PEG in the system as compared to PGA-PEG nanocapsules.

The mean particle size of PGA and PGA-PEG nanocapsules was approximately 200 nm, corresponding to a monomodal and narrow size distribution (polydispersity index=0.1). The zeta potential was indicative of the different composition of these systems. Both PGA and PGA-PEG nanocapsules exhibited a polyanionic charge, whereas nanoemulsion and PEG NE have net positive charge. Considering that nanoemulsion has the same composition as compared to PGA nanocapsules except for the polymer coating, this charge inversion suggests that the electrostatically-

driven coating process is successful. PGA nanocapsules exhibited a negative zeta potential around -40 mV, while PGA-PEG nanocapsules displayed a more neutral zeta potential (-28 mV). This change is a first indication of the presence of PEG shielding the nanocapsules, as observed before for other nanocarriers [22]. Similarly, PEG NE had more neutral zeta potentials as compared to the control nanoemulsion.

Plitidepsin-loaded nanocarriers showed similar physicochemical properties than unloaded ones (Table 1). This might be related to an efficient incorporation of the drug in the oily core of the nanocarriers [23].

TEM images confirmed the approximate values of particle size for PGA and PGA-PEG nanocapsules as measured by PCS, and supported the homogeneity of the particle size distribution (Figure 2). TEM images indicated also that nanocapsules have a rounded and regular morphology, with the presence of a polymeric outer layer.

Table 1: Characterization of size and zeta potential of blank and plitidepsin-loaded nanocapsules and nanoemulsions (Mean ± S.D.; n=3). EE: Encapsulation efficiency; NCs: nanocapsules; NE: nanoemulsion.

Prototype	Plitidepsin conc. (mg/ml)	Size (nm)	P.I.	Zeta potential (mV)	E.E. (%)
NE	-	207 ± 7	0.1	+38 ± 1	-
	0.12	203 ± 7	0.1	+40 ± 1	95
PEG NE	-	200 ± 3	0.1	+26 ± 1	-
	0.12	203 ± 5	0.1	+28 ± 3	98
PGA NCs	-	202 ± 5	0.1	-40 ± 5	-
	0.12	183 ± 6	0.1	-38 ± 1	99
PEG-PGA NCs	-	191 ± 4	0.1	-28 ± 4	-
	0.12	201 ± 5	0.1	-28 ± 3	98

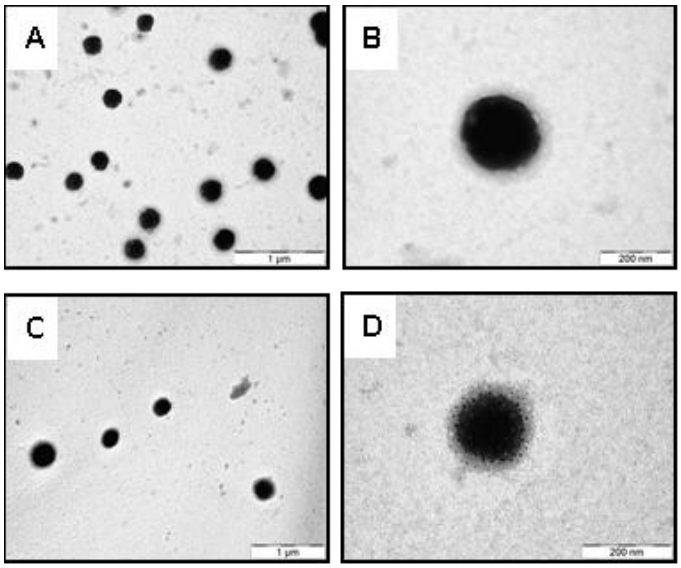


Figure 2: TEM images of PGA and PGA-PEG nanocapsules containing plitidepsin. PGA nanocapsules (A, B); PGA-PEG nanocapsules: (C, D).

Plitidepsin encapsulation and release from PGA and PGA-PEG nanocapsules

The content of plitidepsin in nanoemulsions, PGA and PEG-PGA nanocapsules was determined to establish the encapsulation efficiency of plitidepsin in the nanocapsules. Encapsulation efficiency was very high for PGA and PGA-PEG nanocapsules, 98-99% (Table 1). Plitidepsin encapsulation was also high for the control systems, NE and PEG NE, which suggest the high affinity of the drug for the hydrophobic core.

The release pattern of plitidepsin from PGA and PGA-PEG nanocapsules was studied upon incubation in simulated biological media (PBS with BSA 4% w/w, Figure 3). After one hour, PGA and PEG-PGA nanocapsules were shown to release 60% of their cargo. Afterwards, no further release could be observed for the remaining time of the experiment (24 h). This *in vitro* release profile presents the typical biphasic release characterized by an initial burst until partition equilibrium is reached with the external aqueous phase. The fact that a significant fraction of the drug

remained in the nanocapsules despite the high dilution is a further indication of the high affinity of plitidepsin for the oily core of the nanocapsules [9, 24, 25]. The presence of PEG in the nanocapsules shell did not affect the release properties of the nanocarriers (Figure 3).

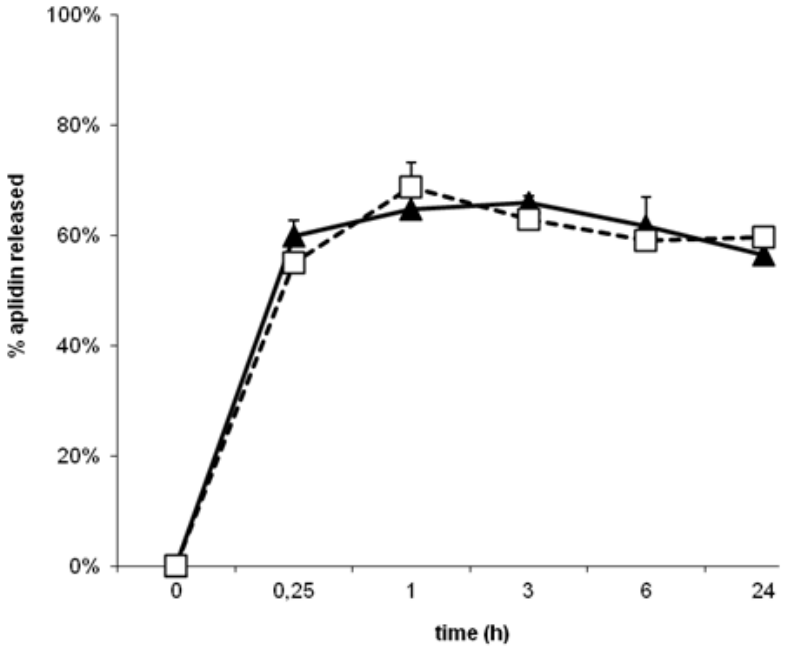


Figure 3: *in vitro* drug release from plitidepsin-loaded nanocapsule formulations in PBS with BSA (4% w/w) medium. PGA nanocapsules (□); PGA-PEG nanocapsules (▲). Data represents the mean ±SD, n=3.

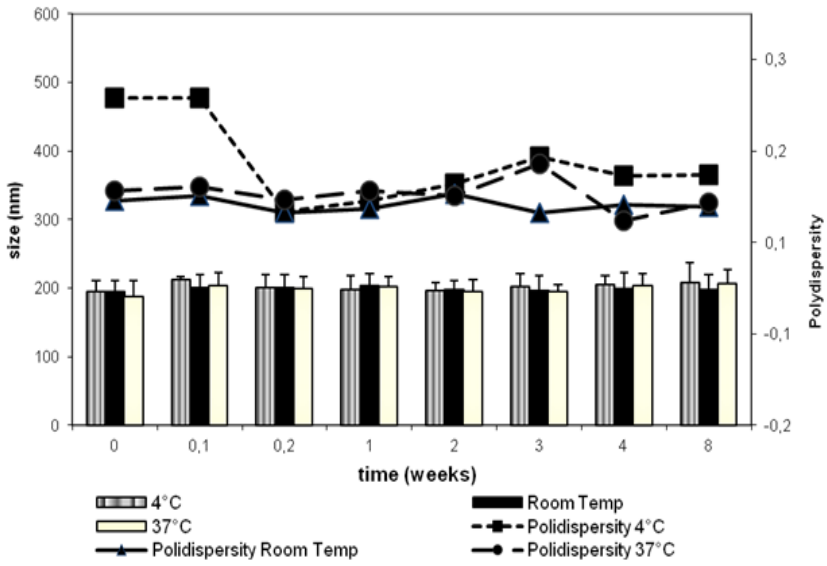
Stability of plitidepsin-loaded PGA and PGA-PEG nanocapsules upon storage

For assessing the stability of the different formulations upon storage, PGA and PGA-PEG nanocapsules preparations were incubated at 4°C, room temperature and 37°C for 8 weeks. Three categories of parameters were assessed at different time points: (i) macroscopic aspect; (ii) particle size, polydispersity and zeta potential; (iii) plitidepsin

concentration in the preparation and encapsulation efficiency. The macroscopic aspect is important to exclude dramatic aggregation or phase separation processes upon storage, including drug precipitation. The preservation of the nanocapsules particle size and polydispersity is critical to ensure that system is suitable for administration, but also that it will maintain its pharmacokinetic properties that can change with nanocarrier size. Plitidepsin concentration and encapsulation efficiency are important to know that the formulation maintains it pharmacological potency over time, and that it will result also in reproducible pharmacokinetics.

The macroscopic analysis showed no evidence of aggregation or sedimentation in the samples. Particle size analysis of PGA and PGA-PEG nanocapsules at different time points in this stability study can be seen in Figure 4A and 4B, respectively. At 4 and room temperature, no significant differences on the mean particle size of plitidepsin-loaded PGA and PGA-PEG nanocapsules were observed during the 8 weeks of the study. Likewise, the polydispersity index of the colloidal systems was also maintained. However, while PGA nanocapsules stored at 37°C did not changed their particle size, PGA-PEG nanocapsules started to aggregate after 4 weeks at this temperature. Zeta potential determinations performed in the different samples confirmed that the surface electrical charge of the nanocarriers did not change over time, another indication of chemical and physicochemical stability of the system (data not shown).

(A)



(B)

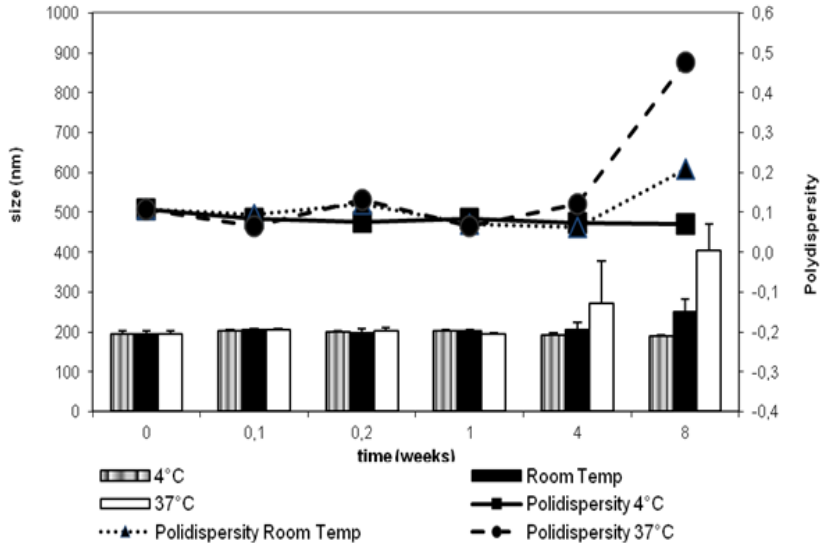


Figure 4: Stability upon storage of plitidepsin-loaded nanocapsules at different temperatures. Plitidepsin-loaded PGA nanocapsules (A), Plitidepsin-loaded PGA-PEG nanocapsules (B). Data represents means ±SD, n=3.

Finally, after 8 weeks storage of plitidepsin-loaded PGA nanocapsules at 4ºC, the drug content and the encapsulation efficiency of the formulation was determined. The results confirmed that plitidepsin was not lost during the storage, and that the encapsulation was maintained above 90%. All these results indicate that PGA and PGA-PEG nanocapsules have excellent stability characteristics, which are critical for their further development as potential industrial formulations.

Freeze-drying studies of plitidepsin-loaded PGA nanocapsules

For better handling and for storing for prolonged periods of time, a freeze-dried formulation of PGA nanocapsules was developed. Non-loaded and plitidepsin-loaded PGA nanocapsules suspensions at different total concentrations (1, 0.75, 0.5 % w/v final concentration) were mixed with trehalose (10% w/v final concentration). Theses mixtures were then freeze-dried, and the particle size of the reconstituted systems compared to the original suspension before freeze-drying. The results showed that PGA nanocapsules could be freeze-dried in 10% trehalose up to the highest concentration tested of 1% w/v with minimum changes in particle size. Also, the recovery of the initial properties of the system after freeze-drying and reconstitution was not affected by the presence of the encapsulated plitidepsin (data not shown).

Toxicity study

As pointed out in the introduction, improving the efficacy/toxicity ratio of current therapies is the ultimate goal of anticancer nanomedicines. In this study, the toxicity of plitidepsin-loaded PGA and PGA-PEG nanocapsules was evaluated upon intravenous (IV) administration to mice. Plitidepsin-loaded nanoemulsion and plitidepsin-loaded PEG NE were also studied to compare with PGA and PGA-PEG coated systems.

Plitidepsin in the reference formulation (Cremophor® EL/ ethanol/ water 15/15/70 w/w/w) was studied as a benchmark. The toxicity for all groups was quantified by comparing the maximum tolerated dose (MTD), defined as the maximum plitidepsin dose resulting in less than 15% loss in body weight and that does not cause lethality.

The MTD for plitidepsin in the reference formulation was 0.3 mg/kg. In contrast, the MTD for plitidepsin in PGA nanocapsules was above the maximum dose administered in this study: 1 mg/kg. For PGA-PEG nanocapsules, nanoemulsion and PEG NE, the MTD was 0.9, 0.9 and 0.95 mg/kg, respectively. Furthermore, no toxicity was observed when the unloaded nanocapsules were administered.

From these results it can be inferred that the MTD of PGA and PGA-PEG nanocapsules is at least 3 times higher than that of the reference formulation. This suggests a much lower toxicity of our nanocarriers compared to the standard vehicle used by the industry for highly hydrophobic anticancer drugs [26]. On the other hand, the similarity in the MTD values between nanoemulsions and nanocapsules suggests that the PGA coating has a limited effect in lowering the toxicity of the nanocarriers.

Pharmacokinetic evaluation

One of the main objectives of encapsulating plitidepsin in PGA and PGA-PEG nanocapsules is to enhance the plasma residence time of the drug, a necessary step to promote its passive targeting to solid tumors [9]. In this context, the next set of studies examined the plitidepsin pharmacokinetics after intravenous administration to mice. The plitidepsin-loaded formulations tested were: PGA, PGA-PEG nanocapsules, nanoemulsion and PEG NE.

The plasmatic concentrations of the different formulations are shown in Figure 5. As it can be observed from the graph, both PGA and PGA-PEG nanocapsules maintained plitidepsin plasmatic levels for longer times as compared to nanoemulsion and PEG NE, which were below the level of detection 8 hours after their IV administration. Besides that, plasmatic concentrations after 48 h also suggested that the elimination of PGA nanocapsules is slightly faster than that of PGA-PEG nanocapsules.

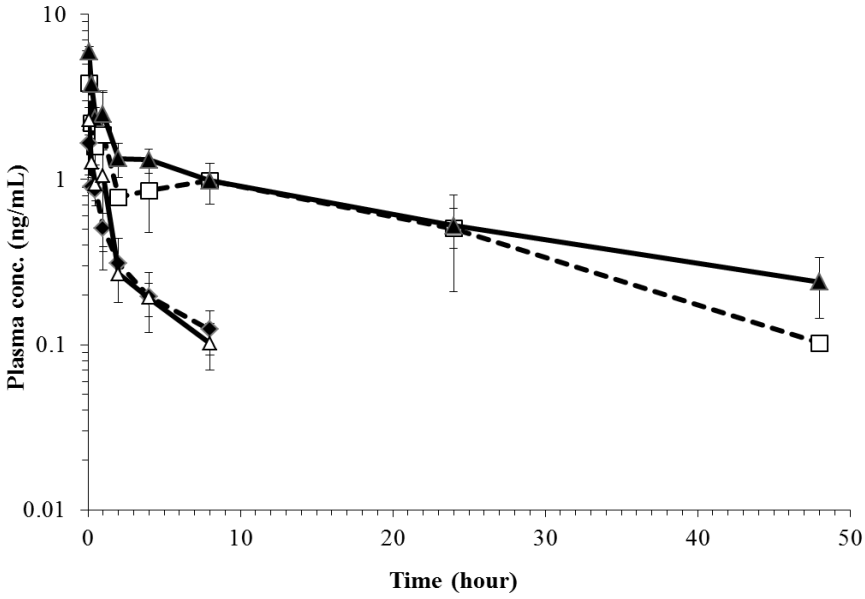


Figure 5: Pharmacokinetic profile of plitidepsin after the IV administration to mice of plitidepsin-loaded PGA nanocapsules (□), PGA-PEG nanocapsules (▲), nanoemulsion (♦) and PEG NE (Δ).

Similar conclusions can be drawn from Table 2, which shows the pharmacokinetic parameters of all formulation groups. PGA and PGA-PEG nanocapsules showed higher half-life times, lower clearance and significantly higher mean residence time (MRT) as compared to nanoemulsion or PEG NE. These parameters clearly point out to an improvement of the residence time of the nanocarriers when coated with PGA as compared to the uncoated nanoemulsions with or without PEG.

Besides this effect in the elimination parameters, it could also be noted that the volume of distribution of the nanocapsules was slightly larger than that of the nanoemulsion, which might be related to a more extensive distribution to peripheral tissues.

Differences between PGA and PGA-PEG nanocapsules could also be detected in the pharmacokinetic analysis. Indeed, pharmacokinetic parameters indicating a more prolonged circulation of PGA-PEG nanocapsules (Table 2). These results pointing out the optimization of the stealth properties of the nanocarriers by the PEG coating layer.

Table 2: Pharmacokinetic parameters of plitidepsin-loaded PGA, PGA-PEG nanocapsules, nanoemulsion and PEG NE after a single IV administration to mice.

Formulation	$t_{1/2}$ (h)	$AUC_{0\rightarrow t}$ (ng*h/ml)	CLp (ml/min/kg)	Vdss (L/kg)	VdB (L/kg)	MRT (h)
NCs PGA	13.3	69.32	224.8	230.4	258.7	17.0
NCs PGA-PEG	18.4	84.1	166.5	240.8	265.8	24.1
NE	1.6	25	507.5	159.2	197.9	5.2
PEG NE	2.3	29.9	462.8	110.1	168.5	3.9

As reported in the literature, colloidal drug carriers are generally recognized by macrophages due to their physicochemical characteristics such as surface charge, particle size, and surface hydrophobicity [27]. Thus, a typical strategy for preparing long-circulating nanocarriers is modifying their surface with PEG, a strategy first developed for liposomes [28] and then applied for other nanocarriers [29, 30]. The utility of this strategy relies on the capacity of PEG to repel opsonins from the surface of the nanocarriers, making the recognition of the nanocarriers by immune cells less likely [6].

Based on the prolonged circulation times observed for PGA-drug conjugates [31] in this work we postulated that this polymer might also be able to provide nanocarriers with a stealth coating. Overall, our results confirm this hypothesis, and indicate that for some systems this strategy might be even more useful than PEGylation (see Figure 5 and Table 2, PGA nanocapsules vs. PEG NE). However, the best results were obtained with systems that combined surface modifications with PGA and PEGylation (PGA-PEG nanocapsules), suggesting that both strategies might work synergistically to provide the carriers with improved biopharmaceutical characteristics.

Conclusions

Novel nanocapsules made of PGA and PGA-PEG were designed as carriers for parenteral administration of the anticancer drug plitidepsin. These nanocapsules are small, polyanionic nanocarriers that show very good plitidepsin encapsulation efficiency. Plitidepsin-loaded PGA and PGA-PEG nanocapsules show highly improved toxicity profiles as compared to plitidepsin conventional formulation. Moreover, they show prolonged plasmatic circulation times that suggest the stealth properties of both nanocarriers. All these properties indicate the interest of PGA and PGA-PEG nanocapsules as delivery systems for the parenteral administration of anticancer drugs.

Acknowledgements

Financial support from CENIT-NANOFAR XS53 project, PharmaMar, Spain, and the Ministry of Sciences and Innovation. Giovanna Lollo has a fellowship from the Ministry of Education of Spain. Marcos Garcia Fuentes acknowledges an Isidro Parga Pondal Fellowship from Xunta de Galicia.

References

1. Park, J.H., et al., Polymeric nanomedicine for cancer therapy. Progress in Polymer Science, 2008. 33(1): p. 113-137.

2. Gelderblom, H., et al., Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. Eur. J. of Cancer, 2001. 37(13): p. 1590-1598.

3. Mora-Huertas, C.E., H. Fessi, and A. Elaissari, Polymer-based nanocapsules for drug delivery. International Journal of Pharmaceutics, 2010. 385(1–2): p. 113-142.

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

5. 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.

6. Torchilin, V., Tumor delivery of macromolecular drugs based on the EPR effect. Adv Drug Deliv Rev, 2011. 63(3): p. 131-5.

7. Maeda, H., Tumor-Selective Delivery of Macromolecular Drugs via the EPR Effect: Background and Future Prospects. Bioconjugate Chemistry, 2010. 21(5): p. 797-802.

8. Huynh, N.T., et al., Lipid nanocapsules: A new platform for nanomedicine. International Journal of Pharmaceutics, 2009. 379(2): p. 201-209.

9. 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.

10. Dash, B.C., et al., The influence of size and charge of chitosan/polyglutamic acid hollow spheres on cellular internalization, viability and blood compatibility. Biomaterials, 2010. 31(32): p. 8188-8197.

11. Jack W, S., Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane. Journal of Controlled Release, 2005. 109(1-3): p. 120-126.

12. Li, C. and S. Wallace, Polymer-drug conjugates: Recent development in clinical oncology. Advanced Drug Delivery Reviews, 2008. 60(8): p. 886-898.

13. Bae, Y. and K. Kataoka, Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers. Advanced Drug Delivery Reviews, 2009. 61(10): p. 768-784.

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

15. Brandon, E.F.A., et al., Structure elucidation of aplidine metabolites formed in vitro by human liver microsomes using triple quadrupole mass spectrometry. Journal of Mass Spectrometry, 2005. 40(6): p. 821-831.

16. 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.

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

18. 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.

19. 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.

20. 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.

21. Lozano M. V., et al., Polyarginine nanocapsules: a new platform for intracellular drug delivery. Submitted.

22. Garcia-Fuentes, M., et al., Application of NMR Spectroscopy to the Characterization of PEG-Stabilized Lipid Nanoparticles. Langmuir, 2004. 20(20): p. 8839-8845.

23. Teixeira, M., et al., Development and characterization of PLGA nanospheres and nanocapsules containing xanthone and 3-methoxyxanthone. European Journal of Pharmaceutics and Biopharmaceutics, 2005. 59(3): p. 491-500.

24. Calvo, P., J.L. Vila-Jato, and M.J. Alonso, Evaluation of cationic polymer-coated nanocapsules as ocular drug carriers. Int. J. Pharm., 1997. 153(1): p. 41-50.

25. Lambert, G., et al., Polyisobutylcyanoacrylate nanocapsules containing an aqueous core for the delivery of oligonucleotides. International Journal of Pharmaceutics, 2001. 214(1-2): p. 13-16.

26. Engels, F.K., R.A.A. Mathot, and J. Verweij, Alternative drug formulations of docetaxel: a review. Anti-Cancer Drugs, 2007. 18(2): p. 95-103.

27. Huynh, N.T., et al., The rise and rise of stealth nanocarriers for cancer therapy: passive versus active targeting. Nanomedicine, 2010. 5(9): p. 1415-1433.

28. Yang, T., et al., Enhanced solubility and stability of PEGylated liposomal paclitaxel: In vitro and in vivo evaluation. International Journal of Pharmaceutics, 2007. 338(1-2): p. 317-326.

29. Plummer, R., et al., A Phase I clinical study of cisplatin-incorporated polymeric micelles (NC-6004) in patients with solid tumours. Br J Cancer, 2011. 104(4): p. 593-598.

30. Oerlemans, C., et al., Polymeric Micelles in Anticancer Therapy: Targeting, Imaging and Triggered Release. Pharmaceutical Research, 2010. 27(12): p. 2569-2589.

31. Jack W, S., Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane. Journal of Controlled Release, 2005. 109(1–3): p. 120-126.

CAPÍTULO 4.

Long circulating PEG-polyglutamic acid nanocapsules for an improved antitumor efficacy: *in vivo* evaluation in a murine solid tumor model

Abstract

A new nanocapsulate system consisting of an oily core surrounded by a high PEGylated polyglutamic acid (PEG-PGA HP) coat was developed and investigated for anticancer drug delivery. A PEG-PGA diblock copolymer containing a high PEG percentage was chosen as a mean to improve the plasma residence time of previously developed PGA nanovehicles and then, to maximize their arrival to the tumor area. These PEGylated nanocarriers were prepared by the solvent displacement technique and could accommodate in their structure a significant amount of the hydrophobic anticancer drug plitidepsin leading to an encapsulation efficiency of 85%. Plitidepsin was released from the nanocapsules exhibiting a biphasic pattern characterized by an initial burst release followed by a slower and continuous release. The *in vivo* fate of the nanocarriers was evaluated after systemic administration into healthy mice, through pharmacokinetic, toxicological, and efficacy studies in tumor-bearing mice. PGA-PEG HP nanocapsules showed long circulating properties leading to an important increase in the t1/2 and AUC of the plitidepsin in blood circulation compared to the drug dissolved a Cremophor® EL solution, as a reference formulation. The maximum tolerated dose (MTD) of plitidepsin loaded PGA-PEG nanocapsules was increased by a 2.5 factor in comparison to the plitidepsin dissolved in Cremophor® EL. The *in vivo* antitumor effect was further confirmed by study tumor inhibition in a xenograft mouse model. PGA-PEG HP nanocapsules showed a similar antitumor activity to the reference formulation at inhibiting tumor growth while reducing toxicity. Moreover, nanocapsules remained stable during storage in suspension for a month at 4ºC, and could be easily reconstituted after freeze-drying in presence of the cryoprotectant trehalose. These promising results highlight the potential of PGA-PEG HP nanocapsules as an effective drug delivery system in cancer therapy.

Introduction

Nanomedicine is an innovative field with immense potential for improving the efficacy of cancer therapeutics [1,2]. Currently, most anticancer drugs used in conventional chemotherapy are rapidly cleared from the blood circulation and, because their small size and their high hydrophobicity, they tend to exhibit a high distribution volume. As they do not differentiate between cancerous and normal tissues, their biodistribution profile generally leads to systemic toxicity and important adverse effects [3]. Using nanoengineering strategies researchers can tailor the unique physical properties of the carriers to modulate their *in vivo* behavior. Tumor-targeted nanopharmaceuticals are ideally designed to allow the anticancer drug to accumulate in the tumor site and to direct it away from those tissues in which toxic effects are not expected [4,5]. Furthermore, nanosystems have the potential advantage of being accumulated and entrapped in tumors (passive targeting) based on the so-called enhanced permeability and retention effect (EPR) (2,6), which refers to the unique physiology of fenestrated vasculature and poor lymphatic drainage of tumors [7-9].

Nanocarriers are versatile devices which can be synthetized from myriad different materials to create numerous nanoarchitectures. Many nanosystems have been examined for clinical use and some of them have already been approved for use in humans. The FDA approval of Doxil®, doxorubicin pegylated liposomes, has opened the doors for the development of other nanoscale drug delivery devices. Since then, over 20 drug-containing nanocarriers have been approved for clinical use including not only liposome but also micelles, nanoparticles or conjugates [7].

Polyaminoacids have raised great expectancy in the development of nanocarriers for anticancer drugs. Among them, the anionic polymer poly-L-glutamic acid (PGA) is a promising candidate because of its

biodegradability and non-toxicity. PGA has been already included in carrier systems relevant to cancer therapy. Xyotax® (PGA-conjugated paclitaxel) has become the first polymer-drug conjugate for the delivery of cytotoxic chemotherapeutic agents to advance to clinical Phase III trials (10,11). Also, en 2008 Phase I and II studies of a PGA derivate, poly-glutamate-camptothecine conjugate (CT 2106) were successfully carried out, http://clinicaltrials.gov/ct2/archive/NCT00291785 (12). An additional interesting property of polyaminoacids relies on the possibility to conjugate them with poly(ethylene glycol) (PEG), thus rendering their surface more hydrophilic and flexible to prevent the uptake by the mononuclear phagocytic system (MPS) (7,13,14).

Plitidepsin is a novel highly hydrophobic antineoplastic drug which has been designed as orphan drug by the EMA and FDA for the treatment of acute lymphoblastic leukemia and multiple myeloma. Despite its efficacy, its low aqueous solubility, instability in the biological environment, and non-specifically distribution in the body, limit its application as chemotherapeutic agent (15,16).

Recently, we reported the design of a novel nanocapsule-type carrier made of polyglutamic acid (PGA) particularly attractive to accommodate hydrophobic anticancer drugs, such as plitidepsin. Surface modification of these nanocarriers by grafting PEG in the polymer blackbone was also carried out as a means to maximize their circulation time. Nevertheless, the systems were rapidly eliminated from the body, probably due to the short length of PEG chains (2 kDa) and the low PEG content (24 % w/w). [27].

Based on this background experience, it was our purpose to develop an alternative high PEGylated nanocapsule´s formulation for hydrophobic anticancer drugs, providing their corona with a diblock copolymer, high molecular weight (20kDa) PEG conjugated with PGA. This novel nanocarrier should present long circulation properties and

should be able to improve the toxicity-efficacy profile of plitidepsin in comparison with the drug dissolved in Cremophor® EL as a reference formulation.

Materials and methods

Chemicals

Plitidepsin was provided by PharmaMar S.A. (Spain). Miglyol 812®, neutral oil formed by esters of capric and caprilyc fatty acid and glycerol, was a gift sample from Sasol Germany GmbH (Germany). Epikuron 170, a phosphatidylcholine enriched fraction of soybean lecithin, was kindly provided by Cargill (Spain). Benzalkonium chloride, Poloxamer 188(Pluronic® F68) and D-(+)-Trehalose dehydrate were purchased from Sigma-Aldrich. Poly-L-glutamic acid-polyethylene glycol (PGA-PEG Mw 35 kDa) was synthetized and supplied by Alamanda Polymers (USA). PGA-PEG was a diblock copolymer with a percentage w/w of PEG (57%). PEG chains length was 20 kDa and the PGA chains length was about 15 kDa.

Preparation of plitidepsin-loaded PGA-PEG high pegylated (HP), nanocapsules

The preparation of PGA-PEG HP nanocapsules was based on a modification of the solvent displacement technique which involved a polymer ionic interaction after solvent diffusion [17]. Briefly, an organic phase made of 30 mg of Epikuron 170, 0.125 ml of Miglyol®, 9.5 ml of acetone, 0.5 ml of ethanol and 7 mg of benzalkonium chloride was poured over an aqueous phase containing 10 mg of the polymer PGA-PEG HP and 50 mg of Poloxamer 188. Solvents were evaporated under vacuum from the suspension to a final volume of 10 ml. Plitidepsin-loaded PGA-

PEG nanocapsules were obtained as previously described dissolving 20 mg of the hydrophobic drug in 0.5 ml of ethanol and following the procedure mentioned.

Characterization of PGA-PEG HP nanocapsules

Particle size and ζ potential of colloidal systems were determined, respectively, by photon correlation spectroscopy and laser Doppler anemometry, using a Zetasizer Nano ZS (Malvern Instruments, UK).

The morphology of the nanocapsules was analyzed by transmission electron microscopy using a Philips CM-12 microscope (FEI Company, Eindhoven). Samples were stained with phosphotungstic acid solution (2 % w/v) and placed on a copper grid with Formvar® films for analysis.

Plitidepsin encapsulation efficiency in PGA-PEG HP nanocapsules was calculated indirectly by the difference between the total amount of plitidepsin in the system and the free drug found in the infranatant of the formulation after the isolation process. The total amount of drug was estimated by dissolving an aliquot of the colloidal suspension of plitidepsin-loaded nanocapsules with acetonitrile. These samples were centrifuged (4000 g, 20 min, 20°C) and the supernatant was analyzed with high-performance liquid chromatography (HPLC) system. The free drug was quantified by the same method following separation of the nanocapsules from aqueous medium by ultracentrifugation (27400 g, 1h, 15°C).

The HPLC system consisted of an Agilent 1100 series instrument equipped with UV detector set at 225 nm. The analytic method employed was previously validated by PharmaMar (Spain) [18]. The parameter ‘encapsulation efficiency’ refers to the percentage of drug that is entrapped with respect to the total amount of drug added in the nanocapsules preparation process.

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

$$E.E.\% = [(A-B)/A] \times 100$$

Where A is the total drug concentration (mg/ml), and B is the drug concentration measured in the external aqueous medium, corresponding to the free drug.

in vitro release study

The release study of plitidepsin from PGA-PEG HP nanocapsules was performed by incubating an aliquot of formulation in PBS (pH 7.4) at an appropriate concentration to assure sink conditions (4.9μg/ml). The vials were placed in an incubator at 37 °C with horizontal shaking. At different intervals (1h, 3h, 6h and 24h), 3 ml of the suspension diluted in PBS were collected and ultra-centrifuged in Herolab® tubes (27400 g, 1h, 15°C). Released plitidepsin was calculated indirectly by determining the total amount of drug present in the system and the free plitidepsin in the supernatant after the ultracentrifugation.

in vivo studies

Animals

Female athymic nu/nu mice and CD-1 male mice between 4 to 6 weeks of age and ranged in weight from 21 to 30 g were purchased from Harlan Laboratories Models, S.L. (Barcelona, Spain). Animals were housed in individually ventilated cages (Sealsafe® Plus, Techniplast S.P.A.), 10 mice per cage, on a 12-hour light-dark cycle at 21-23 ºC and 40-60% humidity. Mice were allowed free access to irradiated standard rodent diet (Tecklad 2914C) and sterilized water. Animals were acclimated for five days prior to being individually tattoo-identified.

Animal protocols were reviewed and approved according to regional Institutional Animal Care and Use Committees (Spain).

Pharmacokinetic evaluation

A pharmacokinetic study was performed in CD-1 male mice (n=40) after a single dose IV of plitidepsin-loaded in PGA-PEG HP nanocapsules and compared with plitidepsin dissolved in Cremophor® EL solution (Cremophor® EL/ Ethanol/ Water 15/15/70 w/w/w) (plitidepsin concentration 0.1 mg/kg of body weight).

On the day of dosing, blood samples were drawn via cardiac puncture at 9 pre-established time points: 5, 15, 30 min and 1, 2, 4, 8, 24, 48 h post-injection. Blood samples were transferred into suitably labeled tubes containing EDTA as anticoagulant. The blood was kept in darkness and ice conditions until it was centrifuged at 3000 rpm for 15 minutes at 5ºC. The plasma obtained was frozen at -20ºC and maintained in darkness until analysis.

Pharmacokinetic Analysis

Plitidepsin concentrations were determined in mouse plasma samples using HPLC/MS/MS after solid-liquid extraction with a mixture of tert-butyl methyl ether (TBME): hexane (1:1, v/v) [18,19]. The pharmacokinetic parameters of ap plitidepsin were performed using a non-compartmental pharmacokinetic method with a WinNonlin 5.2 software.

Toxicity studies

MTD (maximum tolerated dose) of plitidepsin-loaded PGA-PEG HP nanocapsules in healthy CD-1 male mice was investigated after single IV injection. Toxicity of plitidepsin dissolved in a Cremophor® EL

solution (Cremophor® EL/Ethanol/Water 15/15/70 % w/w/w) was also investigated. The formulations were always administered as bolus intravenous injection. The MTD was defined as the highest dose resulting in less than 15% body weight loss and not causing significant lethality or any prominent observable changes during the experiment [20].

The difference in mean body weight was calculated with respect to this of day 1 as:

$$MBW = \frac{mean\ body\ weight_{dayT} - mean\ body\ weight_{day\ 1}}{mean\ body\ weight_{day\ 1}} \times 100$$

Efficacy studies

Xenograft model

MRI-H-121 is a human renal carcinoma originally obtained from the DCT Tumor Bank. Developed by Dr. A. E. Bogden, Mason Research Institute MA and maintained as a serial transplanted tumor line in athymic nude mice. Original tissue came from a patient at University of Massachusetts Medical Center (USA).

in vivo antitumor activity

For the study, 4 to 6 week-old athymic nu/nu mice were subcutaneously implanted into their right flank using a 13G trocar with MRI-H-121 tissue from serial transplanted donor mice. Tissue was debrided of membrane, hemorrhagic and necrotic areas and a 3 mm3 fragments were implanted. When tumors reached 150-200 mm^3, tumor bearing animals (n=10/group) were randomly allocated into the following treatment groups:

1. Plitidepsin dissolved in a Cremophor® EL solution
2. Plitidepsin PGA-PEG HP Nanocapsules
3. Serum saline as a control

The doses and schedules were selected based on MTD determination. All formulations were injected IV bolus in the tail vein. Plitidepsin dissolved in Cremophor® EL was injected at the dose of 0.3 mg/kg during 5 consecutive days, then 4 days free, repeated two times. On the contrary, plitidepsin-loaded PGA-PEG HP nanocapsules were injected at the dose of 0.15 mg/kg during 20 days. The total cumulative dose was the same regardless of the formulation administered.

Tumor volume and mice body weight were measured by 2-3 times per week starting from the first day of treatment (Day 0). Treatments that produced >20% lethality and/or 20% of net body weight loss were considered toxic.

Tumor volume was calculated using the equation:

$$V = \frac{a \times b2}{2}$$

Where a and b were the longest and shortest diameters, respectively.

Animals were euthanized when their tumors reached a volume of 2000 mm^3 and/or severe necrosis was seen.

Antitumor effect was calculated by using ΔT/ΔC (%) defined as a percentage of the change in tumor volume for each treated (T) and placebo (C) group. ΔT/ΔC was calculated on days 7, 14 and 21. Treatment tolerability was assessed by monitoring body weight evolution, clinical signs as well as evidence of local damage at the injection site. All placebo-treated animals died or were sacrificed for ethical reasons from Day 0 to 21. Tumors in this model had a doubling time calculated as 5.7 days (95 % C.I., 5.3 to 6.1).

Stability study of plitidepsin-loaded nanocapsules

The stability of plitidepsin-loaded nanocapsules was evaluated at storage conditions according to terms of time and temperature. Therefore, aliquots of the nanocapsules suspension without dilution were placed in sealed tubes at 4°C for storage. Size, polydispersity index and zeta potential of the nanocapsules were measured for a period of 1 month, whereas the leakage of drug was controlled at the end of the study.

Freeze-drying study

Concentrations of blank PGA-PEG HP nanocapsules (1-0.75-0.5%w/v) and trehalose (5 and 10% w/v) were considered the variables for the lyophilization study. Therefore, 1ml of diluted PGA-PEG nanocapsules were placed in glass vials and frozen in liquid nitrogen. The freeze-drying programme consisted in an initial drying step at -35°C, and secondary drying were temperature was finally equilibrated at 20ºC over a period of 60 h (Labconco Corp., USA).

PGA-PEG HP nanocapsules were recovered by adding 1 ml of ultrapure water to the freeze-dried powders followed by manual resuspension. Finally, their size distribution was measured after resuspension.

Experimental design and statistical analysis

Design, randomization and monitoring of body weight and tumour measurements were performed using NewLab Oncology Software (version 2.25.06.00). The results were expressed in the form of the mean ± standard deviation. Tumor volumes data are presented as medians and interquartile range (IQR). Tumor volume of the compound-treated group

on Day X and Day 0 (TX-T0) was used to determine the activity rating as follows:

$$\Delta T/\Delta C\ \% = (TD\text{-}T0/C0\text{-}CD)*100$$

Activity rating:

- $\Delta T/\Delta C > 50\%$ inactive (-)
- $\Delta T/\Delta C$ >25-50% tumor inhibition (+)
- $\Delta T/\Delta C < 25\%$ TX-T0 >75-125% tumor stasis (++)
- TX-T0 >10-75% partial regression (+++)

Tumor volume from groups following the 1st 2nd and 3rd study weeks were compared using a two-tailed Mann-Whitney U test. In all cases, $p < 0.05$ was accepted as denoting a statistical difference.

Results and Discussion

The main goal of this work was to provide long circulating properties to PGA nanocapsules with the final aim of maximize the passive accumulation of the systems in the tumor site after IV administration. These novel colloidal carriers consist of an oily core in which the active hydrophobic compound is located, and a polymeric coating made of a high pegyated diblock copolymer PGA-PEG HP (Figure 1). The rationale behind the selection of the polymeric shell fulfills the two main important requirements that determine the biodistribution and clearance rate of PEGylated nanosystems. First, many studies have demonstrated that the use of high PEG molecular weights (2000 < PEG chains > 30000 Da) lead nanocarriers to prolong their blood circulation half-lives and reduce their clearance. In addition, an adequate PEG content ensures that PEG chains are in a slightly constricted configuration, then covering the entire surface of nanocarriers [21].

In this study, new high PEGylated PGA-PEG HP nanocapsules, were developed and characterized as vehicles for the antitumor drug plitidepsin. The pharmacokinetic parameters, toxicological properties and therapeutic efficacy of the loaded systems were evaluated and compared with those of an plitidepsin dissolved in a Cremophor® EL solution used as reference formulation.

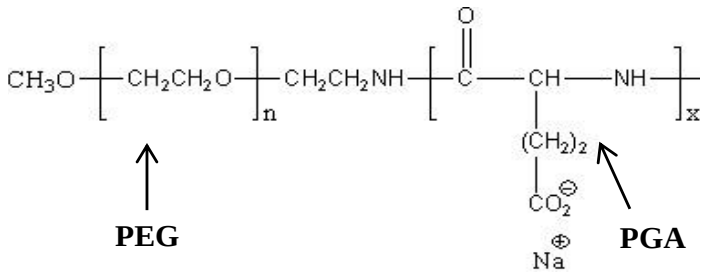


Figure 1: Molecular structure of PGA-PEG block copolymer.

Preparation and characterization of plitidepsin-loaded PGA-PEG HP nanocapsules

PGA-PEG HP nanocapsules were prepared by the solvent displacement technique. This procedure, previously applied to the formation of other types of nanocapsules [22], is a mild technique that does not require the need of high energy sources. By using this method, the deposition of a polymer coating on the oily core is produced once the organic solvent diffuses immediately in the polymer aqueous solution. In our case, the attachment of the PGA-PEG HP to the oily core is driven by the inclusion of the positively charged surfactant, benzalkonium chloride, in the oily phase. The ionic interaction between this surfactant and the carboxylic group of PGA, led to the formation of the PEGylated nanosystems. Benzalkonium chloride was selected on the basis of its acceptable toxicological profile; however the quantity used was the minimum that allowed the formation of a stable system.

The polymer chosen is a block copolymer that contains a high percentage, around 57 w/w, of high MW (20 kDa) PEG. It is expected that its disposition on the external layer create a PEG-hydrated cloud shielding whose conformation ensure that no gaps or spaces on the particle surface are left uncovered [2]. This disposition contributed to create a protective hydrophilic layer bringing stealth properties to the system developed [2,23-25].

The physicochemical properties of blank and plitidepsin-loaded PGA-PEG HP nanocapsules are summarized in Table 1. As can been noted, the use of adequate concentrations of polymer and cationic surfactant results in the formation of homogenous populations of nanocapsules of around 180-190 nm. The results showed that the incorporation of the drug into PGA-PEG HP nanocapsules did not affect markedly the size and the ζ potential of the systems. The zeta potential of the nanocapsules maintained its negative value for both empty and loaded systems, which indicates the inversion from the positive values of the uncoated nanoemulsion (data not shown), confirming the success in the formation of the PGA-PGA HP coating. Furthermore, an encapsulation efficiency value of around 85 % indicated that the oily core could be easily allocate the drug, then confirming the nanocapsules as an ideal vehicle for this hydrophobic drug.

The morphological appearance of the nanocapsules was observed by transmission electron microscopy (Figure 2). The micrographs indicated that PGA-PEG HP nanocapsules have a round shape and a size of less than 200 nm, similar to that obtained by photon correlation spectroscopy.

In a second step we evaluated the release pattern of plitidepsin-loaded PGA-PEG HP nanocapsules. The *in vitro* release profile (data not shown) of loaded nanocapsules indicated that the system exhibits a biphasic profile characterized by an initial burst effect with about 80 % of

drug released within the first hour followed by a second phase in which no further drug release was observed. This profile characterized by an initial fast release phase, typical of other nanocapsules and nanoemulsion, indicates that the release process is governed by the oil-water partition and that the polymer coating does not affect the release rate [22,26].

Table 1: Physicochemical characteristics of blank and plitidepsin-loaded PGA-PEG nanocapsules.(Mean ± S.D.; n=3).NCs: nanocapsules

Formulation	Size (nm)	P.I.	ζ Pontential (mV)	Enc. Efficiency (%)
Blank NCs	180 ± 4	0.1	-20 ± 4	-
Plitidepsin -loaded PGA-PEG HP NCs	190 ± 15	0.1	-24 ± 5	85 ± 4

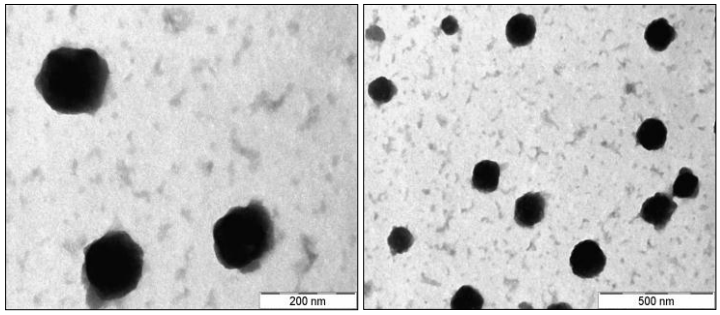


Figure 2: TEM images of plitidepsin-loaded PGA-PEG HP nanocapsules.

in vivo studies

Pharmacokinetic evaluation

The elimination rate of plitidepsin formulated in PGA-PEG HP nanocapsules and Cremophor® EL from the blood circulation was evaluated in CD-1 mice. The formulations were administered IV through a single bolus injection (0.1 mg/kg plitidepsin). As shown in Figure 3,

PGA-PEG nanocapsules efficiently prolong the *in vivo* residence time of the encapsulated drug. In contrast, plitidepsin Cremophor® EL formulation provided after 8 hours plasma levels which were under the limit of detection. Finally, approximately 10% of the injected dose of plitidepsin-loaded nanocapsules remained in circulation after 48 h of injection.

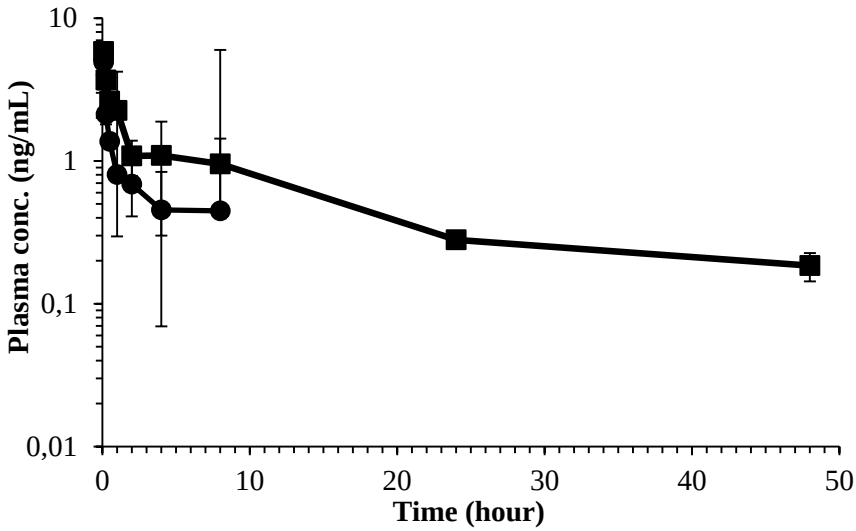


Figure 3: Plasma concentration-time profile of plitidepsin following IV injection in mice of PGA-PEG HP nanocapsules (■) and Cremophor® EL(●).

Pharmacokinetics parameters were extracted from the analysis with the WinNonlin 5.2 software. As showed in Table 2, PGA-PEG HP nanocapsules exhibited a 2 fold higher half-life (17.02 h) of plitidepsin compared with apldin dissolved in a Cremophor® EL (8.1 h). Besides, the peak plasma concentration (Cmax) achieved from nanocapsules was higher. The plasma AUC of plitidepsin-loaded nanocapsules was 5 fold greater than the one obtained with plitidepsin dissolved Cremophor® EL and, the MRT was considerably longer. Moreover, plitidepsin-loaded PGA-PEG HP nanocapsules showed a 3 times lower plasmatic cleareance

(52.7 ml/min/kg) and lower volume of distribution (65.9 L/kg) compared with plitidepsin dissolved in a Cremophor® EL (CLp 157 L/h/kg and Vdss 98.10 L/kg).

Table 2: The main pharmacokinetic characteristics of plitidepsin-loaded PGA-PEG nanocapsules and plitidepsin Cremophor® EL after a single IV injection in mice.

Formulation	$t_{1/2}$ (h)	$AUC_{0\rightarrow t}$ (ng*h/ml)	CLp (ml/min/kg)	Vdss (L/kg)	VdB (L/kg)	MRT (h)
Cremophor® EL	8.1	57.9	157.0	98.1	106.9	10.5
PGA-PEG HP NCs	17.0	274.7	52.7	65.9	77.7	20.8

Overall, the results of the blood kinetic profile highlight the long-circulating properties of PGA-PEG HP nanocapsules. These data are in good agreement whit that reported by Gonzalo et al which revealed that pegylated nanocapsules improve the pharmacokinetic parameters of PGA nanocapsules after single IV injection. Gonzalo et al prove that the presence of the steric hydradance over the shell of the nanocapsules contributed to the enhancement of the pharmacokinetic parameters [27]. However, in this work we demonstrate that the use of a diblock copolymer consisting of PEG at high density enhanced plitidepsin half-life of elimination, AUC and Cmax in the range of 3 to 5-folds compared with the low pegylated formulations. High pegylated nanocapsules were retained longer, as their distribution volume and clearance pattern were slowed down.

Increasing pegylation of nanoparticles surface, already reported by [28,29], considerable increase the half- life time of the drug encapsulate and decrease the uptake from the MPS containing organs. The first systems that adopted this strategy were liposomes. Yang et al found

that PEGylated liposomes increased the biological half-life of paclitaxel from 5.05 h to 17.8 h compared to the conventional liposomes.

Further, Huynh et al reported that the incorporation of long PEG chains linked to the nanoparticles provide high plasmatic level of the encapsulated drug, increasing the possibility to reach the tumor via EPR effect [30,31]. Likewise, pegylated nanocapsules enhanced the pharmacokinetic parameters of the encapsulated docetaxel. Khalid et al demonstrated that these changes in the pharmacokinetic parameters were accompanied by a substantial deposition of drug in the tumor via passive targeting [32].

Toxicological evaluation

Toxicological evaluation was aimed at establishing the maximum tolerated dose (MTD) of plitidepsin-loaded in PGA-PEG HP nanocapsules following IV injection in CD-1 male mice. The results were compared with plitidepsin dissolved in Cremophor® EL.

The MTD of PGA-PEG nanocapsules was 0.75 mg/kg, 2.5 times higher than that of Cremophor® EL solution (0.3 mg/kg). These results indicate that nanocapsules are better tolerated than the control and could be administered at higher doses. Furthermore, no mortality and no changes in body weight were observed (data not shown). This reduction of toxicity exhibited by PGA-PEG HP nanocapsules could be attributed to two major evidences. First, the lower toxicity of the excipients used in the formulation render PGA-PEG nanocapsules suitable for multiple administrations, eluding the well-known serious adverse effects caused by Cremophor® EL. Besides, the encapsulation of apidin into PGA-PEG nanocapsules modified the biodistribution of the encapsulated drug avoiding its accumulation in in the healthy tissues.

Antitumor activity

The *in vivo* antitumor efficacy of plitidepsin-loaded PGA-PEG HP nanocapsules was evaluated in a human renal xenograft mouse model (MRI-H-121). A comparative study was performed by dividing animals into 3 groups according to the treatment received and the schedule of administration established.

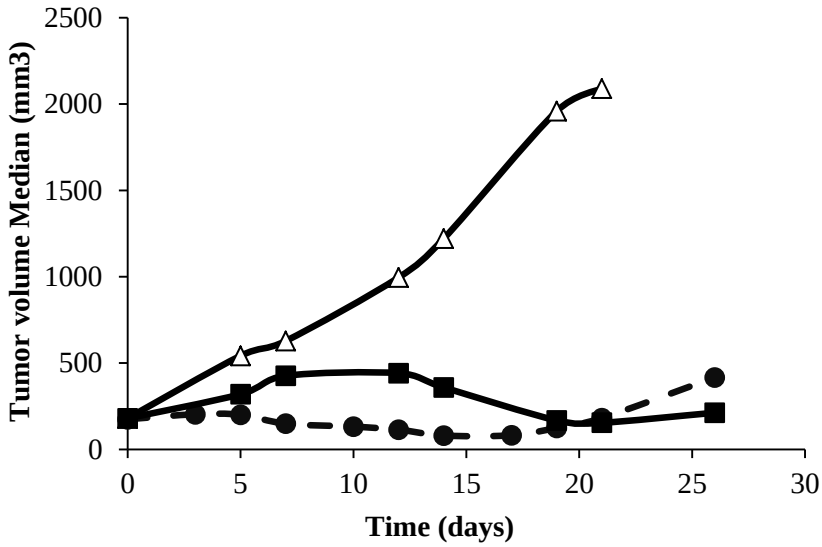


Figure 4: Evolution of tumor volume following IV administration of a single-dose of plitidepsin-loaded PGA-PEG HP nanocapsules (■) and Cremophor® EL (●) in a subcutaneously implanted human renal xenograft mouse model (MRI-H-121).Serum represent the control (Δ).

A strong antitumor activity was seen after the treatment with both plitidepsin formulated in Cremophor® EL and PGA-PEG HP nanocapsules (Figure 4). Moreover, there is a significative difference ($p < 0.001$) in tumor volume at the day 14 and 21 for plitidepsin-loaded PGA-PEG nanocapsules and Cremophor® EL with the respect to the control, indicating that both formulations had a similar antitumor activity (Table 3). Tumor stasis was recovered at the end of the experiment after the

treatment with plitidepsin-loaded PGA-PEG HP nanocapsules and Cremophor® EL.

The activity rating and the antitumor effect, calculated as ΔT/ΔC, reported in Table 4 highlight the differences in the mechanism of action of the two formulations. Plitidepsin dissolved in Cremophor® EL showed a rapid onset of action (delayed tumor growth), but this effect decrease at the day 21 of the experiment. This result suggests that when plitidepsin was dissolved in Cremophor® EL, its antitumor efficacy was due not only to the action of the drug but there was also a contribution of the active ingredient Cremophor® EL. In fact the MTD value of nanocapsules was 2.5 fold lower than the Cremophor® EL. However, in the case of nanocapsules, the cytotoxic activity was mainly induced by the plitidepsin encapsulated. Furthermore, no mortality or changes in body weight was observed throughout study period when the nanocapsules were administered, while one animal was found dead in the plitidepsin Cremophor® EL group on day 15.

Table 3: Tumor Volumes (TV) obtained in tumor bearing MRI-H-121 xenografts mice for plitidepsin-loaded PGA-PEG HP nanocapsules and plitidepsin dissolved in Cremophor® EL. NCs: nanocapsules.

Formulation	Day	TVmm^3 median (IQR)	[a]P	[b]P	Mortality
Cremophor® EL	7	149 (133.9-268.6)	0.0001		0/10
	14	80.1 (52.7-127.7)	< 0.0001		0/10
	21	181.3 (144.0-264.9)	< 0.0001		1/10
PGA-PEG HP NCs	7	426.1 (376-5561.6)	NS		0/10
	14	358.8 (275.8-486.0)	< 0.0001		0/10
	21	154.5 (80.5-236.8)	< 0.0001	NS	0/10

Data are presented as median and interquartile range (IQR)

[a]P value for Mann –Whitney U test (control group compared against the rest)
[b]P value for Mann –Whitney U test (CEW formulation compared against the Cremophor® EL free formulation)
NS, not statistically significance.

Table 4: Antitumor effect parameters and activity ranking of plitidepsin-loaded PGA-PEG HP nanocapsules, plitidepsin dissolved in Cremophor® EL after IV injection in mice bearing MRI-H121 xenograft. NCs: nanocapsules.

Formulation	Dose (mg/kg)	ΔT/ΔC %			Activity ranting		
		Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Cremophor® EL	0.30	-5.8	-9.1	0.3	++	+++	++
PGA-PEG HP NCs	0.15	55.0	17.2	-1.3	-	+	++

Tumor inhibition (+); Tumor stasis (++); Partial regression (+++).

Considering the antitumor activity, the reduction of toxicity, which offer the possibility of administer the nanocapsules daily, we could suggest that a more adequate biodistribution profile for the PGA-PEG HP nanocapsules compared to the Cremophor® EL formulation occurs, lending to the passive accumulation of the drug in the pathological site.

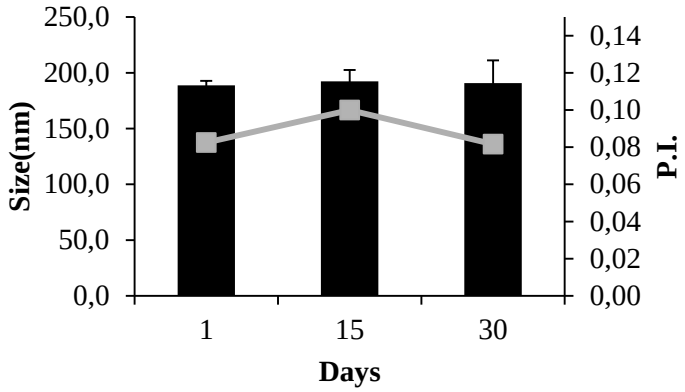
Besides, the antitumor behavior observed with PGA-PEG HP nanocapsules follow the same trend described for PEGylated nanocarriers that demonstrated enhancing the antitumor activity of encapsulated taxanes when were compared with the respective commercial forms [33,34]. Heureaux et al reported that the entrapment of paclitaxel into Pegylated lipidic nanocapsules result in an improvement of the therapeutic efficacy associate with a better survival [35]. Furthermore, they have shown that using this nanotechnology approach it was possible to administrate more doses without any premedication. In line with these ends Yuk et al demonstrated that multi core shell nanoparticles are also effective in inhibiting tumor growth compared with the commercial docetaxel formulation [36].

All these results are expected to be related with the intrinsic properties of the nanocapsules developed as tissue concentration driven by the EPR effect [37].

Stability and freeze-drying studies of PGA-PEG HP nanocapsules

The stability of the nanocapsules under storage during 1 month at 4 °C in terms of size, zeta potential and leakage of the drug, was also assessed. There was no modification on the particle size neither on the zeta potential of the nanocapsules, which maintained their original values throughout the study(Figure 5).Additionally no leakage of plitidepsin could be observed, which remarks the effectiveness of PGA-PEG HP nanocapsules as a carrier of the hydrophobic anticancer drug

(a)



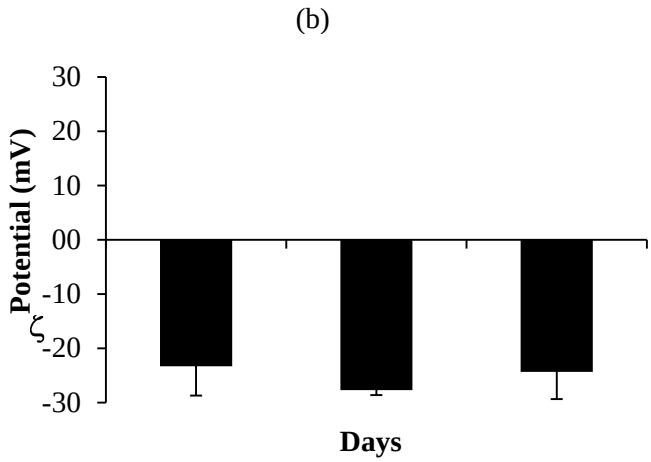


Figure 5: Particle size (a) and zeta potential (b) evolution of plitidepsin-loaded PGA-PEG nanocapsules at storage condition (1month, 4°C). (Mean ± S.D.; n=3).

In a second step we explored the optimal freeze-drying conditions for the conversion of the aqueous suspension of nanocapsules into a powder. The cryoprotectant trehalose was incorporated in the study at two different concentrations (5 and 10% w/v) and also the concentration of blank nanocapsules was tested at four levels (1-0.75-0.5-0.25 % w/v). The results indicated that the recovery of the initial properties of PGA-PEG HP nanocapsules upon freeze-drying and reconstitution varied with the concentration of nanocapsules and cryoprotectant. The size of PGA-PEG HP nanocapsules remained close to the initial values after lyophylization at a trehalose concentration of 10%. However, when the cryoprotectant concentration decreased, the size of the resuspended nanocapsules was slightly higher (Figure 6).

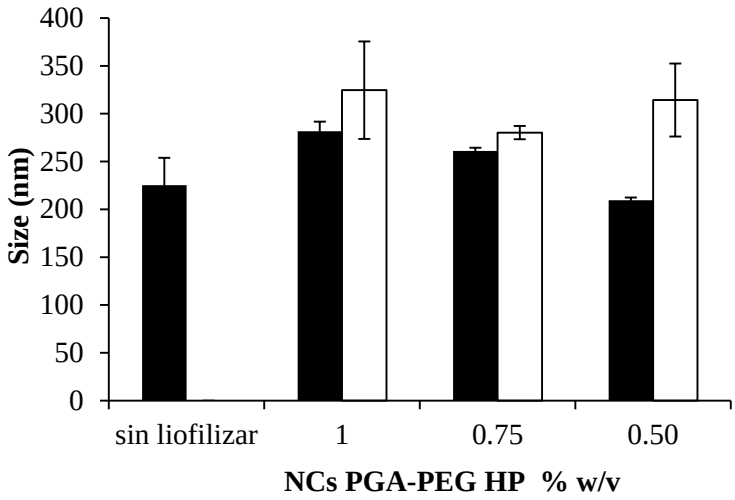


Figure 6: Particle size of the reconstituted freeze dried blank PGA-PEG nanocapsules using trehalose. Different concentrations (w/v) of nanocapsules were lyophilized using trehalose at 5 (□) or 10 (■) % w/v (Mean ± S.D.; n=3).

Conclusions

Here we successfully developed novel high PEGylated nanocapsules as novel carrier for the antitumor drug plitidepsin. Results from the present study demonstrated that coating nanocapsules with a high PEG diblock copolymer improve pharmacokinetic parameters, conferred long circulating properties and lend to the passive accumulation of nanocapsules at the tumor site. Moreover plitidepsin-loaded PGA-PEG nanocapsules were well tolerated and less toxic than plitidepsin formulated in Cremophor® EL. *in vivo* antitumor activity in the MRI-H-121 (renal) xenograft tumor model also revealed an important suppression of tumor growth compared to control after a single intravenous administration. All these data clary indicated the feasibility and the advantages of PGA-PEG HP nanocapsules as a novel platform for the features cancer chemotherapy.

Acknowledgements

Financial support from CENIT-NANOFAR XS53 project, PharmaMar, Spain, and the Ministry of Sciences and Innovation. Giovanna Lollo has a fellowship from the Ministry of Education of Spain. Marcos Garcia Fuentes acknowledges an Isidro Parga Pondal Fellowship from Xunta de Galicia.

References

1. Blanco E, Hsiao A, Mann AP, Landry MG, Meric-Bernstam F, Ferrari M. Nanomedicine in cancer therapy: Innovative trends and prospects. Cancer Science 2011;102(7):1247-1252.

2. Wang M, Thanou M. Targeting nanoparticles to cancer. Pharmacological Research 2010 8;62(2):90-99..

3. Sinha R, Kim GJ, Nie S, Shin DM. Nanotechnology in cancer therapeutics: bioconjugated nanoparticles for drug delivery. Molecular Cancer Therapeutics 2006 August 01;5(8):1909-1917..

4. Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev 2002 9/13;54(5):631-651.

5. Kateb B, Chiu K, Black KL, Yamamoto V, Khalsa B, Ljubimova JY, et al. Nanoplatforms for constructing new approaches to cancer treatment, imaging, and drug delivery: What should be the policy? Neuroimage 2011 1;54(Supplement 1):S106-S124..

6. Maeda H, Bharate GY, Daruwalla J. Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. European Journal of Pharmaceutics and Biopharmaceutics 2009 3;71(3):409-419.

7. Danhier F, Feron O, Préat V. To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. J Controlled Release 2010 12/1;148(2):135-146.

8. Mora-Huertas CE, Fessi H, Elaissari A. Polymer-based nanocapsules for drug delivery. Int J Pharm 2010 1/29;385(1-2):113-142.

9. van Vlerken L, Vyas T, Amiji M. Poly(ethylene glycol)-modified Nanocarriers for Tumor-targeted and Intracellular Delivery. Pharm Res 2007 -08-01/;24(8):1405-1414; 1414.

10. Li C, Wallace S. Polymer-drug conjugates: Recent development in clinical oncology. Adv Drug Deliv Rev 2008 5/22;60(8):886-898.

11. Singer JW. Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane. J Controlled Release 2005 12/5;109(1-3):120-126.

12. Pasut G, Veronese FM. Polymer–drug conjugation, recent achievements and general strategies. Progress in Polymer Science 2007 9;32(8-9):933-961.

13. Huynh NT, Roger E, Lautram N, Benoît J, Passirani C. The rise and rise of stealth nanocarriers for cancer therapy: passive versus active targeting. Nanomedicine 2010 11/01; 2011/05;5(9):1415-1433

14. Yoshizawa Y, Kono Y, Ogawara K, Kimura T, Higaki K. PEG liposomalization of paclitaxel improved its *in vivo* disposition and anti-tumor efficacy. Int J Pharm ;In Press, Corrected Proof.

15. Mitsiades CS, Ocio EM, Pandiella A, Maiso P, Gajate C, Garayoa M, et al. Aplidin, a Marine Organism–Derived Compound with Potent Antimyeloma Activity In vitro and *In vivo*. Cancer Research 2008 July 01;68(13):5216-5225.

16. Oliveira C, Garcia EM, López-Olmeda JF, Sánchez-Vázquez FJ. Daily and circadian melatonin release in vitro by the pineal organ of two nocturnal teleost species: Senegal sole (Solea senegalensis) and tench (Tinca tinca). Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 2009 7;153(3):297-302.

17. Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm 1989 10/1;55(1):R1-R4.

18. Brandon EFA, van Ooijen RD, Sparidans RW, Lázaro LL, Heck AJR, Beijnen JH, et al. Structure elucidation of aplidine metabolites formed in vitro by human liver microsomes using triple quadrupole mass spectrometry. Journal of Mass Spectrometry 2005;40(6):821-831..

19. Yin J, Aviles P, Lee W, Ly C, Floriano P, Ignacio M, et al. Development of a liquid chromatography/tandem mass spectrometry assay for the quantification of Aplidin®, a novel marine-derived antineoplastic agent, in human plasma. Rapid Communications in Mass Spectrometry 2003;17(16):1909-1914.

20. Zhigaltsev IV, Winters G, Srinivasulu M, Crawford J, Wong M, Amankwa L, et al. Development of a weak-base docetaxel derivative that can be loaded into lipid nanoparticles. J Controlled Release 2010 6/15;144(3):332-340.

21. Owens III DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm 2006 /1/3/;307(1):93-102..

22. Lozano MV, Torrecilla D, Torres D, Vidal A, Dominguez F, Alonso MJ. Highly Efficient System To Deliver Taxanes into Tumor Cells: Docetaxel-Loaded Chitosan Oligomer Colloidal Carriers. Biomacromolecules 2008 08/01; 2011/09;9(8):2186-2193

23. Mosqueira VCF, Legrand P, Gulik A, Bourdon O, Gref R, Labarre D, et al. Relationship between complement activation, cellular uptake

and surface physicochemical aspects of novel PEG-modified nanocapsules. Biomaterials 2001 11/15;22(22):2967-2979.

24. Wang X, Wang Y, Chen ZG, Shin DM. Advances of Cancer Therapy by Nanotechnology. Cancer Res Treat 2009 /3/;41(1):1-11.

25. Mosqueira VCF, Legrand P, Morgat J, Vert M, Mysiakine E, Gref R, et al. Biodistribution of Long-Circulating PEG-Grafted Nanocapsules in Mice: Effects of PEG Chain Length and Density. Pharm Res 2001 -10-01/;18(10):1411-1419; 1419.

26. Prego C, Torres D, Fernandez-Megia E, Novoa-Carballal, Alonso MJ. Chitosan PEG nanocapsules as new carriers for oral peptide delivery: Effect of chitosan pegylation degree. J Controlled Release 2006 /4/10/;111(3):299-308.

27. Gonzalo T., Lollo G., G.F.M., Correa J., Riguera R. , Fernández-Megía E., Calvo P., Torres D., Alonso M.J., Polyglutamic acid and polyglutamic acid-polyethyleneglycol nanocapsules: a new nanocarrier for parenteral delivery of anticancer drugs. 2012. In preparation.

28. Yang T, Cui F, Choi M, Cho J, Chung S, Shim C, et al. Enhanced solubility and stability of PEGylated liposomal paclitaxel: In vitro and *in vivo* evaluation. Int J Pharm 2007 6/29;338(1–2):317-326..

29. Crosasso P, Ceruti M, Brusa P, Arpicco S, Dosio F, Cattel L. Preparation, characterization and properties of sterically stabilized paclitaxel-containing liposomes. J Controlled Release 2000 1/3;63(1–2):19-30..

30. Lacoeuille F, Hindre F, Moal F, Roux J, Passirani C, Couturier O, et al. *In vivo* evaluation of lipid nanocapsules as a promising colloidal carrier for paclitaxel. Int J Pharm 2007 11/1;344(1–2):143-149.

31. Huynh NT, Passirani C, Saulnier P, Benoit JP. Lipid nanocapsules: A new platform for nanomedicine. Int J Pharm 2009 9/11;379(2):201-209.

32. Khalid M, Simard P, Hoarau D, Dragomir A, Leroux J. Long Circulating Poly(Ethylene Glycol)-Decorated Lipid Nanocapsules Deliver Docetaxel to Solid Tumors. Pharmaceutical Research 2006;23(4):752-758.

33. Kim SC, Kim DW, Shim YH, Bang JS, Oh HS, Kim SW, et al. *In vivo* evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. J Controlled Release 2001 5/14;72(1–3):191-202.

34. Danhier F, Lecouturier N, Vroman B, Jérôme C, Marchand-Brynaert J, Feron O, et al. Paclitaxel-loaded PEGylated PLGA-based nanoparticles: In vitro and *in vivo* evaluation. J Controlled Release 2009 1/5;133(1):11-17.

35. Hureaux J, Lagarce F, Gagnadoux F, Rousselet M, Moal V, Urban T, et al. Toxicological Study and Efficacy of Blank and Paclitaxel-Loaded Lipid Nanocapsules After i.v. Administration in Mice. Pharmaceutical Research 2010;27(3):421-430.

36 Yuk SH, Oh KS, Koo H, Jeon H, Kim K, Kwon IC. Multi-core vesicle nanoparticles based on vesicle fusion for delivery of chemotherapic drugs. Biomaterials 2011 11;32(31):7924-7931.

37 Lee JY, Bae KH, Kim JS, Nam YS, Park TG. Intracellular delivery of paclitaxel using oil-free, shell cross-linked HSA – Multi-armed PEG nanocapsules. Biomaterials 2011 11;32(33):8635-8644.

CAPÍTULO 5.

Pegylated polyglutamic acid nanocapsules as carriers for anticancer drug delivery: *in vivo* proof-of-principle

Abstract

Recently we reported the development of novel biodegradable nanocapsules consisting of an oily core and a polymer shell made of polyglutamic acid (PGA) and a high pegylated PGA-polyethylenglycol block copolymer (PGA-PEG HP). In this work our aim has been to carry out the *in vivo* proof-of-principle of these nanocapsules as carriers for anticancer drug delivery. For this, we selected specific prototypes containing a fluorescent marker or docetaxel as a model drug. In a first instance, we evaluated the *in vivo* blood kinetics of fluorescently labeled PGA and PGA-PEG HP nanocapsules. PGA-PEG HP nanocapsules showed an important increase in the half-life and AUC as compared to PGA nanocapsules. Secondly, we determined the antitumor efficacy of docetaxel loaded PGA-PEG HP nanocapsules in an *in vivo* U87MG glioma mouse model. The results indicated that nanocapsules displayed an antitumor effect that was comparable to that of Taxotere® (60% tumor growth inhibition with respect to the untreated mice), while their efficacy in terms of survival rate was significantly increased. These evidences highlight the potential of these novel nanocapsules as a new drug delivery platform in cancer therapy.

Introduction

From the number of anticancer drug delivery vehicles approved so far for clinical use it has become clear that oncological nanomedicines hold promise as a strategy to overcome the important drawbacks associated to conventional formulations of anticancer agents. These drawbacks refer mainly to their low water solubility and overwhelming toxicity associated to the lack of selectivity for cancer cells. Often, this toxicity problem has been further enhanced by the use of excipients and solvents, which are themselves responsible of additional serious side effects [1].

Drug delivery nanostructures offer suitable means to improve current cancer chemotherapy by solving these water solubility problems and also by modulating the pharmacokinetic and biodistribution of cytotoxic drugs. Namely, they have the possibility to passively extravasate the fenestrated vasculature of tumor tissues and accumulate in cancer tumor cells. However, this accumulation can only be possible if the nanocarrier has the ability to escape the mononuclear phagocytic system (MPS). This is the behavior that has been reported for the so-called "stealth" nanocarriers, which are characterized for having a hydrophilic corona around them [2]. The hydrophilic surface has been classically conferred by the use of PEGylated biomaterials (either polymers or phospholipids), however, recently there is a tendency to explore the other hydrophilic polymers. In this line, polyaminoacids have also been found to reduce MPS uptake and increase the blood circulation time of liposomes [3-5]

In addition to the reported stealth properties, polyaminoacids are gaining increasing importance because of their interesting physicochemical properties and promising safety profile [6]. In particular, the anionic polymer poly-L-glutamic acid (PGA) has raised great expectancy as it is known to be highly susceptible to lysosomal

degradation [7]. Some authors have also evidenced the benefit of introducing PEG chains in the polymer architecture, either in the form of block or grafted copolymers [8-10]. Overall, the potential and safety of these polymers are well exemplified by the two formulations, PGA conjugates and PGA-PEG micelles containing anticancer drugs, which are under clinical development (Xyotax® and NC-6004) [11-13]. In the case of micelles, the PEGylation of PGA has been reported to further enhance the long circulating properties of the nanocarriers [14].

Because of this interesting background information, we decided to explore the potential of PGA as a biomaterial for the development of novel nanocarriers, named as PGA nanocapsules intended for the delivery of anticancer drugs. Up to now, we have shown that PGA nanocapsules, which consist of an oily core surrounded by a PGA shell, have an important capacity for the encapsulation of different hydrophobic drugs such as docetaxel and plitidepsin [15]. Moreover, we have also found that these nanocarriers provide an efficient intracellular drug delivery.

Based on this previous experience, our purpose in this work has been to assess the proof-of-principle of PGA and PGA-PEG high pegylted (HP) nanocapsules from an *in vitro* and *in vivo* perspective. For this, we have evaluated *in vitro* the biocompatibility of PGA and PGA-PEG HP nanocapsules in terms of complement activation and hemolysis. Furthermore, *in vivo* blood kinetic profiles, antitumor efficacy and survival rate after intravenous injection to mice were evaluated. For these studies we chose, docetaxel, an hydrophobic anticancer agent, as a model drug.

Materials and Methods

Chemicals

Docetaxel (from Flucka), Miglyol 812®, neutral oil formed by esters of caprylic and capric fatty acid and glycerol, was a gift sample from Sasol Germany GmbH (Germany). Epikuron 170, a phosphatidylcholine enriched fraction of soybean lecithin, was provided by Cargill (Spain). Benzalkonium chloride, Poloxamer 188 (Pluronic® F68) and Polyglutamic acid (Mw 15-50 kDa) were purchased from Sigma-Aldrich. Polyglutamic acid-polyethylenglycol (PGA-PEG Mw 35 kDa) was supplied by Alamanda Polymers (USA). PGA-PEG was a diblock copolymer with a percentage w/w of PEG of about 57. PEG chains length was 20 kDa and the PGA chains length was about 15 kDa. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (Did) (Did Em 644 nm; Ex 664 nm) was obtained from Molecular Probes-Invitrogen (USA).Taxotere® was provided by the Hospital Pharmacy of Angers.

Preparation of Nanocapsules

The preparation of PGA and PGA-PEG HP nanocapsules was based on a modification of the solvent displacement technique as previously reported by our group [16]. The method involved a polymer ionic interaction after solvent diffusion. Briefly, an organic phase was formed by dissolving 30 mg of Epikuron 170 in 0.5 ml of ethanol, followed by 125 µl of Mygliol®812 and 7 mg of cationic surfactant, benzalkonium chloride, in 9 ml of acetone solution. This organic phase was immediately poured over 20 ml of a mix solution of poloxamer (0.25 % w/v) and PGA or PGA-PEG HP (1 mg/ml). Finally, solvents were evaporated under vacuum from the suspension to a final constant volume

of 10 ml. Nanoemulsions were also obtained by the method previously described. Unlike nanocapsules, nanoemulsions are only formed by the hydrophobic cores without the polymer cover. We prepare anionic and cationic nanoemulsions which differ for the presence of the cationic surfactant benzalkonium chloride.

As control for complement study we have used a cationic nanoemulsion, while the anionic nanoemulsion was the control for the evaluation of the blood kinetic profile.

The encapsulation of the fluorescent probe DiD into PGA, PGA-PEG HP nanocapsules and anionic nanoemulsions was achieved by adding aliquots of DiD dissolved in ethanol to the organic phase prior to the formation of the nanocapsules. By the same way docetaxel (DCX) was encapsulate into PGA-PEG HP nanocapsules.

Characterization of PGA nanocapsules

Particle size and polydispersion index were determined by photon correlation spectroscopy (PCS). Samples were diluted to an appropriate concentration in deionized water and each analysis was carried out at 25ºC with an angle detection of 173º. 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 into nanocapsules

In order to achieve the incorporation of DCX into PGA-PEG HP nanocapsules, 0.5 ml of the DCX stock ethanol solution (conc. 20 mg/ml) were added to the organic phase and the process was continued as described previously. PGA-PEG HP nanocapsules were concentrated up

to a final volume of 5 ml in order to obtain a final drug concentration of 2 mg/ml. [17]

The encapsulation efficiency of DCX in PGA-PEG HP 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 PGA-PEG HP nanocapsules with acetonitrile. This sample was centrifuged during 20 min at 4000g 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 PGA-PEG HP nanocapsules from the aqueous medium by ultracentrifugation at 27400g x g, 15°C, 1h.

DCX was assayed by a slightly modified version of the method proposed by Lee et al. [18]. 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.

Fluorescent, DiD, encapsulation into nanocapsules

Fluorescent labeled formulations, PGA, PGA-PEG HP nanocapsules and DiD loaded anionic nanoemulsion, were obtained replacing 0.5 ml of ethanol of the organic phase with 0.5 ml of DiD stock solution in ethanol (2.5 mg/ml). The final concentration of DiD in the nanocapsules and anionic nanoemulsion was around 100 µg/ml. DiD encapsulation efficiency in all the systems was determined indirectly by the difference between the total amount of fluorescent probe in the formulations and the free dye measured in the infranatant of the nanocapsules and nanoemulsions after ultracentrifugation (27400g 15°C, 1h). At the end of the ultracentrifugation aliquots of infranatant were diluted with acetonitrile and analyzed by UV spectrophotometry (646 nm). To determinate the total amount of the probe present in the systems aliquots of nanocapsules and nanoemulsioons suspension were diluted with acetonitrile and analyzed at λ=646 nm.

Hemolysis test

Hemolytic potential of PGA and PGA-PEG HP nanocapsules was determinate and compared with the cationic nanoemulsion in rat blood. The blood of female Wistar rats was obtained by cardiac puncture. Sodium citrate, pH 7.4, was diluted with blood (1:10) before adding PBS. This mixture was centrifuged (700 g, 10 min, 20°C) three times, discarding the supernatant and adding PBS. Then the erythrocyte were diluted with PBS (3% w/v) and stored at 4°C. A sample of 150 µl of the erythrocyte stock dispersion was added to 150 µl of nanocapsules suspensions (2 % w/v and 1 % w/v diluted in PBS) and led to incubate under shaking at 37°C for 1 hour. Then, the samples were centrifuged (750 g, 3min, 20°C) in order to remove intact erythrocytes and debris, 100 µl of the supernatant were added to 2000 µl of a mixture of absolute

ethanol and conc. HCl (40/1 (v/v)) and centrifuged again. This mixture dissolved all components and avoided precipitation of hemoglobin. The absorption of the supernatant was measured by UV spectroscopy (λ=398 nm) against blank samples. Results were set in relation to control samples of 0% lysis (PBS) and 100% lysis (bidistilled water) [19]. The hemolytic percentage was calculated according to the equation below [20]:

$$lysis\ \%\ = \frac{A_{sample} - A_{blank}}{A_{water} - A_{blank}} \times 100$$

in vitro complement activation study

Complement activation was evaluated in normal human serum (NHS) (provided by the Establissment Francais du Sang, CHU, Angers, France) by measuring the residual hemolytic capacity of the complement system after contact with the different particles [21]. The technique consisted in determining the amount of serum able to lyse 50% of a fixed number of sensitized sheep erythrocytes with rabbit anti-sheep erythrocyte antibodies (CH50), according to the procedure described elsewhere [22] . Complement activation was expressed as a function of the surface area. Nanocapsules and nanoemulsions surface areas were calculated as previously described [23], using the equation: S=n4πr^2and V=n(4/3)(πr^3)leading to S=3m/rρ where S is the surface area (cm2) and V the volume (cm3) of n spherical beads of average radius r (cm), m the weight (µg) and φ the volumetric mass (µg/cm3). All experiments were performed in triplicate [24].

Blood Kinetic study

in vivo plasmatic elimination kinetic study

Animal care was administered in strict accordance to French Ministry of Agriculture regulations. The treatment was according to Morielle et al [24] as follows: 150 µl of fluorescent PGA, PGA-PEG HP nanocapsules and anionic NE were injected in the tail vein of six-week old female Swiss mice (9-12 weeks 20-22 g) (Ets Janvier, Le Genest-St-ile, France). The fluorescence was measured at time 30, 60, 120, 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). To obtain the fluorescence at time 0, aliquot of fluorescent nanocapsules were diluted with a blood sample at the same *in vivo* concentration. 150 µl of the supernatant were deposited in a black, 96-well plate (Greiner Bio-one, Germany). Plasma residual fluorescence was measured from the supernatant of centrifuged blood taken from three rats receiving 150 µL of a physiological saline solution. The results were analyzed with the Ascent software for Fluorscan (Thermo Fischer Scientific, France). Did fluorescence was counted by a Fluorscan at emission wavelength of 644 nm with an excitation wavelength of 664 nm. 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 [25]. Fluorescence was expressed in fluorescence units (FU) and was calculated as: FU sample – FU empty. 100% of fluorescence was considered as the value at t = 0 min [24].

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 (t1/2 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 cell line

U87MG glioma cell line (ATCC, Manassas, VA) were obtained from the European Collection of Cell Culture (UK, Nº94110705). The cells were cultured at 37ºC/5% CO2 in Dulbecco modified eagle medium (DMEM) with glucose and L-glutamine (BioWhitakker, Belgium) containing 10% fetal calf serum (FCS) (BioWhitakker) and 1% antibiotic and antimycotic solution (Sigma, France). On the implantation is day, cells were trypsinised and resuspended into minimal essential medium (EMEM), without FCS or antibiotics, to the final desired concentration [26].

Subcutaneous glioma model and therapy schedule

Animals were manipulated under isoflurane/oxygen anaesthesia. Tumor bearing mice were prepared by injecting subcutaneously a suspension of 1 x 106 U87MG glioma cells in 150 µl of Hanks Balanced Saline Solution (HBSS) into the right flank of athimic nude mice (6 weeks

old females, 20-24g, purchased from Ets Janvier, Le Genest-St-ile, France). 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:

$$V = \frac{\pi}{6} \times width^{2} \times length$$

When tumors reached a calculated average volume of approximately 200 mm3, 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. Animals were treated (Day 0) by a single intravenous (IV) injection of different treatments, 150 µl via lateral tail vein as follows: physiological saline solution (0.9% NaCl), docetaxel loaded PGA-PEG HP nanocapsules (2 mg/Kg mouse) and Taxoter$^{e®}$ (2 mg/Kg mouse).

Tumor size was measured twice weekly after the IV of the treatments. At day 25, mice were then isolated and weighed. The different treated groups were compared in terms of mean survival time in days after U87GM cell implantation. The percentage increase in survival time (% IST) was determined relative to the mean survival of untreated controls as presented in the following equation:

$$\%IST = \frac{Mean_T - Mean_C}{Mean_C} \times 100$$

Where MeanT was the mean of survival time of the treated group and MeanC was the median/mean of the survival time of the control group [27, 28].

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, and mean survival time (days), long term survivors (%) and increase in survival time (IST_{mean}%).

Results and discussion

Recently we have presented the design and the development of a novel type of polyaminoacid nanocapsules, PGA nanocapsules, and their modification with a high pegylated PGA-PEG HP copolymer as a promising platform in anticancer drug delivery [15, 29]. As described in the introduction, our goal in this work has been to provide the first proof-of-principle of these novel nanocarriers as anticancer drug delivery vehicles. For this, we have performed a number of studies, which include: (i) the evaluation of some biocompatibility properties of PGA and PGA-PEG HP nanocapsules; (ii) the determination of the blood kinetic parameters of fluorescent labeled PGA and PGA-PEG HP nanocapsules (iii) the determination of the efficacy and toxicity profile of DCX-loaded PGA-PEG HP in a U87MG glioma mice model, using the commercial formulation Taxotere®, as a control.

The characteristic of PGA and PGA-PEG HP nanocapsules and control nanoemulsions

PGA and PGA-PEG HP nanocapsules were prepared with the same materials and using the solvent displacement technique previously reported [15]. They were loaded separately with DiD and DCX and, then, characterized for their size, zeta potential and encapsulation efficiency. As shown in Table 1, irrespective of the loaded compound and the polymer coating, the nanocapsules formed monodispersed populations with a mean size around 200 nm. As expected, PGA and PGA-PEG HP nanocapsules were both negatively charged, but their zeta potential values were dependent on the polymer coating. The lower values observed for PGA-PEG HP nanocapsules as compared to that of PGA nanocapsules (-20 mV vs. -39 mV) was attributed to the shielding effect of PEG chains around the surface of the systems. Table 1 also shows the charge inversion observed for PGA and PGA-PEG HP nanocapsules with the respect to the cationic nanoemulsion (+40 mV), thus evidencing the formation of a continuous shell of polymer around the cationic oily nanodroplets. The anionic nanoemulsion exhibits as well a negative potential (-40 mV) due to the lack of positively charged surfactants in their composition. Finally, with respect to the loading properties of the system, the results indicate that both, the fluorescent probe and DCX, were efficiently encapsulated (67-90 %) without altering the original characteristics of nanocapsules.

Table 1: Physicochemical characteristics of blank and DiD- and DXC-loaded PGA, PGA-PEG HP nanocapsules. The characteristics of control cationic and anionic nanoemulsions are also shwon. P.I.: polydispersity index, Values are given as mean ± SD; n=3. NCs: nanocapsules and NE: nanoemulsion.

Formulation	Size (nm)	P.I.	ζ Potential (mV)	Enc. Eff (%)
PGA NCs	183 ± 6	0.1	-39 ± 4	-
PGA-PEG HP NCs	180 ± 4	0.1	-20 ± 4	-
Anionic NE	207 ± 7	0.1	-38 ± 1	-
Cationic NE	227 ± 8	0.1	+40 ± 4	-
DiD-loaded PGA NCs	179 ± 3	0.1	-31 ± 2	67 ± 5
DiD-loaded PGA-PEG HP NCs	194 ± 2	0.1	-15 ± 3	70 ± 8
DiD-loaded Anionic NE	214 ± 5	0.1	-28 ± 6	79 ± 10
DCX-loaded PGA-PEG HP NCs	200 ± 3	0.1	-20 ± 4	90 ± 2

The hemolysis activity of nanocapsules and control nanoemulsions

This analysis was performed in order to have an estimation of the cytotoxicity of the nanocapsules after IV administration. The release of hemoglobin was used to quantify the membrane damaging properties of the systems assayed. The results showed that both PGA and PGA-PEG HP nanocapsules had no hemolytic behavior when diluted with erythrocytes at 1 and 0.5 % w/v, being the hemolysis values under 3%. No detectable disturbance of the red blood cell membranes could be observed with these nanocapsules. In contrast, cationic nanoemulsions, used as a control, had a high hemolytic activity (60%) at both concentrations tested. Therefore, these results indicate that the presence of a PGA or PGA-PEG HP shell around the oily nanodroplets counteracts the inherent hemolytic activity of cationic emulsions, thus providing the system with an adequate biocompatibility.

The complement activation properties

It is known that following injection into the blood circulation, foreign particles can be rapidly cleared by phagocytosis by the MPS. This process involves first the recognition of these particles by opsonins, such as complement protein, which upon adsorption onto the nanoparticles facilitate their recognition and elimination by MPS. In this study we evaluated the complement activation properties of the nanocapsules as a way to preliminary predict the *in vivo* fate of nanocarriers after intravenous administration. With this objective in mind, we determined the extent of interactions between nanoparticles and the complement system using the CH50 technique. CH50 measures the hemolytic capacity of a fixed amount of normal human serum towards 50% of antibody-sensitized sheep erythrocytes after exposure with different concentrations of blank PGA and PGA-PEG HP nanocapsules compared with cationic nanoemulsions as control.

As presented in Figure 1, PGA and PGA-PEG HP nanocapsules exhibited a very weak no dose-dependent hemolytic activity. These results are in accordance with those previously reported in the literature. In fact, it is known that the complement activation activity of particulated matter is governed by three main parameters: composition, size and surface properties, being larger and positively charged particles more prone to lead to the complement activation [30]. In addition, it is known that the use of anionic polyaminoacids and polyethylene glycol increase the surface hydrophilicity and reduce complement activation [31, 32]. Accordingly, in our study we observed that negatively charged PGA and PGA-PEG HP nanocapsules (mean size of 200 nm) exhibited a very weak complement activation ability, whereas the cationic control emulsion triggered a rapid complement activation[30, 33].

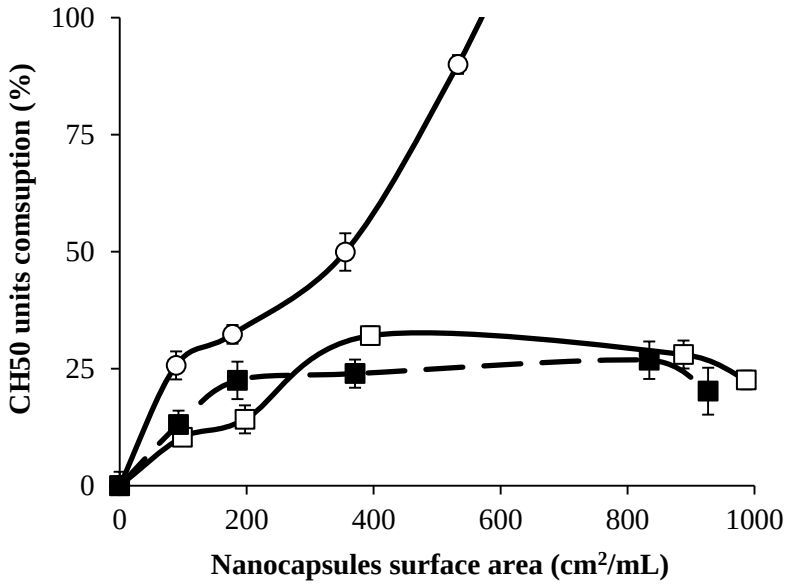


Figure 1: Complement activation profile measured as lytic capacity of the serum (% CH50 units) towards antibody-sensitized sheep erythrocytes after exposure to of PGA (□) and PGA-PEG HP nanocápsulas (■) compared with the cationic nanoemulsion used as a control (○). Complement consumption was evaluated as a function of the nanoparticles surface area (cm^2).

in vivo studies

The results presented in Figure 2 illustrate the plasma kinetics profiles of fluorescent PGA, PGA-PEG HP nanocapsules and that of a control anionic nanoemulsion upon IV administration to mice. The results indicate the percentage of fluorescence content of blood samples. The 100% fluorescence value was determined upon mixing labeled formulations with blood. The results indicate that the nanoemulsion was removed from the blood circulation within a few minutes, with only 15% of the total fluorescence remaining in plasma at 30 min after i.v. injection. In contrast, PGA and PGA-PEG HP nanocapsules remained at a high concentration (about 40% of injected dose) after 3 h post-injection.

Finally, approximately 8% of the injected dose of PGA nanocapsules remained in circulation 24 h after the injection, while in the case of PGA-PEG HP the total amount present in the blood was around 20% (significantly higher compared with the nanoemulsion).

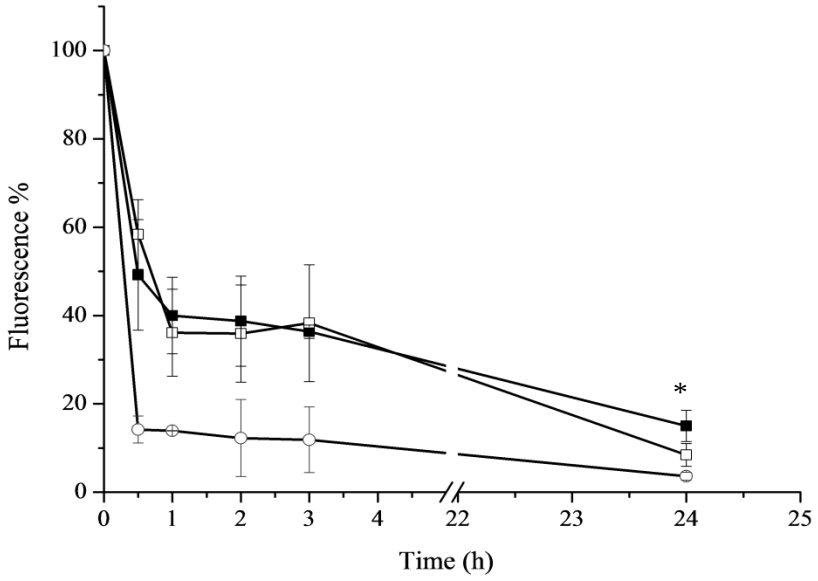


Figure 2: Percentage of nanocarriers-associated fluorescence remaining in plasma after single bolus injection of PGA (□) and PGA-PEG HP nanocapsules (■) in Swiss mice. The injected dose of DiD was 1 mg/kg of animal weight. The fluorescence observed upon administration of the control nanoemulsion (○) is also showed as a control. Each data point represents the group mean ± SD of the percentage of injected dose. *P < 0.05.

The blood kinetics parameters are summarized in Table 2. It can be noted that both, PGA and PGA-PEG HP nanocapsules, clearly differ in their kinetics behavior from that of the control nanoemulsion. Particular remarcable is the difference in the half-life and mean residence time (MRT), whose values are double than those observed for the control nanoemulsion. Moreover, the values of the area under the curve (AUC) rose from 11 mg/ml.h for nanoemulsion to 38 mg/ml.h for PGA

nanocapsules and finally to 50 mg/ml*h in the case of PGA-PEG HP nanocapsules.

Overall, these results illustrate the protective role of the PGA coating vis a vis the phagocitic uptake of the nanocarrier by the MPS.

Moreover, in agreement with previous literature reports, the results indicate that by modifying the PGA with PEG it is possible to further enhance this protective role [13, 34]. This is understood by the formation of a more protective shield that is able to prevent the rapid elimination from the blood circulation through steric repulsion to opsonins. According to a theoretical calculation, we have found that the distance between two PEG chains on the external surface of the nanocapsules is around 3 nm, a value that has been previsouly resported for other long circulating systems [35]. Briefly, the PEGylation of the PGA is expected to enhance the possibility for the nanocarrier to reach the target site via passive targeting.

Table 2: The main parameters illustrating the plasma elimination kinetics of DiD loaded PGA, PGA-PEG HP nanocapsules and anionic nanoemulsion after a single i.v. injection in Swiss mice. Each data point represents the group mean ± SD. NCs: nanocapsules and NE: nanoemulsion.

Formulation	**t ½ distribution α (h) (0-1h)**	**t ½ elimination β (h) (1-24h)**	**MRT (h) (0-24h)**	**AUC (mg/ml*h) (0-24h)**
PGA NCs	0,66	10,02	9,72	38,02
PGA-PEG HP NCs	**0,74**	**16,08**	**17**	**50,65**
NE	0,34	8,17	6,59	11,43

Antitumor activity

The promising plasma kinetics results obtained for PGA-PEG HP nanocapsules encouraged us to go further in the *in vivo* proof-of-principle and to evaluate their efficacy in U87MG glioma bearing mice model. For this study we used the commercial docetaxel formulation, Taxotere® as a control.

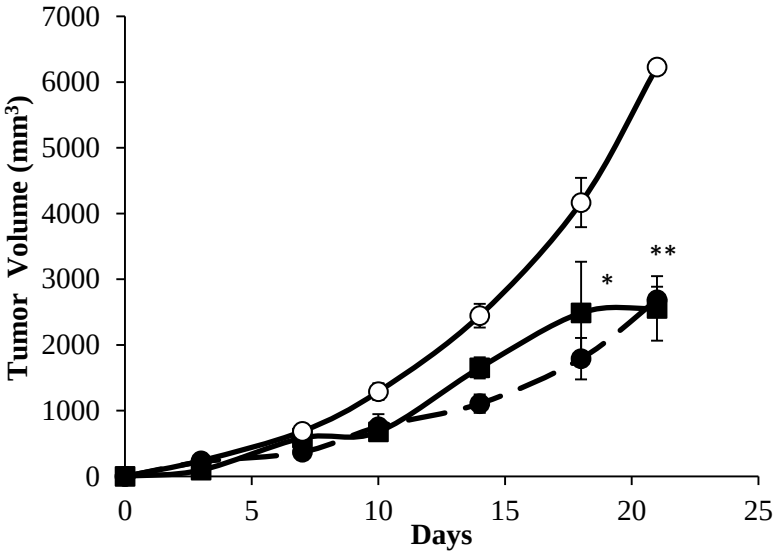


Figure 3: Evolution of tumor volume following IV administration of a single-dose of DCX-loaded PGA-PEG HP nanocapsules (■) and Taxotere® (●) in a subcutaneous U87MG glioma mouse model. Control group received 0.9% NaCl solution (○).Statistical analysis by pairs shows significant differences on Day 18 and 21 in tumor growth of mice treated with PGA-PEG nanocapsules or Taxotere® as compared to control (non-treated mice). *P < 0.05 **P < 0.01 t-Student test. All data are reported as means ± S.E.; n = 6.

The results presented in Figure 3 show the growth of the subcutaneous tumor over the time and tumor volume at days 18 and 21 as compared to the initial volume (at the day of the treatment). It can be noted that in the control group the tumor grew exponentially, reaching a

tumor volume of about 6x103 mm^3 at day 20. In contrast, the IV injection of a single dose of either, DCX-loaded PGA-PEG HP nanocapsules or the control, Taxotere®, significantly decreased tumor growth compared to the control group ($p<0.01$). Interestingly, towards the end of the study we observed a tendency for the mice tumor treated with nanocapsules to reach a plateau, whereas that of mice treated with Taxotere®, continue to increase. These results are in accordance with the long-circulating properties of PGA-PEG HP nanocapsules and their potential to favor the concentration of the drug in the tumor by passive targeting.

in vivo toxicity study

As an additional relevant analysis for the *in vivo* proof-of-principle of PGA-PEG HP nanocapsules, we evaluated the toxicity of the systems by monitoring the mice survival times. The Kaplan-Meier survival plots are shown in Figure 4. For the calculation of the mean survival times, we used a censured model, according to which a censure event was a tumor growth that was more than 10% of mouse weight.

Figure 4 shows the survival probability values measured by the Kaplan-Meier method. These results indicate that non-treated mice did not survive beyond 18 days, whereas the survival time increased significantly upon treatment with Taxotere® and more importantly upon treatment with DCX-loaded PGA-PEG HP nanocapsules. More precisely, the results in Table 9 show that mice treated with commercial Taxotere® increased the mean survival time in 50% with respect to the control, whereas those treated with the PGA-PEG HP nanocapsules exhibited a 61% increase of this parameter. Overall, these results evidenced the reduced toxicity of the nanocapsules as compared to the commercial formulation.

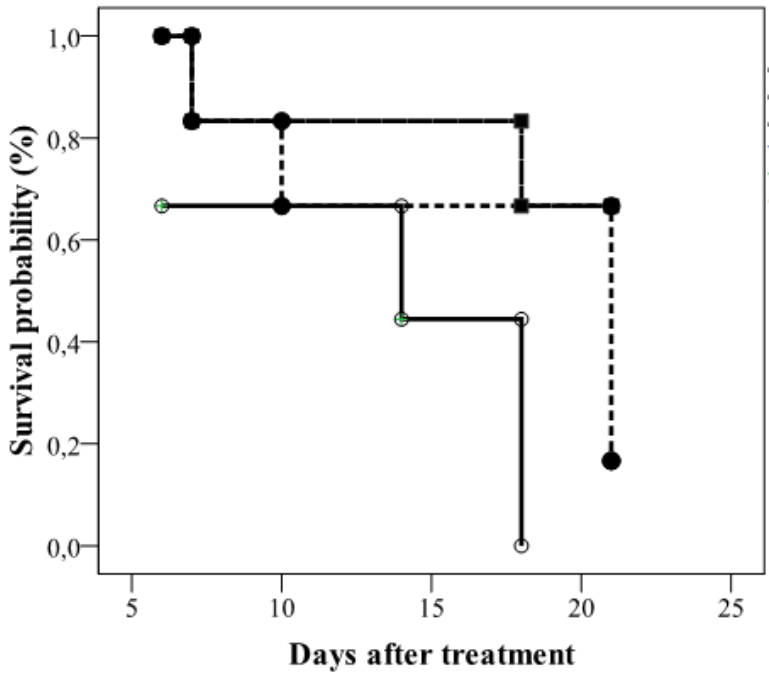


Figure 4: Kaplan-Meier survival curves of subcutaneous U87MG glioma tumor bearing mice following treatment with Taxotere® (●) or DCX-loaded PGA-PEG HP nanocapsules (■). Non-treated mice were used as a control (○).

Table 3: Mean survival time of U87 glioma-bearing mice that received an IV injection of DCX-loaded PGA-PEG HP nanocapsules or Taxotere®. Non treated mice (receiving an injection of serum) were used as a control. Number of mice per group: 6.

Treatment	Mean survival time (days)		Increase in survival time (IST) (%)	
	Survival time range	**Mean ± SD**	**IST $_{mean}$**	***p*-Value *vs* control**
DCX-loaded PGA-PEG HP NCs	7-18	18.1 ± 2	61.6	0.066
Taxotere®	7-21	16.8 ± 2	50	0.147
Control (non-treated)	7-17	11.2 ± 2	-	-

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

Conclusion

The results of this work represent the first *in vivo* proof-of-principle of PGA and PGA-PEG HP nanocapsules as anticancer drug delivery nanocarriers. Concretely, these results show that these nanocarriers have good blood compatibility and a very low complement activity. They also show that the PGA shell protects the nanocarrier from uptake by MPS, providing them with a blood long circulating behavior, and that his effect could be further enhanced using the block copolymer PGA-PEG. Finally, DCX-loaded PGA-PEG HP nanocapsules exhibited an antitumor effect that was comparable to that of the commercial formulation, whereas their toxicity measured by the mice survival times-was significantly reduced. Consequently, PGA-PEG HP nanocapsules are presented here as a new delivery technology for hydrophobic anticancer drugs.

Acknowledgements

The work has been supported by Lymphonanocarriers for the treatment of metastatic cancer, European Commission FP7 EraNet - EuroNanoMed Program and Instituto Carlos III (PS09/02670). Giovanna Lollo has a fellowship from the Ministry of Education of Spain. Marcos Garcia Fuentes acknowledges an Isidro Parga Pondal Fellowship from Xunta de Galicia.

References

1. Alexis, F., et al., Nanoparticle Technologies for Cancer TherapyDrug Delivery, M. Schäfer-Korting, Editor 2010, Springer Berlin Heidelberg. p. 55-86.

2. Vladimir, T., Tumor delivery of macromolecular drugs based on the EPR effect. Advanced Drug Delivery Reviews, 2011. 63(3): p. 131-135.

3. 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.

4. 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 (BBA) - Biomembranes, 2007. 1768(3): p. 737-743.

5. 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.

6. Nair, L.S. and C.T. Laurencin, Biodegradable polymers as biomaterials. Progress in Polymer Science, 2007. 32(8-9): p. 762-798.

7. Chun, L., Poly(l-glutamic acid)–anticancer drug conjugates. Advanced Drug Delivery Reviews, 2002. 54(5): p. 695-713.

8. Du, J.-Z., et al., Evaluation of Polymeric Micelles from Brush Polymer with Poly(ε-caprolactone)-b-Poly(ethylene glycol) Side

Chains as Drug Carrier. Biomacromolecules, 2009. 10(8): p. 2169-2174.

9. Guo, S., et al., Ternary complexes of amphiphilic polycaprolactone-graft-poly (N,N-dimethylaminoethyl methacrylate), DNA and polyglutamic acid-graft-poly(ethylene glycol) for gene delivery. Biomaterials, 2011. 32(18): p. 4283-4292.

10. Szczepanowicz, K., et al., Formation of Biocompatible Nanocapsules with Emulsion Core and Pegylated Shell by Polyelectrolyte Multilayer Adsorption. Langmuir, 2010. 26(15): p. 12592-12597.

11. Sabbatini, P., et al., A phase II trial of paclitaxel poliglumex in recurrent or persistent ovarian or primary peritoneal cancer (EOC): A gynecologic oncology group study. Gynecologic Oncology, 2008. 111(3): p. 455-460.

12. Plummer, R., et al., A Phase I clinical study of cisplatin-incorporated polymeric micelles (NC-6004) in patients with solid tumours. Br J Cancer, 2011. 104(4): p. 593-598.

13. Li, C. and S. Wallace, Polymer-drug conjugates: Recent development in clinical oncology. Advanced Drug Delivery Reviews, 2008. 60(8): p. 886-898.

14. Bae, Y. and K. Kataoka, Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers. Advanced Drug Delivery Reviews, 2009. 61(10): p. 768-784.

15. Gonzalo T. Lollo G., G.F.M., Correa J., Riguera R. , Fernández-Megía E., Calvo P., Torres D., Alonso M.J., Polyglutamic acid and polyglutamic acid-polyethyleneglycol nanocapsules: a new

nanocarrier for parenteral delivery of anticancer drugs. In preparation, 2012.

16. 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.

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.

18. Lee, S.H., S.D. Yoo, and K.H. Lee, Rapid and sensitive determination of paclitaxel in mouse plasma by high-performance liquid chromatography. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 1999. 724(2): p. 357-363.

19. Schubert, M.A. and C.C. Müller-Goymann, Characterisation of surface-modified solid lipid nanoparticles (SLN): Influence of lecithin and nonionic emulsifier. European Journal of Pharmaceutics and Biopharmaceutics, 2005. 61(1-2): p. 77-86.

20. Lu, J., S.C. Owen, and M.S. Shoichet, Stability of Self-Assembled Polymeric Micelles in Serum. Macromolecules, 2011. 44(15): p. 6002-6008.

21. Kazatchkine, M.D. and M.P. Carreno, Activation of the complement system at the interface between blood and artificial surfaces. Biomaterials, 1988. 9(1): p. 30-35.

22. Vonarbourg, A., et al., Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake.

Journal of Biomedical Materials Research Part A, 2006. 78A(3): p. 620-628.

23. Passirani, C., et al., Interactions of nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate) with the complement system. Life Sciences, 1998. 62(8): p. 775-785.

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

25. Calvo, P., et al., Long-Circulating PEGylated Polycyanoacrylate Nanoparticles as New Drug Carrier for Brain Delivery. Pharmaceutical Research, 2001. 18(8): p. 1157-1166.

26. Huynh, N., et al., Treatment of 9L Gliosarcoma in Rats by Ferrociphenol-Loaded Lipid Nanocapsules Based on a Passive Targeting Strategy via the EPR Effect. Pharmaceutical Research: p. 1-10.

27. Huynh, N., et al., Treatment of 9L Gliosarcoma in Rats by Ferrociphenol-Loaded Lipid Nanocapsules Based on a Passive Targeting Strategy via the EPR Effect. Pharmaceutical Research, 2011: p. 1-10.

28. 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.

29. Lollo G., C.P., Avilés P., Guillén M.J., García-Fuentes M., Torres D., Alonso M.J., Long circulating PEG-polyglutamic acid nanocapsules for an improved antitumor efficacy: *in vivo* evaluation in a murine solid tumour model. In preparation, 2012.

30. Vonarbourg, A., et al., Parameters influencing the stealthiness of colloidal drug delivery systems. Biomaterials, 2006. 27(24): p. 4356-4373.

31. Li, Y., et al., Delivery of nanomedicines to extracellular and intracellular compartments of a solid tumor. Advanced Drug Delivery Reviews, (0).

32. Dash, B.C., et al., The influence of size and charge of chitosan/polyglutamic acid hollow spheres on cellular internalization, viability and blood compatibility. Biomaterials, 2010. 31(32): p. 8188-8197.

33. Moghimi, S.M., et al., Material properties in complement activation. Advanced Drug Delivery Reviews, 2011. 63(12): p. 1000-1007.

34. Yasuhiro, M., Poly (amino acid) micelle nanocarriers in preclinical and clinical studies. Advanced Drug Delivery Reviews, 2008. 60(8): p. 899-914.

35. Mosqueira, V.C.F., et al., Biodistribution of Long-Circulating PEG-Grafted Nanocapsules in Mice: Effects of PEG Chain Length and Density. Pharmaceutical Research, 2001. 18(10): p. 1411-1419.

CAPÍTULO 6.

A novel approach for oral delivery of peptides:polyarginine nanocapsules

Abstract

We have previously reported the development of novel nanocapsules made of polyarginine (PARG) specifically designed for overcoming cellular barriers. In this work, our goal has been to investigate the potential of these nanocarriers for oral drug delivery. With this purpose in mind, we first selected an antitumoral amphiphilic peptide and explored ways to efficiently encapsulate it within the nanocapsules. The results indicated that it was possible to achieve an adequate encapsulation efficiency using an adapted solvent displacement technique. In a second instance, we evaluated *in vitro* the stability of the nanocarrier in simulated intestinal fluid as well as their interaction with the model epithelial cell line Caco-2. The results showed that PARG nanocapsules were stable in the simulated fluids and exhibited the ability to increase in a transient manner the transepithelial electric resistance. Moreover, we observed that PARG nanocapsules were internalized by the monolayer in a very efficient manner without evident signs of cytotoxicity. Finally, we performed an *in vivo* fluorescent imaging study, from which we could conclude that fluorescently-labeled PARG nanocapsules were retained in the intestine for at least three hours upon oral administration. Overall the results of this work point out the interest of PARG nanocapsules as potential transmucosal carriers for oral drug delivery.

Introduction

Over the last decades, a significant number of active macromolecules, mainly peptides, have been produced either by biotechnology or chemical synthesis. These macromolecules are characterized by their instability in biological media and their limited ability to overcome mucosal barriers. As a consequence they cannot be administered orally, representing a great limitation for their clinical development [1, 2]. The oral route is the one preferred by patients because of its convenience for self-administration and its non-invasive nature. Unfortunately, oral peptide delivery still remains a major pharmaceutical challenge and a dire technological need for industry. As a result, a substantial amount of research has been oriented to the development of strategies to make the oral administration of peptides feasible [3, 4].

An ideal drug delivery system for oral administration should protect the drug from the aggressive environment typical of the gastric and intestinal cavities, maximize the interaction of the intact drug with the intestinal mucosa, prolong its intestinal residence time and reversibly increase the permeability of the mucosal epithelium to enhance drug absorption [5]. Considering that some nanocarriers are believed to present such features [6], they have been explored for oral drug delivery applications [7, 8].

Our group has shown that nanocapsules, defined as vesicular systems in which an oily core is surrounded by a polymeric shell, represent a promising platform to increase the oral bioavailability of drugs. Because of their reservoir structure, nanocapsules are expected to protect the encapsulated drug from the enzymatic attack [9]. On the other hand, the capacity of nanocarriers to overcome mucosal barriers is very much dependent on the nature of the coating material [10]. In particular, we observed that chitosan nanocapsules are able to adhere to a mucus-producing model epithelium (Caco-2-HT29 co-culture) [11].

Within this frame, biopolymers such as peptides, proteins and polysaccharides are gaining increasing attention for the design of novel drug delivery systems, and in the context of nanocapsules, as biomaterials for their coating layer. In particular, our interests has recently shifted to polyaminoacids, which are highly functional molecules structurally similar to homo polypeptides, and thus, completely biodegradable by human enzymes [12]. Among them, Poly-L-arginine (PARG), a homopolymer belonging to the Cell Penetrating Peptides (CPP) family, is considered a promising biomaterial for the design of drug delivery systems [13-16].

The cell-penetrating properties of arginine-rich polyaminoacids have been attributed to the presence of guanidine functional groups in its side chain, which interacts directly with cell surface domains and subsequently facilitates cellular internalization [17-19]. Besides this penetration enhancement ability, PARG can interact with the tight junction proteins, occluding and E-cadherin, favoring the paracellular transport of hydrophilic drugs [13]. All these properties are deemed to be responsible for the capacity of PARG to improve the nasal delivery of drugs [20, 21].

Taking into account this background, we have developed recently a new type of nanocapsules coated with PARG [22]. In the present work, we studied the capacity of this nanocarrier for drug encapsulation, and its potential for oral drug delivery. As a drug of choice for these studies, we encapsulated the amphiphilic peptide PM02734. This is a novel marine-derived drug synthetized by PharmaMar S.A. (Spain) with demonstrated antitumor activity in breast, colon, pancreas, lung and prostate cancer cell lines [23].

Materials and methods

Materials

Polyarginine (PARG, MW 5000-15000 Da) and poloxamer 188 were purchased from Sigma-Aldrich (Spain). Miglyol® 812 purchased from Sigma-Aldrich (Spain). Epikuron® 170, was donated by Cargill (Spain). The peptide drug PM02734 was obtained from PharmaMar S.A. (Spain). DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) and fluorescein-DHPE (N-(fluorescein-5-thiocarbamoyl)-1.2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (fluorescein-DHPE) were supplied by Invitrogen. All the solvents employed were of analytical grade and supplied by Merck.

Preparation of PARG nanocapsules

PARG nanocapsules were obtained by a modification of the solvent displacement technique based on a polymer ionic interaction upon solvents diffusion. The general method to obtain PARG nanocapsules can be described as follows: 0.125 ml of Miglyol® 812, 30 mg of Epikuron® 170 were dissolved in 9 ml of acetone and 0.5 ml of ethanol. This organic phase was poured over an aqueous phase (20 mL) containing the surfactant poloxamer 188 (0.25% w/w) and PARG (15 mg). Finally, the organic solvents were removed under vacuum to a final volume of 10 ml. A nanoemulsion was used as a control for the cell uptake studies. Nanoemulsions were prepared similarly to nanocapsules, but without PARG in the aqueous phase.

Physicochemical characterization of PARG nanocapsules

Particle size and polydispersity index were determined by photon correlation spectroscopy (Zetasizer NanoZS, software version 5.10,

Malvern Instruments, Malvern, U.K.). The zeta potential values were calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry. The morphological analysis of the nanocapsules was performed by transmission electron microscopy (TEM, CM12 Philips, The Netherlands). For TEM imaging, samples were stained with 2% w/v phosphotungstic acid solution, and placed on a copper grid with Formvar® films for analysis.

PM02734 encapsulation into PARG nanocapsules

PM02734 loaded nanocapsules were prepared by including 2.5 mg or 10 mg of the active compound in the ethanol solution before adding the other components of the organic phase. Then, the same method described above was followed to prepare drug loaded nanocapsules. PARG nanocapsules with the highest drug concentrations (PM02734 concentration 1.6 mg/mL) were obtained by the same way, but solvents were evaporated under vacuum to a final volume of 6.5 mL.

PM02734 encapsulation efficiency of PARG nanocapsules was determined indirectly by the difference between the total amount of the active compound in the formulation and the free drug measured in the aqueous phase. The total amount of drug was estimated by dissolving an aliquot of PARG nanocapsules suspension with acetonitrile. This sample was centrifuged during 20 min at 4000 g and the supernatant, measured with HPLC. The free drug was determined by ultracentrifuging an aliquot of the PARG nanocapsules (1h, 27400 g, 15°C). Nanocapsules accumulated in the top (cream), leaving a clear drug solution in the bottom of the tubes. This clear solution was extracted, diluted with the HPLC mobile phase and centrifuged (4000g, 20 min) before analysis by HPLC.The encapsulation efficiency was calculated according with the relationship:

Encapsulation Efficiency (%) = [Total drug conc. – Free drug conc.]/[Total drug conc.] × 100

HPLC analysis

The HPLC system consisted of an Agilent 1100 Series instrument equipped with a UV detector set at 225 nm and a Simmetry Shield TM RP 18 column (4.6 x 150 mm i.d., pore size 3.5 μm Waters®). The mobile phase consisted of a mixture of acetonitrile with 0.04% v/v trifluoroacetic acid (phase A) and water with 0.04% v/v trifluoroacetic acid (phase B). The flow rate was 0.6 ml/min and the analysis was carried out in gradient from 45% to 72% of phase A over 30 minutes, and then, 72% of phase A for 10 minutes.

Fluorescent probe encapsulation into PARG nanocapsules

The incorporation of the fluorescent probe fluorescein-DHPE in PARG nanocapsules was achieved by adding aliquots of the dye stock solution in ethanol to the organic phase before to the formation of the nanocapsules. Fluorescent nanoemulsions were used as controls. To calculate the encapsulation efficiency of fluorescein-DHPE loaded PARG nanocapsules the systems were centrifuged (27400 g, 1h, 15°C) and the free fluorescein was detected by fluorimetry (λEx= 496 nm, λEm =519 nm).

Nanocapsules loaded with the fluorescent dye DiD, used in the *in vivo* imaging studies were prepared as described before. However, in this case, 0.5 ml of DiD stock solution in ethanol (2.5 mg/ml) was added to the organic phase before nanocapsule preparation (to a final concentration of 100 μg/ml). To calculate the encapsulation efficiency of DiD loaded nanocapsules, the system was previously centrifuged (20000 g, 1h, 4°C) to eliminate the non-encapsulated dye. After this process, the

encapsulation efficiency was calculated indirectly by the difference between the total amounts of DiD in the formulation and the free dye found in the solution below the cream. The total amount of DiD was estimated by dissolving an aliquot of the colloidal suspension of nanocapsules with acetonitrile. These samples were analyzed by UV (λ = 646 nm).

Stability study of PM02734-loaded PARG nanocapsules in simulated physiological fluids

Drug loaded nanocapsules were incubated at 37°C in simulated gastric (USP 35, pH 1.2) or intestinal medium (USP 35, pH 6.8) prepared without enzymes. Then, aliquots of the suspensions were incubated at 37ºC and the size and polydispersity index of the nanocapsules was measured at different times for a period of 2 h.

Stability upon storage of PM02734-loaded PARG nanocapsules

The stability of loaded PARG nanocapsules suspension at 4 °C was followed for a period of 15 days by measuring the particle size and polidispersity index of the colloidal system. The percentage of drug encapsulated in the nanocapsules was also determined after 15 days of storage at 4°C, in order to determine the stability of the drug loaded in the PARG nanocapsules.

in vitro release study

The release of PM02734 from PARG nanocapsules was performed by incubating a sample of each formulation with intestinal fluid without enzymes (USP 35, pH 6.8) at sink conditions. The vials were placed in an incubator at 37º C with horizontal shaking. An aliquot of 4 ml

of the suspension was collected at different time intervals (1, 2, 3, 4 h), and ultracentrifuged in Herolab® tubes (Herolab GmbH, Germany). The amount of drug released was calculated directly by determining the amount of free drug in the aqueous phase as described above. As a control, the total amount of drug in the suspension was also determined.

Culture of Caco-2 cells

Human colon adenocarcinoma cells (Caco-2 cells) were cultivated on 80 cm2 flasks (Nunc, Denmark) using Dulbecco's modified Eagle medium-high glucose (DMEM) supplemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids (x100), penicillin (100U/ml) and streptomycin (100 μg/ml) (all obtained from Sigma-Aldrich). Cells were maintained at 37ºC in a humidified atmosphere with CO2. The culture medium was changed every two days for approximately 5-6 days until cells reached approximately 80-90% confluence. Then, cells were trypsinized, subcultured and seeded at 2.5x105 cells per flask. For these experiments, cells with passage number 65 were used.

Cytotoxicity studies

Cells were cultivated on 96-well plates (Nunc, Denmark) with a cell density of 1.4 x 104 cells/cm^2 for approximately two days, in order to obtain a homogeneous cell monolayer. For the actual study, the medium was replaced by PARG nanocapsules and the control nanoemulsion in cell culture medium at concentrations between 30 μg/cm^2 and 606 μg/cm^2. DMEM was used as a positive control. Cells monolayers were kept in contact with the samples for 2 hours, at 37ºC and 5% CO2. After this time, the samples were removed and the cell viability was determined by an MTT assay performed according to the manufacturer instructions [19]. The absorbance (λ=490 nm) of the samples was measured with a BioRad

680 spectrophotometer, and corrected for the background absorbance (λ=655 nm). The percentage of cell viability was calculated by comparing the absorbance of the samples exposed to the nanocapsules as compared to the control.

Measurement of the transepithelial electrical resistance (TEER)

The measurement of the transepithelial electrical resistance (TEER) was performed on 6-well Transwell cell culture chambers (Costar, USA) using a Millicell®-ERS system (Millipore, Spain). Cells were seeded onto the apical compartment of a Transwell at a cell density of 2.5 x 105 cell/well. The culture medium was changed every other day and the cells were kept for 21 days, in order to obtain a tight cell monolayer.

Transepithelial electrical resistance (TEER) of the cell monolayers was measured just before adding the nanoemulsions, as a reference. Then, cell medium in the apical chambers was changed for PARG nanocapsules or nanoemulsion in cell medium or for fresh cell medium, used as a control. Two concentrations of each nanosystem were tested, 0.1 mg/ml and 1mg/ml, leading to a final dose per surface of 32 and 320 μg/cm^2, respectively. The samples were incubated for 120 min and TEER values were recorded at 30, 60 and 120 minutes. After this time, the tested samples were removed and replaced by fresh medium in order to check the TEER values at 24 h after exposure to the nanocapsules. Each TEER value was calculated as a percentage related to the initial TEER value.

Qualitative uptake studies

Caco-2 cells were seeded at a density of 5.5 x 104 cells/cm^2 on sterile coverslips placed in 24-well plates and left to grow until a

monolayer was formed. After this, the apical culture medium was removed and cells were incubated with the fluorescent nanosystems. Free fluorescent dye was used as a control. After incubation for 2 hours, cells were washed three times with PBS (Sigma-Aldrich, Spain). Then, the cells were fixed for 10 min with 4% p-formaldehyde in PBS at room temperature, rinsed twice with fresh PBS and permeabilized for 20 min with 1% Bovine Serum Albumin (BSA)/ 0.1% Triton X-100 solution in PBS. After permeabilization, the cytoskeleton of the cells was stained with 5% Phalloidin-Bodipy® 650/665 solution in PBS for 60 min at 4ºC. After staining, the monolayers were thoroughly washed with PBS, the coverslips separated from the wells, and the sample embedded in one drop of the mounting medium Fluorsave® (Carbiochem, US). Samples were kept overnight at 4ºC and examined under a confocal laser scanning microscope (Leica TCS-SP2, Leica Microsystems).

in vivo fluorescence imaging of DiD-loaded PARG nanocapsules

in vivo fluorescence imaging was performed with a non-invasive imaging device using IVIS (Calliper, LEITAT, Spain). This system provides high signal-to-noise images of fluorescence signals emerging from within living animals. The animals were fasted for 24h before nanocarriers administration. Then, 0.5 ml of isolated DiD labeled PARG nanocapsules (at a concentration of 40 mg/ml) were given orally to mice. For imaging, animals were placed prone in a light-tight chamber, where a controlled flow of 1.5% isoflurane in air was administered through a nose cone via a gas anesthesia system. Then, mice were monitored by real-time Near Infra-Red (NIR) fluorescence intensity in the whole body. A gray-scale reference image was obtained under low-level illumination. The images were monitored 1, 3 and 24 h after oral administration. Optical excitation was carried out at 644 nm, and the emission wavelength was

detected at 664 nm. All experimental results were repeated on at least three different animals. Representative pictures are shown.

Results and discussion

As indicated in the introduction, the purpose of this work was to explore the potential of PARG nanocapsules for the oral administration of macromolecules. These nanocarriers are reservoir-type systems with an oily core and a PARG coating. The selection of PARG has been motivated by previous works which indicated the capacity of arginine rich peptides to improve the electrostatic interaction of nanocarriers with intestinal mucosa [18]. Furthermore, the inclusion of a lipid core was based on the recognized ability of lipids to protect macromolecules as well as to enhance intestinal permeability [29, 30]. We have previously reported the design of PARG nanocapsules [22] and found that this system has the capacity to penetrate inside cells and to promote the intracellular absorption of antitumoral drugs.

In this work, PARG nanocapsules were prepared and characterized to load an amphiphilic anticancer macromolecule: PM02734. Furthermore, PARG nanocapsules were tested for their capacity to interact with model intestinal epithelia in a Caco-2 cell model and finally *in vivo* upon oral administration. It is assumed that the main mechanisms supporting nanocarrier-mediated oral delivery of macromolecules are: (i) protection of the drug encapsulated from the harsh environment of the gastrointestinal tract, (ii) nanocarrier accumulation in the intestinal epithelium and (iii) the permeabilizing effect of the polymer. In this paper, we investigate all these potential mechanisms for PArg nanocapsules.

Preparation and characterization of peptide-loaded PARG nanocapsules

Nanocapsules were successfully obtained by the solvent displacement technique, conveniently adapted in order to induce the deposition of PARG onto the oil nanodroplets [22]. This technique has the advantages of being simple, quick and very mild as it avoids the use of high energy sources [23].

The nanocapsules developed showed a nanometric size below 200 nm, and low polydispersity index, evidencing the presence of a monodisperse particle population (Table 1). Interestingly, the incorporation of PARG to the formulation resulted in a significant reduction of particle size (-50 nm) as compared to the reference nanoemulsion (composition equal to the oily cores). Thus, suggesting the stabilizing role of PARG. Moreover, the surface charge of the PARG nanocapsules was positive, a result that is attributed to the presence of PARG on the surface of the nanocarriers. A size increase was observed upon encapsulation of PM02734, into the nanocapsules, whereas such increase was not apparent upon encapsulation of DiD or fluorescein-DHPE. Irrespective of this change in particle size the polydispersity of the nanocapsules remained unimodal. The loading of a high amount of peptide or the fluorescent marker DiD (125 µg/mL) was also accompanied with a reduction in the zeta potential values, thus suggesting a partial localization of these molecules on the nanocapsules surface. In contrast, when the amount of peptide encapsulated was low (250 µg/mL), the zeta potential value was not altered, thus suggesting the good entrapment of the peptide within the core of the nanocapsules.

Moreover, the results presented in (Table 1) indicate that the peptide PM02734 could be encapsulated into PARG nanocapsules with a reasonable efficiency (50% encapsulation efficiency). This moderate loading value could be related to the amphiphilic character of the peptide.

As expected, the encapsulation of the fluorescent probes, DiD and fluorescein-DHPE, was higher due to their solubilization in the oily core.

Table 1: Size, polydispersity index and zeta potential of blank, peptide and fluorescent probeloaded PARG nanocapsules (NCs). Nanoemulsion represents a control. Values are Means ± SD, n=3

Formulation	[Active] mg/mL	Size (nm)	P.I.	ζ Potential (mV)	Enc. Eff. (%)
Nanoemulsion	-	189 ± 6	0.1	-50 ± 7	
PARG NCs	-	138 ± 9	0.1	+47 ± 8	
Peptide-loaded PARG NCs	0.25	186 ± 7	0.1	+55 ± 6	54.1 ± 4
Peptide-loaded PARG NCs	1.6	178 ± 15	0.1	+30 ± 11	45.7 ± 7
DiD-labeled PARG NCs	0.125	129 ± 2	0.1	+25 5 1	75.0 ± 5
Fluorescein-DHPE labeled PARG NCs	0.80	140 ± 1	0.1	+52 ± 1	79.2 ± 1

The morphology of PM02734-loaded PARG nanocapsules was studied by transmission electron microscopy (TEM). The TEM images confirmed the particle size measured by PCS and the homogeneity of the particle's population. The images also show that PARG nanocapsules exhibit a rounded shape and a fluffy corona, which might be attributed to the polymer shell (Figure 1).

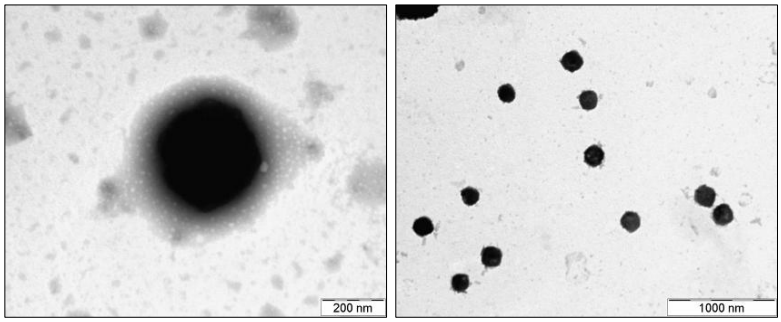


Figure 1: TEM images of PM02734-loaded PARG nanocapsules.

Stability and peptide release studies in simulated physiological fluids.

One of the key points to assess the potential of nanocarriers for oral drug delivery is the preservation of their stability in gastrointestinal fluids and the protection of the encapsulated drug along the gastrointestinal tract. Having these premises in mind, we evaluated the stability of the nanocapsules in simulated gastric and intestinal fluids. As shown in Figure 2, PARG nanocapsules remained perfectly stable in simulated gastric fluids for 2 h. On the other hand, we observed a continuous but slight size increase after one hour of incubation in simulate intestinal fluid, although the overall size increase was limited (up to 300 nm). This acceptable stability behavior could be explained by the high pKa of the guanidine group (pKa ~12.5) in the PARG chain, which leads to the protonation of PARG over all the ranges of physiological pH [14]. This positive charge could contribute to the stabilization of the nanocapsules in both, gastric and intestinal fluids, and simultaneously favor their interaction with the intestinal mucosa [30, 31].

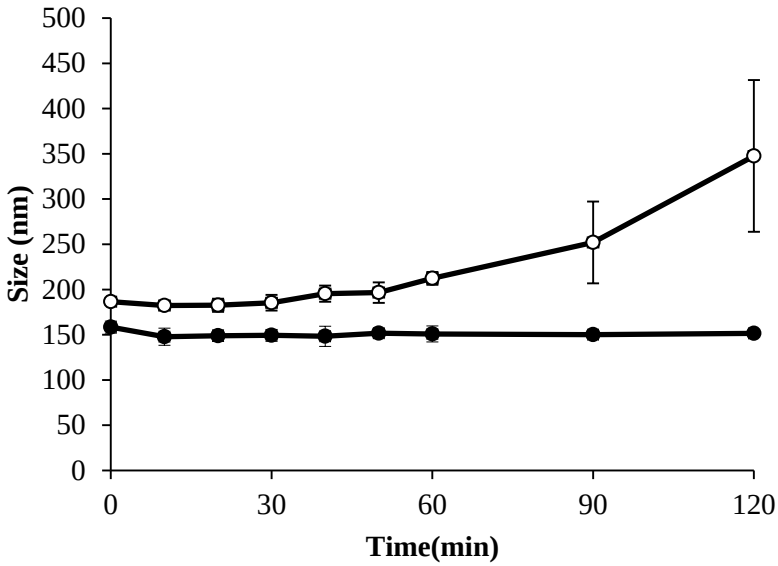


Figure 2: Evolution of the particle size of PARG nanocapsules measured after incubation in simulated gastric (●) and intestinal fluid (○). Values are presented as Mean ±SD, n=3

The stability pattern observed for the nanocapsules is in agreement with their *in vitro* release behavior in simulated intestinal medium. Indeed, we observed that PARG nanocapsules released only 10% of the total drug encapsulated after 4 hours (data not shown). Appropriate control experiments confirmed the presence of the peptide associated to the oily core at all-time points during the release experiment. This indicates that the colloidal system is not modified dramatically upon dilution in this simulated fluid, and also, that PM02734 has a strong tendency to remain associated to PARG nanocapsules.

Stability of peptide-loaded PARG nanocapsules upon storage

In a next step, we evaluated the stability of the peptide-loaded PARG nanocapsules upon storage for up to 15 days at 4 ºC. The results indicated that the mean size of the nanocapsules and the polydispersity index were not modified (Figure 3). Moreover, the amount of

encapsulated peptide remained stable during storage for at least up to 15 days.

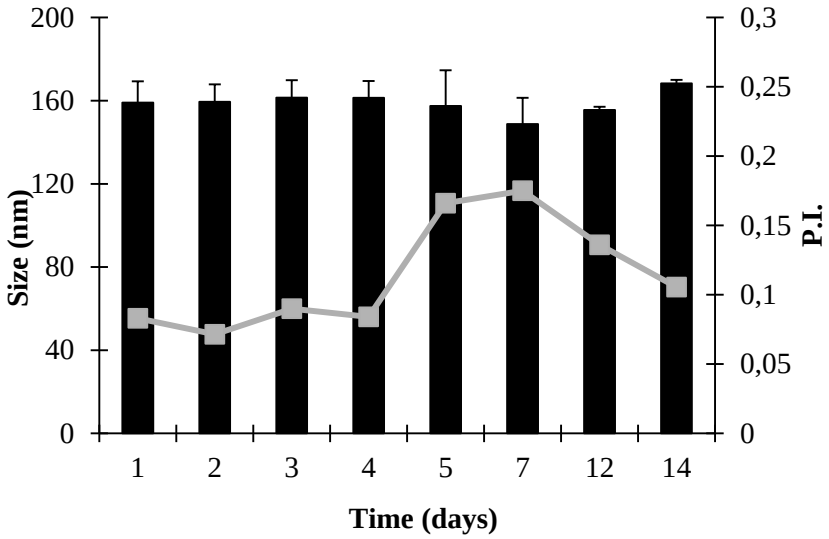


Figure 3: Particle size and polydispersity index (P.I.) evolution of PARG nanocapsules upon storage at 4°C during 2 weeks. Data is presented as means ± S.D., n=3.

Cytotoxicity of PARG nanocapsules in Caco-2 cells

The cytotoxicity of PARG nanocapsules was evaluated by MTT assay in the Caco-2 cell model. Figure 4 shows that in the range of concentrations tested, both PArg nanocapsules suspension and control nanoemulsion had limited effect in cell viability, with values of cell survival typically above 80% of the control (DMEM cell medium). Only the highest concentration of PARG nanocapsules caused a further reduction of cell viability, reaching the 70% of the control. In summary, PARG nanocapsules exhibited a low cytotoxicity profile, indicating their acceptability for oral drug delivery.

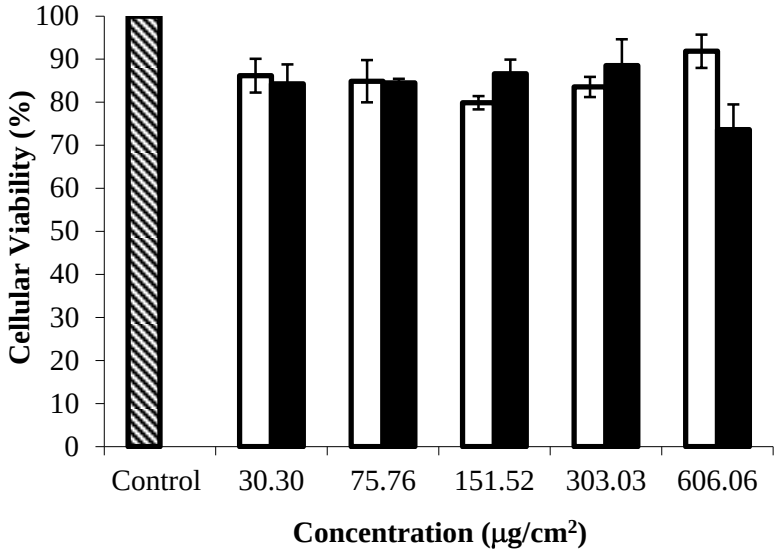


Figure 4: Cytotoxicity of PARG Nanocapsules (■) and nanoemulsion (□) in Caco-2 cell line (Mean ± SD, n=2).

Effect of PARG nanocapsules on the transepithelial electrical resistance (TEER)

Taking into account the reported interaction of PARG with the tight junctions [20] we found it critical to investigate if this ability was maintained for the selected PARG structured in nanocapsules. Modifications in the membrane permeability related to the opening of the tight junctions are normally associated to reductions in the TEER values [31, 32]. Thus, we decided to monitor the TEER values upon exposure of the Caco-2 cell monolayer to nanocapsules and corresponding control (DMEM cell medium). The results indicated that both nanoemulsion and PARG nanocapsules at the lowest concentration tested (32 μg/cm^2) did not affect the TEER value after two hours of contact with the cell monolayer (Figure 5-1). In contrast, PARG nanocapsules at a high dose (320 μg/cm^2) caused a significant reduction of the TEER already after 30 minutes of contact with the monolayer (Figure 5-2). This effect reached

30% reduction in the TEER, indicating opening of the tight junctions. In addition, 24 h after the removal of the nanocapsules the initial TEER value was recovered, which indicates that perturbation of the monolayer is transitory and disappears by removing the nanosystems. Taking into account that the nanocapsule's concentrations used in this experiments did not comprise cell viability, this TEER reduction could be directly associated to the ability of PARG nanocapsules to open the tight junctions.

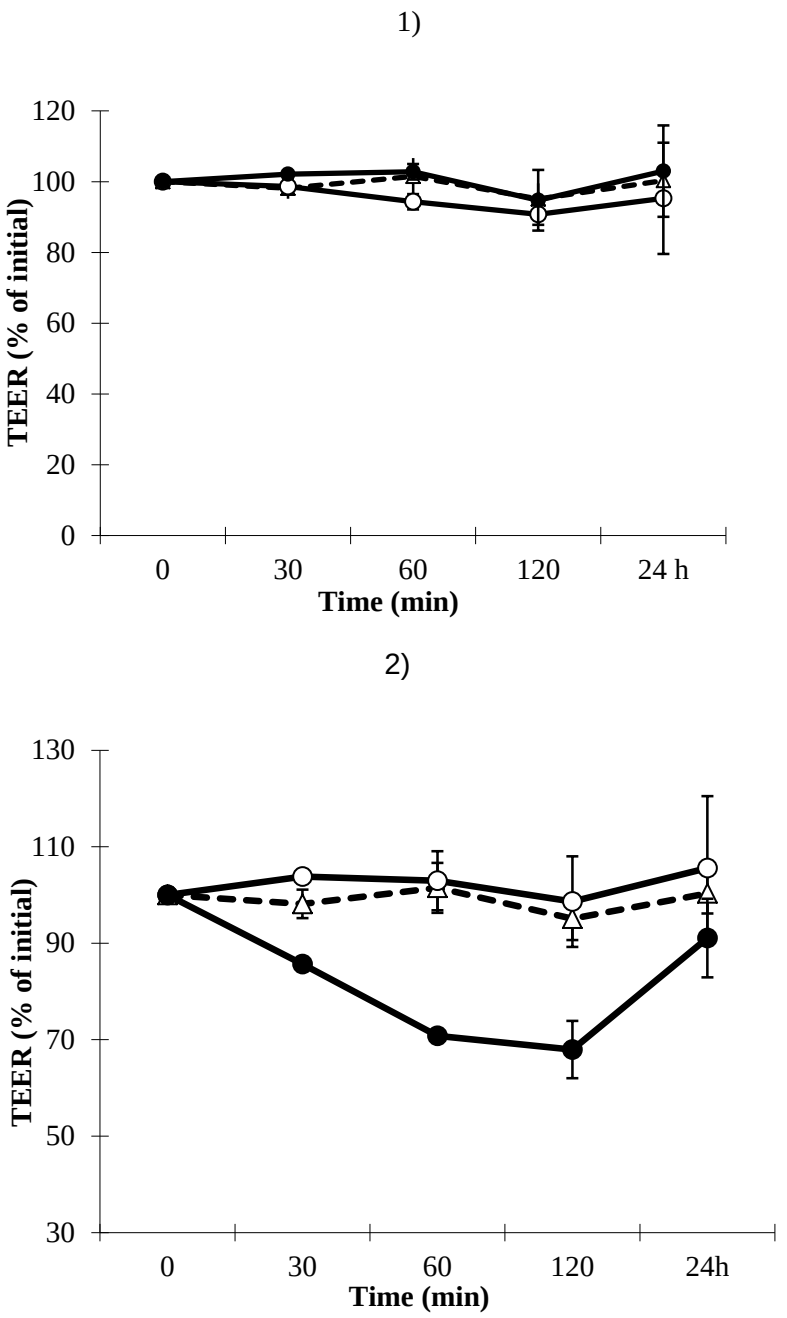


Figure 5: Transepithelial electrical resistance (TEER) of Caco-2 monolayer after incubation with control nanoemulsions (○) and PARG nanocapsules (●) at two different doses: (1) 32 μg/cm^2 and (2) 320 μg/cm^2 and its control (Δ) (DMEM cell medium).

Interaction of PARG nanocapsules with the Caco-2 cells monolayer

Polyarginine enter cells more effectively than other polycationic homopolymers such as those composed by lysine, ornithine or histidine [19, 33]. This property has been used to enhance the transport of associated macromolecules through lipidic membranes [28, 34]. The confocal micrographs presented in Figure 6-1 and Figure 6-2 show the images of consecutive optical cross-sections of Caco-2 cells (x-y, x-z and y-z projections) exposed to PARG nanocapsules and nanoemulsions for up to 2 hours. The fluorescent nanocarriers are shown in green, whereas the cell cytoskeleton, labeled with a fluorescent phalloidin conjugate is shown in red. The results in Figure 6- 1 show that the nanocapsules are located inside the cells and also in the boundary regions among cells. The analyses of the xz and zy projections also indicate the presence of the nanocapsules in both the apical (a) and the basolateral (b) side. The results displayed in Figure 6 illustrate the different behavior of the nanoemulsion as compared to the nanocapsules. In fact, the nanoemulsion particles were preferentially located on the surface of the monolayer; there were no evidence of their intracellular penetration. From these results we could infer that the PARG coating is essential for the intimate interaction followed by intracellular penetration of PARG nanocapsules into the Caco-2 cells. Overall, these results lead us to speculate about the potential *in vivo* penetration enhancement properties of PARG nanocapsules and their interest for oral administration of macromolecules. However, we should be cautious with the interpretation of these data due Caco-2 model monolayer does not have the mucus layer, a relevant barrier for the interaction of nanocarriers.

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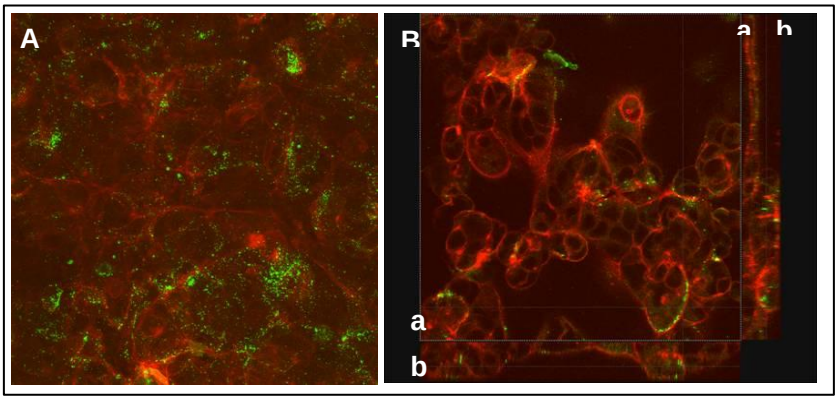

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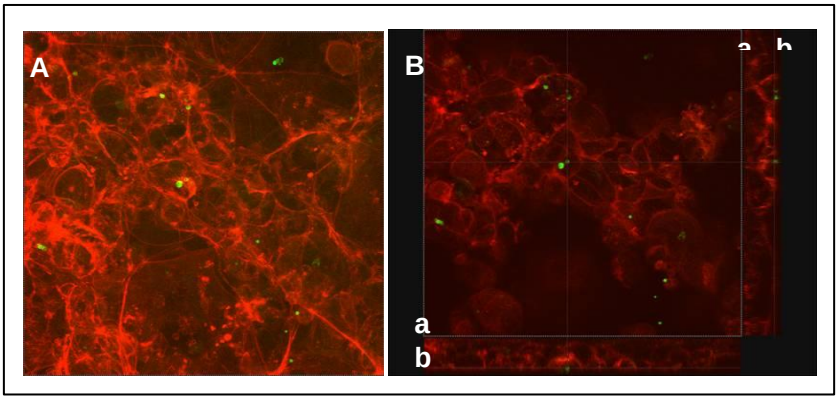


Figure 6: Confocal microscopic images of Caco-2 cells after incubation for 2 h at 37 ºC of PARG nanocapsules (1), and nanoemulsion (2). Optical cross-sections xy-axis (A) with xz- and yz- projections (B) are shown. Green: fluorescein-DHPE PArg nanocapsules; Red: cytoskeleton

Gastrointestinal retention of fluorescently labeled nanocapsules

The residence time of DiD-labeled PARG nanocapsules in the gastrointestinal tract was evaluated by Near Infrared (NIR) fluorescence imaging following oral administration to mice. DiD-loaded nanoemulsions were used as a control Figure 7 show representative images of the animals at 1 h, 3 h, and 24 h after the oral administration of the nanocarriers. Fluorescence signals in the abdominal region were evident from 1 h to 3 h after the administration of PARG nanocapsules

(Figure 7-1). This signal appeared more intense than the one obtained with the control nanoemulsion (Figure 7-2). At 24 h after the administration of both fluorescent nanocarriers the signal was not detectable.

To corroborate the presence of PARG nanocapsules in the gastrointestinal tract, the animals were sacrificed and the fluorescence intensity of the intestine was analyzed following the necropsy. As showed in Figure 8, PARG nanocapsules were rapidly cleared in the esophagus and stomach, and were predominantly located in the small intestine for up to 3 hours.

Based on these observations, we could speculate that PARG nanocapsules have a certain affinity for the intestinal epithelia probably related to presence of multiple cationic guanidine functional groups of the polymer backbone. This interpretation would be in agreement with previous works showing that nanocarriers coated with polycationic polymers, e.g. chitosan, are more efficient for oral drug delivery that nanocarriers coated with neutral hydrophilic polymers [20, 33, 35]. In this respect, the results of this work would represent the first preliminary evidence of this special ability of PARG nanocapsules. However, more systematic studies would be necessary in order to understand the mechanistic issues underlying the interaction of PARG nanocapsules with the intestinal mucosa.

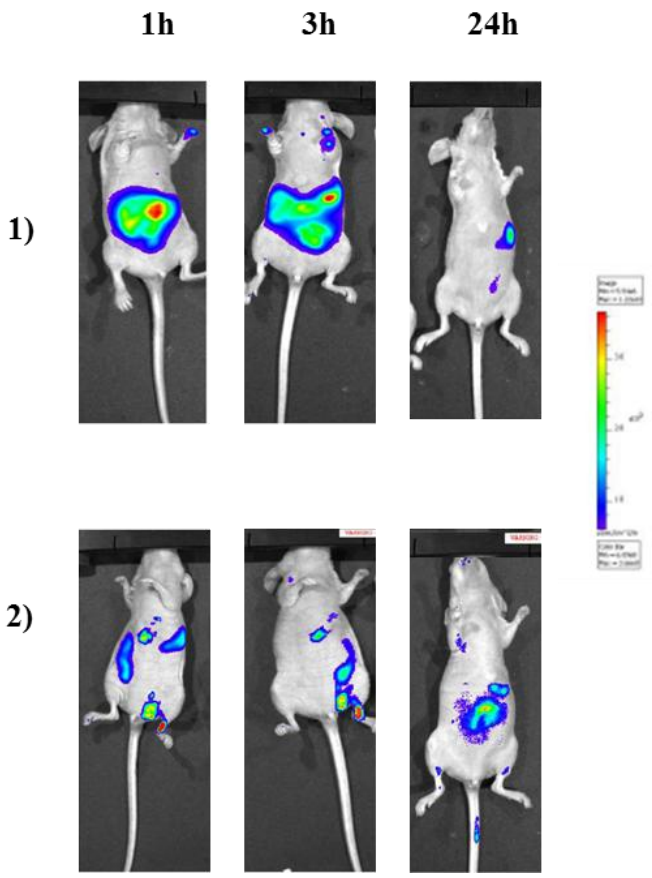
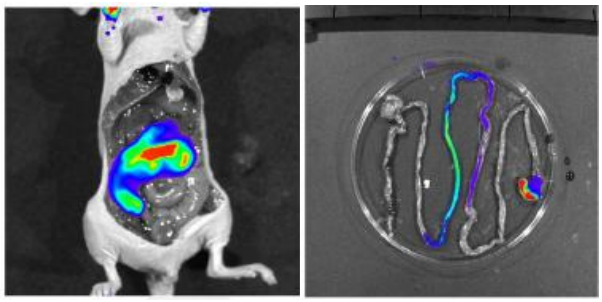


Figure 7: Fluorescence images of representative animals at 1hour, 3hours and 24 hours following oral administration of Did-loaded PARG nanocapsules (1) and nanoemulsion (2).

1)



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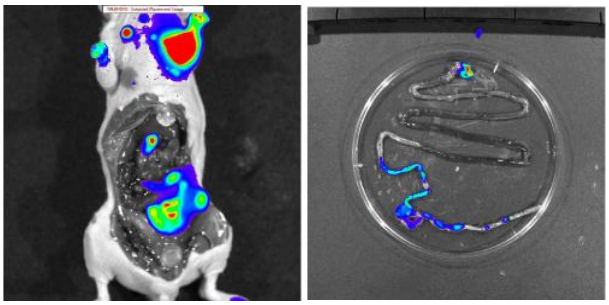

Figure 8: Fluorescence images of whole animal and the isolated intestinal tract after oral administration of DiD-loaded PARG nanocapsules. 1 hour post-administration (1) and 3 hours post-administration (2).

Conclusions

The results of this study that PARG nanocapsules exhibit interesting properties regarding their potential use as oral peptide delivery vehicles. Namely, (i) they are able to entrap large complex molecules; they are stable in simulated gastrointestinal fluids; (ii) they interact with the intestinal model epithelium (*in vitro*) increasing the transepithelial resistance and crossing the cellular barriers without signs of cytotoxicity; (iii) they are retained in the intestinal tract (*in vivo*) for at least three

hours. Additional studies are to be performed in order to fully understand the mechanistic issues behind these promising features.

Acknowledgements

Financial support from CENIT-NANOFAR XS53 project, PharmaMar, Spain, and the Ministry of Sciences and Innovation. Giovanna Lollo has a fellowship from the Ministry of Education of Spain. Marcos Garcia-Fuentes acknowledges an Isidro Parga Pondal Fellowship from Xunta de Galicia.

References

1. Morishita, M. and N.A. Peppas, Is the oral route possible for peptide and protein drug delivery. Drug Discovery Today, 2006. 11(19-20): p. 905-910.

2. Martins, S., et al., Lipid-based colloidal carriers for peptide and protein delivery - liposomes versus lipid nanoparticles. International Journal of Nanomedicine, 2007. 2(4): p. 595-607.

3. Kumar, T.R.S., K. Soppimath, and S.K. Nachaegari, Novel delivery technologies for protein and peptide therapeutics. Current Pharmaceutical Biotechnology, 2006. 7(4): p. 261-276.

4. Csaba, N., M. Garcia-Fuentes, and M.J. Alonso, The performance of nanocarriers for transmucosal drug delivery. Expert Opinion on Drug Delivery, 2006. 3(4): p. 463-478.

5. Plapied, L., et al., Fate of polymeric nanocarriers for oral drug delivery. Current Opinion in Colloid & Interface Science, 2011. 16(3): p. 228-237.

6. Garcia-Fuentes, M., D. Torres, and M.J. Alonso, Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules. Colloids and Surfaces B-Biointerfaces, 2003. 27(2-3): p. 159-168.

7. Makhlof, A., et al., A mucoadhesive nanoparticulate system for the simultaneous delivery of macromolecules and permeation enhancers to the intestinal mucosa. Journal of Controlled Release, 2011. 149(1): p. 81-88.

8. Pouton, C.W. and C.J.H. Porter, Formulation of lipid-based delivery systems for oral administration: Materials, methods and strategies. Advanced Drug Delivery Reviews, 2008. 60(6): p. 625-637.

9. Roger, E., et al., Biopharmaceutical parameters to consider in order to alter the fate of nanocarriers after oral delivery. Nanomedicine, 2010. 5(2): p. 287-306.

10. Garcia-Fuentes, M., Prego, C., Torres, D., Alonso, M.J., Polymer-coated lipid nanostructures as carriers for oral peptide delivery. Bulletin Technique Gattefossè, 2004. 97: p. 51-61.

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

12. Nair, L.S. and C.T. Laurencin, Biodegradable polymers as biomaterials. Progress in Polymer Science, 2007. 32(8–9): p. 762-798.

13. Di Colo, G., Y. Zambito, and C. Zaino, Polymeric enhancers of mucosal epithelia permeability: Synthesis, transepithelial penetration-enhancing properties, mechanism of action, safety issues. Journal of Pharmaceutical Sciences, 2008. 97(5): p. 1652-1680.

14. Oyarzun-Ampuero, F.A., et al., A new drug nanocarrier consisting of polyarginine and hyaluronic acid. European Journal of Pharmaceutics and Biopharmaceutics, 2011. 79(1): p. 54-57.

15. Brooks, N.A., et al., Cell-penetrating peptides: Application in vaccine delivery. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2010. 1805(1): p. 25-34.

16. González-Aramundiz, J.V., et al., Polypeptides and polyaminoacids in drug delivery. Expert Opinion on Drug Delivery, 2012. 9(2): p. 183-201.

17. Kamei, N., et al., Permeation characteristics of oligoarginine through intestinal epithelium and its usefulness for intestinal peptide drug delivery. Journal of Controlled Release, 2008. 131(2): p. 94-99.

18. Morishita, M., et al., A novel approach using functional peptides for efficient intestinal absorption of insulin. Journal of Controlled Release, 2007. 118(2): p. 177-184.

19. Mitchell, D.J., et al., Polyarginine enters cells more efficiently than other polycationic homopolymers. Journal of Peptide Research, 2000. 56(5): p. 318-325.

20. Ohtake, K., et al., Poly-l-Arginine Enhances Paracellular Permeability via Serine/Threonine Phosphorylation of ZO-1 and Tyrosine Dephosphorylation of Occludin in Rabbit Nasal Epithelium. Pharmaceutical Research, 2003. 20(11): p. 1838-1845.

21. Miyamoto, M., et al., Improved nasal absorption of drugs using poly-l-arginine: effects of concentration and molecular weight of poly-l-arginine on the nasal absorption of fluorescein isothiocyanate–dextran in rats. European Journal of Pharmaceutics and Biopharmaceutics, 2001. 52(1): p. 21-30.

22. Lozano M. V., et al., Polyarginine nanocapsules: a new platform for intracellular drug delivery. Submitted.

23. Calvo, P., J.L. Vila-Jato, and M.J. Alonso, Evaluation of cationic polymer-coated nanocapsules as ocular drug carriers. International Journal of Pharmaceutics, 1997. 153(1): p. 41-50.

24. Fessi, H., et al., Nanocapsule formation by interfacial polymer deposition following solvent displacement. International Journal of Pharmaceutics, 1989. 55(1).

25. 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.

26. Chen, C.C., et al., Effects of lipophilic emulsifiers on the oral administration of lovastatin from nanostructured lipid carriers: Physicochemical characterization and pharmacokinetics. European Journal of Pharmaceutics and Biopharmaceutics, 2010. 74(3): p. 474-482.

27. Sonaje, K., et al., Biodistribution, pharmacodynamics and pharmacokinetics of insulin analogues in a rat model: Oral delivery using pH-Responsive nanoparticles vs. subcutaneous injection. Biomaterials, 2010. 31(26): p. 6849-6858.

28. Porter, C.J.H., et al., Enhancing intestinal drug solubilisation using lipid-based delivery systems. Advanced Drug Delivery Reviews, 2008. 60(6): p. 673-691.

29. des Rieux, A., et al., Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. Journal of Controlled Release, 2006. 116(1): p. 1-27.

30. García-Fuentes, M., D. Torres, and M.J. Alonso, Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules. Colloids and Surfaces B: Biointerfaces, 2003. 27(2-3): p. 159-168.

31. Florence, A.T. and N. Hussain, Transcytosis of nanoparticle and dendrimer delivery systems: evolving vistas. Advanced Drug Delivery Reviews, 2001. 50, Supplement 1(0): p. S69-S89.

32. Prego, C., et al., Transmucosal macromolecular drug delivery. Journal of Controlled Release, 2005. 101(1-3): p. 151-162.

33. Takechi, Y., et al., Physicochemical Mechanism for the Enhanced Ability of Lipid Membrane Penetration of Polyarginine. Langmuir, 2011. 27(11): p. 7099-7107.

34. Sakuma, S., et al., Oligoarginine-linked polymers as a new class of penetration enhancers. Journal of Controlled Release, 2010. 148(2): p. 187-196.

35. Zaki, N.M., et al., Rapid-onset intranasal delivery of metoclopramide hydrochloride: Part I. Influence of formulation variables on drug absorption in anesthetized rats. International Journal of Pharmaceutics, 2006. 327(1–2): p. 89-96.

DISCUSIÓN GENERAL

Discusión General

En los últimos años se ha asistido al desarrollo de nuevos nanomedicamentos que han aportado importantes ventajas en el tratamiento de enfermedades oncológicas[68]. Muchos nanosistemas, como liposomas, micelas, y conjugados, se encuentran actualmente en estudios clínicos avanzados[69]. Otras nanoestructuras, como las nanocápsulas, son objeto de investigación debido a su enorme potencial[70]. Las ventajas de las nanocápsulas residen en su peculiar estructura: el núcleo oleoso permite incorporar una gran cantidad de fármaco y aportan un ambiente inerte para su almacenamiento. La cubierta polimérica, por otro lado, permite modular las propiedades biofarmacéuticas del nanosistemas según su naturaleza. Esta estructura modular es por lo tanto enormemente flexible, ya que permite diseñar núcleos y recubrimientos poliméricos adaptados a aplicaciones específicas. Las nanocápsulas constituidas por triglicéridos de cadena media y cubierta polimérica han sido empleadas con éxito para la encapsulación de moléculas de baja solubilidad en agua como los antitumorales[71].

En este contexto, y teniendo en cuenta los antecedentes e hipótesis recogidos en secciones anteriores, en el presente trabajo se ha estudiado el potencial de las nanocápsulas de dos poliaminoácidos como vehículos para la administración parenteral y oral de fármacos antitumorales. La discusión puede así dividirse en dos partes principales en atención al sistema estudiado y a su aplicación:

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68 **Hervella, P., V. Lozano, et al.** (2008). "Nanomedicine: New Challenges and Opportunities in Cancer Therapy." J. Biomed. Nanotechnol. **4**(3): 276-292.

69 **Alexis, F., E. M. Pridgen, et al.** (2010). Nanoparticle Technologies for Cancer Therapy Drug Delivery. M. Schäfer-Korting, Springer Berlin Heidelberg. **197:** 55-86.

70 **Huynh, N. T., C. Passirani, et al.** (2009). "Lipid nanocapsules: A new platform for nanomedicine." Int. J. Pharm. **379**(2): 201-209.

71 **Lozano, M. V., D. Torrecilla, et al.** (2008). "Highly Efficient System To Deliver Taxanes into Tumor Cells: Docetaxel-Loaded Chitosan Oligomer Colloidal Carriers." Biomacromolecules **9**(8): 2186-2193.

1. Desarrollo de nanocápsulas de PGA (acido poli-L-glutámico) y PGA-PEG (poli-L-glutámico-polietilenglicol) para la administración parenteral de fármacos antitumorales

2. Desarrollo de nanocápsulas de PARG (poliarginina) para la administración oral de fármacos antitumorales

Desarrollo de nanocápsulas de PGA y PGA-PEG para la administración parenteral de fármacos antitumorales

Con el objetivo de alcanzar mejoras del ratio eficacia/toxicidad de fármacos antitumorales administrados por vía intravenosa, se propuso el diseño de nanoestructuras capaces de: (i) solubilizar fármacos antitumorales en elevadas concentraciones, con buena eficacia de encapsulación y utilizando materiales biocompatibles; (ii) mejorar el tiempo de residencia plasmática de fármacos antitumorales, como prerrequisito para mejorar la vectorización pasiva del fármaco a tumores sólidos. Como nanovehículos capaces de adecuarse a estos requerimientos, se diseñaron las nanocápsulas recubiertas PGA y de PGA conjugado con polietilenglicol, PGA-PEG. El PGA es un polímero hidrófilo y biodegradable constituido por unidades de ácido L-glutámico con carga negativa[72]. Gracias a su excelente perfil de biocompatibilidad, el PGA es un importante biomaterial para el desarrollo de nuevas formulaciones para la liberación de fármacos. Además, varios estudios han mostrado que las nanoestructuras de PGA tienen es un tiempo de residencia en plasma bastante elevad [73]. Debido a estas características

[72] **Chun, L.** (2002). "Poly(l-glutamic acid)–anticancer drug conjugates." Advanced Drug Delivery Reviews **54**(5): 695-713.

[73] **González-Aramundiz, J. V., M. V. Lozano, et al.** (2012). "Polypeptides and polyaminoacids in drug delivery." Expert Opinion on Drug Delivery **9**(2): 183-201.

favorables, el PGA se encuentra presente en nanostructuras en avanzado estudio clínico[74,75].

Además, en el presente trabajo también hemos conjugado el PGA con PEG, con el objetivo de modificar la superficie del nanosistema, así aumentar todavía el tiempo de circulación plasmática de los nanovehículos desarrollados[76,77,78]. Dichas mejoras en la farmacocinética de las nanocápsulas se logran reduciendo la opsonización en la superficie de los nanosistemas, que llevan a una reducción en su captura por parte del sistema retículo endotelial[79]. Los copolímeros PEGilados empleados en el presente trabajo tienen dos diferentes estructuras y grado de PEGilación. Los porcentajes de PEG han sido de 24 % p/p para el derivado "grafted" (PGA-PEG) de baja PEGilación y de 57 % p/p para el derivado "dibloque" de alta PEGilación (PGA-PEG HP).

Preparación y caracterización físico-química de las nanocápsulas

Las nanocápsulas de PGA y PGA-PEG con distintas estructura y grado de PEGilación se prepararon mediante la técnica de desplazamiento de disolvente, un procedimiento adaptado previamente por nuestro grupo para otros polímeros catiónicos como el quitosano[80]. Para la formación de las nanocápsulas, al tratarse de polímeros aniónicos y necesitar una interacción electrostática para su deposición en la superficie, fue necesaria

74 **Jack W, S.** (2005). "Paclitaxel poliglumex (XYOTAX™, CT-2103): A macromolecular taxane." Journal of Controlled Release **109**(1-3): 120-126.

75 **Oerlemans, C., W. Bult, et al.** (2010). "Polymeric Micelles in Anticancer Therapy: Targeting, Imaging and Triggered Release." Pharmaceutical Research **27**(12): 2569-2589.

76 **Bae, Y. and K. Kataoka** (2009). "Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers." Advanced Drug Delivery Reviews **61**(10): 768-784.

77 **Bae, Y. and K. Kataoka** (2009). "Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers." Advanced Drug Delivery Reviews **61**(10): 768-784.

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

79 **Maeda, H., G. Y. Bharate, et al.** (2009). "Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect." European Journal of Pharmaceutics and Biopharmaceutics **71**(3): 409-419.

80 **Calvo, P., B. Gouritin, et al.** (2001). "Long-Circulating PEGylated Polycyanoacrylate Nanoparticles as New Drug Carrier for Brain Delivery." Pharmaceutical Research **18**(8): 1157-1166.

la incorporación de un tensioactivo catiónico. Se eligió el cloruro de benzalconio (BKC) por su conocido perfil regulatorio, aunque pudimos comprobar que dicho tensoactivo puede ser intercambiado por otros como el cloruro de cetilpiridinio[81]. Básicamente, el proceso de preparación de las nanocápsulas consiste en la formación espontanea de una suspensión de partículas debido a la mezcla de los disolventes orgánicos con un no-solvente en el que están disueltos el polímero y el poloxámero 188. De este modo se forman gotas de aceite de Mygliol, lecitina y de tensioactivo catiónico, contenidos en la fase orgánica, sobre las que se dispone el polímero mediante interacción iónica entre la carga positiva del BKC y la carga negativa del PGA. Como se había mencionado, se prepararon dos tipos de nanocápsulas de PGA-PEG, unas con un polímero tipo "grafted" y con baja PEGilación y otras tipo "dibloque" con elevada PEGilación.

Utilizando la misma técnica se obtuvieron dos tipos de nanoemulsiones empleadas como control en los experimentos. Las nanoemulsiones presentaban la misma composición que las nanocápsulas de PGA o de PGA-PEG, excepto por la ausencia de dicha cubierta polimérica. Las nanoemulsiones PEGiladas consistían en la misma composición que las nanoemulsiones, pero se añadía la cantidad exacta de estearato de PEG que permite incorporar tantas cadenas de dicho polímero como hay en las nanocápsulas de PGA-PEG.

Los nanosistemas preparados fueron caracterizados en términos de tamaño y potencial zeta (Tabla 1). Todos los sistemas obtenidos muestran un tamaño nanométrico cercano a los 200 nm. Este tamaño de partícula es en principio adecuado para permitir la potencial extravasación de los nanosistemas a través del endotelio venoso tumoral, pero no en los

[81] **Alonso, M. J., D. Torres, et al.** (2011). Nanocápsulas con cubierta polimérica. USC. Spain. Reference number: P603ES00

capilares sanos[82]. Por lo tanto, el tamaño de los nanosistemas podría permitir la vectorización pasiva de los fármacos a tumores sólidos.

En cuanto a la carga superficial, las nanocápsulas muestran una inversión de potencial con respecto a la nanoemulsión catiónica que indica la presencia del polímero alrededor del nanosistema. Los sistemas con PGA tienen un potencial más negativo, alrededor de -40 mV, respecto a aquellos recubiertos con copolímeros PEGilados. Concretamente las nanocápsulas de PGA-PEG mostraron un potencial zeta de -28 mV y las nanocápsulas de PGA-PEG HP de -20 mV. Esta reducción en términos absolutos del potencial zeta es debida al recubrimiento de PEG[83] y es una primera indicación de las diferentes propiedades químicas de los sistemas a nivel de superficie.

Tabla 1: Propiedades físico-químicas de las nanocápsulas de PGA y PGA-PEG blancas. (Media ± D.E.); NE: Nanoemulsión, NCs: Nanocápsulas, [a] Indice de Polidispersion.

Formulación	Tamaño (nm)	I.P.[a]	ζ (mV)
NE aniónica	207 ± 7	0.1	-38 ± 1
NE catiónica	227 ± 8	0.1	+40 ± 4
NCs PGA	202 ± 5	0.1	-49 ± 1
NCs PGA-PEG	191 ± 4	0.1	-28 ± 4
NCs PGA-PEG HP	180 ± 4	0.1	-20 ±4

[82] **Fang, J., H. Nakamura, et al.** (2011). "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 **63**(3): 136-151.

[83] **Garcia-Fuentes, M., D. Torres, et al.** (2004). "Application of NMR Spectroscopy to the Characterization of PEG-Stabilized Lipid Nanoparticles." Langmuir **20**(20): 8839-8845.

Preparación y caracterización físico-química de las nanocápsulas de PGA y PGA-PEG cargadas con la sonda fluorescente (DiD) y con los fármacos antitumorales plitidepsina y docetaxel

Para la realización de estudios *in vivo* las nanocápsulas de PGA y PGA-PEG HP fueron cargadas con la sonda fluorescente DiD. Las características físico-químicas de los sistemas se muestran en la Tabla 2. Se puede observar que la incorporación del DiD no modifica ni el tamaño ni el potencial zeta de las nanocápsulas con respecto a las formulaciones blancas. Además, debido a sus características anfifílicas, el DiD pudo ser encapsulado en el nanosistema con eficacias cercanas al 70% o superiores.

Tabla 2: Propiedades físico-químicas de las nanocápsulas de PGA, PGA-PEG HP y nanoemulsión cargadas con la sonda fluorescente DiD. (Media ± D.E.); NE: Nanoemulsión, NCs: Nanocápsulas, [a] Índice de polidispersión.

Formulación	Tamaño (nm)	I.P. [a]	ζ (mV)	Eff. Encap. (%)
NE Aniónica cargadas con DiD	214 ± 5	0.1	-28 ± 6	79 ± 10
NCs PGA cargadas con DiD	179 ± 3	0.1	-31 ± 2	67 ± 5
NCs PGA-PEG HP cargadas con DiD	194 ± 2	0.1	-15 ± 3	70 ± 8

Para la realización de estudios de farmacocinética, toxicidad y eficiacia *in vivo* las nanocápsulas de PGA, PGA-PEG y PGA-PEG HP se cargaron con un fármaco hidrófobo antitumoral, la plitidepsina (APL). Adicionalmente, las nanocápsulas PGA-PEG HP fueron cargadas con otro fármaco antitumoral, el docetaxel (DCX). En las propiedades físico-químicas recogidas en la Tabla 3, se puede observar que los sistemas cargados con ambos fármacos no presentan diferencias con los nanovehículos blancos. Para este experimento se añadieron otras

formulaciones control: la nanoemulsión y la nanoemulsión PEGilada. Como se puede observar, todos los sistemas mostraron eficacias de encapsulación siempre superiores al 85%, tanto para la plitidepsina como para el docetaxel. Considerando la elevada hidrofobia de ambos fármacos y las eficacias de encapsulación observadas se puede deducir que el núcleo oleoso que caracteriza a las nanocápsulas y a las nanoemulsiones es muy adecuado para incorporar este tipo de moléculas.

Tabla 3: Propiedades físico químicas de las nanocápsulas de PGA, PGA-PEG y PGA-PEG HP cargadas con plitidepsina (PLT), Docetaxel (DCX). La nanoemulsión aniónica y la nanoemulsión PEGilada fueron utilizadas como control (Media ± D.E.); NE: Nanoemulsión, NCs: Nanocápsulas, [a] índice de polidispersión, F: Farmaco.

Formulación	F.	Tamaño (nm)	I.P. [a]	ζ (mV)	E. E. (%)
NE catiónica	APL	207 ± 7	0.1	+38 ± 1	95 ±3
NE PEG	APL	201 ± 5	0.1	+28 ± 3	98 ± 2
NCs PGA	APL	183 ± 6	0.1	-38 ± 1	98 ± 2
NCs PGA-PEG	APL	201 ± 5	0.1	-28 ± 3	98 ± 2
NCs PGA-PEG HP	APL	190 ± 15	0.1	-24 ± 5	85 ± 4
NCs PGA-PEG HP	DCX	200 ± 3	0.1	-20 ± 4	90 ± 2

Estudios de estabilidad y liofilización de las nanocápsulas de PGA y PGA-PEG HP

Estudios de estabilidad en almacenamiento demostraron que las nanocápsulas de PGA y PGA-PEG HP pueden ser almacenadas por dos meses o más en diversas condiciones (más detalles e los capítulos 2 y 3). Con el objetivo de mejorar aún más las posibilidades de almacenamiento y de conseguir preparados fáciles de manejar, se desarrollaron formulaciones de nanocápsulas de PGA y PGA-PEG HP en forma de polvo mediante liofilización.

En el caso de las nanocápsulas de PGA, se procedió a la liofilización de distintas concentraciones de nanocápsulas blancas y cargadas con el fármaco plitidepsina (0.5, 0.75, 1 % p/v) en presencia del crioprotector trehalosa (5% y 10 % p/v). Los resultados obtenidos confirmaron que se puede liofilizar nanocápsulas de PGA hasta el 1 % p/v, con 10% de trealosa como crioprotector, sin obtener cambios en las características físico-químicas de los sistemas reconstituidos respecto a los valores iniciales. Por el contrario, las nanocápsulas de PGA no pudieron ser reconstituidas de manera adecuada para los sistemas con 5% de trealosa.

Para las nanocapsulas de PGA-PEG HP se procedió a la liofilización de distintas concentraciones de nanocápsulas blancas (0.25, 0.5, 0.75, 1 % p/v) en presencia de dos concentraciones del crioprotector trehalosa, 5 y 10 % p/v (Figura 1). Las nanocápsulas de PGA-PEG HP pudieron ser reconstituidas sin alteraciones en sus características físico-químicas en todo el rango de concentraciones de sistema y crioprotector estudiados. Sin embargo, si se observó la necesidad de utilizar un crioprotector para poder reconstituir de forma adecuada la suspensión de nanocápsulas. Como había sido observado anteriormente, la combinación de trehalosa y el PEG como polímero de recubrimiento proporcionan un aumento de la estabilidad de las nanocápsulas una vez liofilizadas y permiten su reconstitución sin la alteración de las características iniciales[84].

[84] **Layre, A.-M., P. Couvreur, et al.** (2006). "Freeze-Drying of Composite Core-Shell Nanoparticles." Drug Development and Industrial Pharmacy **32**(7): 839-846.

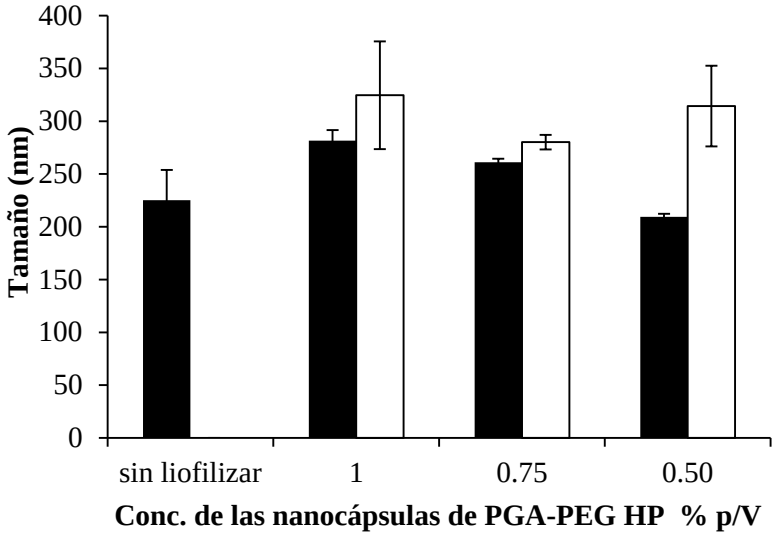

Figura 1: Tamaño de partícula tras la reconstitución de los liofilizados de las nanocápsulas PGA-PEG HP en presencia de trehalosa 10% p/v (■) y de las nanocápsulas PGA-PEG HP en presencia de trehalosa 5 % p/v (□).

Estudios de hemólisis

La evaluación de la capacidad hemolítica es importante para determinar el daño a la membrana de los glóbulos rojos producido por la administración IV de los nanosistemas[85]. En este estudio, se determinó la capacidad hemolítica *in vitro* de los nanosistemas mediante su incubación en presencia de glóbulos rojos, y la posterior medición de la hemoglobina liberada. Se ensayaron las nanocápsulas de PGA, las nanocápsulas de PGA-PEG HP y las nanoemulsiones. Los resultados han evidenciado que ninguno de los sistemas de nanocápsulas es hemolítico. Sin embargo, los sistemas no recubiertos como la nanoemulsión resultaron en un importante efecto hemolítico del 60% a las mismas concentraciones ensayadas con las nanocápsulas (Capítulo 3). Estos resultados sugieren la

[85] **Lu, J., S. C. Owen, et al.** (2011). "Stability of Self-Assembled Polymeric Micelles in Serum." Macromolecules **44**(15): 6002-6008.

elevada hemocompatibilidad de las nanocápsulas de PGA y PGA-PEG HP desarrolladas.

Estudios de activación del complemento

Tras ser administración por vía IV los nanosistemas pueden ser reconocidos y eliminados rápidamente por parte de las células monucleares fagocíticas del sistema retículo endotelial[86]. Este proceso implica un primer reconocimiento de las partículas mediante la adsorción de opsoninas -proteínas del complemento- a su superficie. La opsonización favorece el reconocimiento de los nanostistemas por las células del sistema inmune y su aclaramiento del torrente sanguíneo. Los ensayos *in vitro* de activación del complemento pueden ser utilizado para predecir las interacciones de las nanocápsulas con el sistema inmune. Esta determinación se realiza midiendo la capacidad residual de activación del complemento tras su incubación con dosis crecientes de nanosistemas. La capacidad residual de activación permite calcular el consumo del sistema de complemento producido por la incubación con el nanosistema, relacionándose directamente dicho consumo con el proceso de opsonización *in vivo*.

Como se muestra en la Figura 2, las nanocápsulas de PGA y de PGA-PEG HP producen una débil activación del complemento alcanzando apenas un valor del 30% del consumo de unidades de CH50 (concentración de suero que es capaz de producir la lisis del 50% de un preparado estándar de eritrocitos sensibilizados con anticuerpos antieritrocito). Por otro lado, la nanoemulsión da lugar a una fuerte y rápida activación. La interacción de los nanosistemas con las proteínas del complemento varía en función del tamaño, carga superficial del sistema y

[86] **Meerasa, A., J. G. Huang, et al.** (2011). "CH50: A Revisited Hemolytic Complement Consumption Assay for Evaluation of Nanoparticles and Blood Plasma Protein Interaction." Current Drug Delivery **8**(3): 290-298.

naturaleza de la cubierta polimérica. En este sentido, se sabe que partículas con tamaño elevado, potencial zeta positivo y superficies hidrófobas promueven una rápida activación del complemento[87,88]. Todas las partículas ensayadas tienen un tamaño similar, cercano a 200 nm, pero difieren en su potencial zeta, siendo muy positivo para la nanoemulsión y negativo para las nanocápsulas de PGA y PGA-PEG HP. El carácter polianiónico junto con la elevada hidrofilia de la cubierta polimérica parece reducir la interacción de las nanocapsulas de PGA o PGA-PEG HP con el complemento[89]. Curiosamente, no se observaron diferencias en la capacidad de activación del complemento entre las nanocápsulas de PGA y las nanocápsulas de PGA-PEG HP. Dado que el PEG es el polímero más estudiado respecto a su capacidad para repeler opsoninas, estos resultados parecen indicar el interés del PGA como biomaterial de recubrimiento en el diseño de nanosistemas con residencia plasmática elevada.

87 **Vonarbourg, A., C. Passirani, et al.** (2006). "Evaluation of pegylated lipid nanocapsules versus complement system activation and macrophage uptake." Journal of Biomedical Materials Research Part A **78A**(3): 620-628.

88 **Moghimi, S. M., A. J. Andersen, et al.** (2011). "Material properties in complement activation." Advanced Drug Delivery Reviews **63**(12): 1000-1007.

89 **Morille, M., T. Montier, et al.** (2010). "Long-circulating DNA lipid nanocapsules as new vector for passive tumor targeting." Biomaterials **31**(2): 321-329.

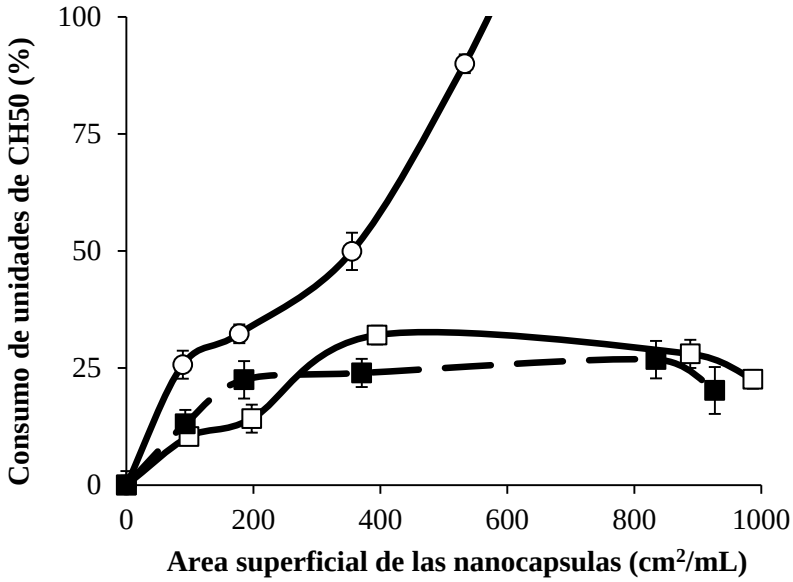

Figura 2: Consumo de las unidades del sistema de complemento (CH50) frente a la dosis de nanopartículas (expresado en superficie/cm^2). Los sistemas ensayados fueron: PGA (□), PGA-PEG HP (■) y las nanoemulsiones (○). (Media ± D. E.)

Estudios *in vivo*

Estudios de cinética plasmática de las nanocápsulas de PGA y PGA-PEG HP cargadas con DiD

Una vez preparados los sistemas de PGA y PGA-PEG HP cargados con la sonda fluorescente DiD, se procedió a la evaluación de la cinética plasmática *in vivo*. Para ello, las nanocápsulas fueron inyectadas por vía IV en ratones Swiss de 9-12 semanas (20-22 g). Como formulación control se utilizó una nanoemulsión aniónica cargada con el mismo marcador. A intervalos de tiempo determinados, 30 min, 1 h, 3 h y 24 h, se tomaron muestras de sangre mediante punción cardíaca.

Como se puede apreciar en la Figura 3, las nanocápsulas de PGA y PGA-PEG HP permanecen por periodos mucho más prolongados en la circulación plasmática. Así, por ejemplo, aproximadamente un 40% de la

dosis de fluorescencia en las nanocápsulas podía ser detectado en plasma al cabo de 3 horas de la administración. Por el contrario, menos de un 10% de la fluorescencia inicial podía ser detectada a ese mismo tiempo en el caso de la nanoemulsión aniónica.

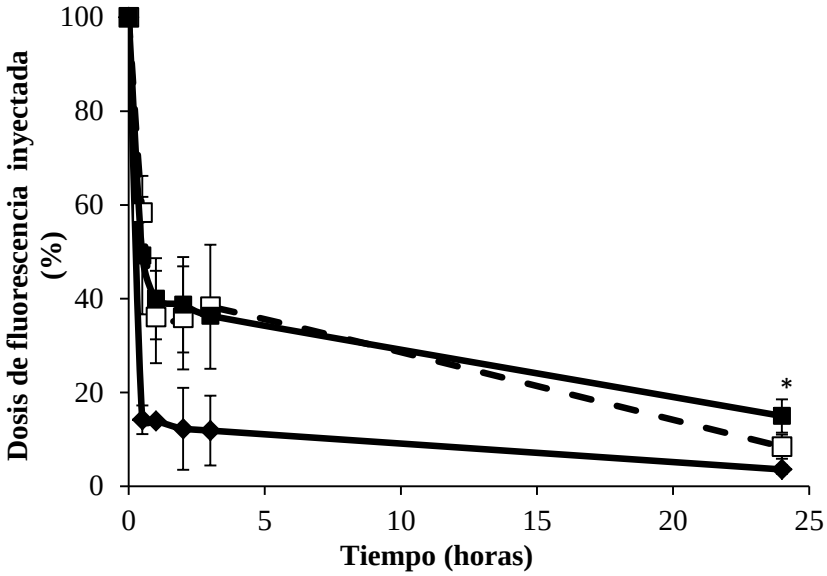

Figura 3: Niveles de fluorescencia en plasma a diferentes tiempos tras la administración por vía IV en ratones de las nanocapsualas de PGA (□) o PGA-PEG HP (■). El porcentaje de la dosis inyectada (concentración de DiD en mg/kg del peso total del animal a cada tiempo en relación a la concentración a tiempo cero) está expresado en función del tiempo. La nanoemulsión aniónica (♦) fue utilizada como control. Cada punto representa la media del porcentaje de la dosis inyectada ± D.E., n=3. *P < 0.05.

Tabla 4: Parámetros de la cinética plasmática de las nanocápsulas de PGA, PGA-PEG HP y de la nanoemulsión aniónica utilizada como control tras administración IV en ratones. NE: Nanoemulsión, NCs: Nanocápsulas.

Formulación	$t_{1/2\,\beta}$ h	MRT h	AUC mg/ml*h
NE aniónica	8.17	6.59	11.43
NCs PGA	10.02	9.72	38.02
NCs PGA-PEG HP	16.08	17	50.65

$t_{1/2\,\beta}$: 1-24h; MRT: 0-24H; AUC: 0-24h.

La cinética de eliminación en sangre de las nanocápsulas y sus parámetros han sido evaluados y determinados a través de un modelo no compartimental utilizando el paquete estadístico Kinetica 5.1 (Thermo Fischer Scientific, Francia, Tabla 4). Los datos presentados en la Tabla 4 evidencian las diferencias existentes en la cinética de eliminación en sangre de las distintas formulaciones. Se observa una mayor permanencia en plasma de los sistemas de nanocápsulas en comparación con el control. Destacan los valores de la vida media, MRT y AUC de las nanocápsulas de PGA-PEG HP que son superiores a las nanocápsulas de PGA. Los parámetros farmacocinéticos confirman el interés del recubrimiento con PGA y PEG para el diseño de nanosistemas de largo tiempo de circulación plasmática.

Como se ha descrito ampliamente en la literatura, la característica principal para conseguir un sistema de larga permanencia plasmática es una elevada hidrofilia de la superficie. Las nanocápsulas de PGA-PEG HP presentan al polímero en la superficie del sistema formando una cubierta tipo nube. Dicha cubierta le confiere propiedades furtivas capaces de prevenir la rápida eliminación de este sistema de la circulación

plasmática, favoreciendo la repulsión estérica de las opsoninas[90]. Siguiendo consideraciones similares a las realizadas en la literatura para otros sistemas recubiertos con PEG[91] hemos realizado algunos cálculos teóricos para estimar la separación entre las cadenas de PEG en la superficie. Estos cálculos resultaron en una distancia de 3 nm entre cadenas de PEG adyacentes, una distancia adecuada para conferir al polímero una estructura tipo cepillo, que es el régimen conformacional más eficiente para repeler la adsorción de proteínas[92,93].

Evaluación farmacocinética de las nanocápsulas de PGA y de PGA-PEG cargadas con plitidepsina

En una siguiente etapa se estudió el comportamiento de los sistemas utilizando análisis farmacocinéticos de formulaciones con el fármaco plitidepsina. Se estudió el perfil farmacocinético de la plitidepsina encapsulada en nanocápsulas de PGA, PGA-PEG y PGA-PEG HP, tras su administración IV en la vena de la cola de ratones CD-1. Los análisis farmacocinéticos fueron llevados a cabo en dos ensayos separados gestionados por PharmaMar S.A.

En el primer ensayo se determinaron las curvas de eliminación plasmática de la plitidepsina encapsulada en las nanocápsulas de PGA y PGA-PEG comparándolas con las de la plitidepsina en la nanoemulsión y la nanoemulsión recubierta con PEG (Figura 4). La dosis administrada de

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90 **Vonarbourg, A., C. Passirani, et al.** (2006). "Parameters influencing the stealthiness of colloidal drug delivery systems." Ibid. **27**(24): 4356-4373.

91 **Garcia-Fuentes, M., D. Torres, et al.** (2004). "Application of NMR Spectroscopy to the Characterization of PEG-Stabilized Lipid Nanoparticles." Langmuir **20**(20): 8839-8845.

92 **Mosqueira, V. C. F., P. Legrand, et al.** (2001). "Biodistribution of Long-Circulating PEG-Grafted Nanocapsules in Mice: Effects of PEG Chain Length and Density." Pharmaceutical Research **18**(10): 1411-1419.

25 **Wang, M. and M. Thanou** (2010). "Targeting nanoparticles to cancer." Pharmacological Research 62(2): 90-99.

plitidepsina en los nanosistemas fue de 0.4 mg/kg de peso del ratón, mientras que la de la nanoemulsión y de la nanoemulsión PEGilada fue de 0.1 mg/kg de peso de ratón. Como se muestra en la Figura 4 la cubierta polimérica otorga un incremento en el tiempo de permanencia en plasma de la plitidepsina encapsulada, como consecuencia del aumento en la residencia de los nanosistemas. Además, en la gráfica se aprecia que cuando la plitidepsina está encapsulada en las nanocápsulas de PGA-PEG se consiguen niveles plasmáticos de fármaco más altos con respecto a la formulación sin PEGilar.

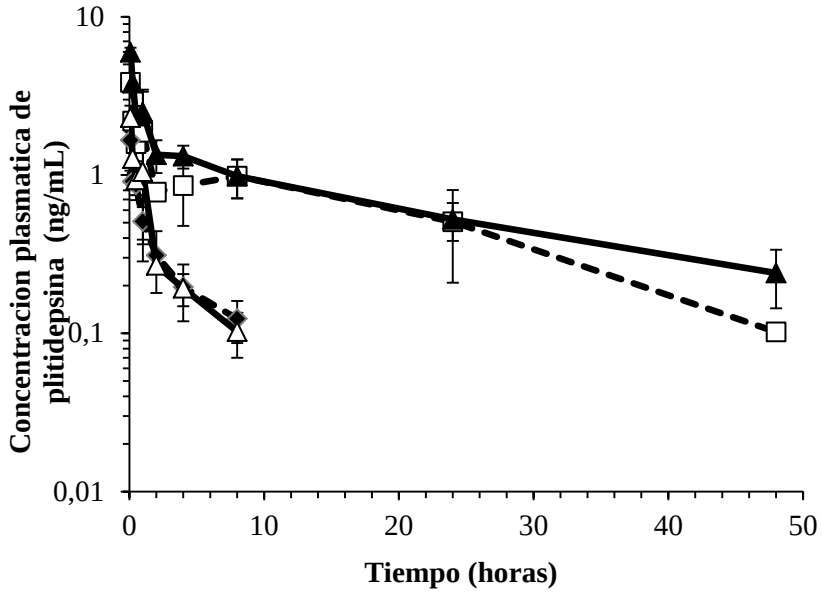

Figura 4: Evolución de la concentración de plitidepsina a lo largo del tiempo tras la administración IV en ratones de nanocápsulas de PGA (□) y PGA-PEG (▲), nanoemulsión (♦) y nanoemulsión PEG (Δ).Cada punto representa la media ± D.E n =.3

Los parámetros farmacocinéticos más representativos frente a la dosis tras una única administración de plitidepsina en los distintos nanosistemas están recogidos en la Tabla 5. Se aprecia una diferencia importante entre los parámetros farmacocinéticos de la plitidepsina encapsulada en las nanocápsulas y en las nanoemulsiónes, siendo

consistentes los parámetros con una residencia plasmática mucho más elevada para las primeras formulaciones Además, se observó un efecto del recubrimiento con PGA-PEG, con aumentos en los tiempos de vida media, AUC, MRT. Asimismo las nanocápsulas presentaron mayores volúmenes de distribución lo que parece indicar una mayor distribución a tejidos periféricos en comparación con las nanoemulsiones.

Tabla 5: Parámetros farmacocinéticos de las nanocápsulas de PGA, PGA-PEG, nanoemulsión y nanoemulsión PEG tras la administración IV en ratones Swiss.

Formulación	$t_{1/2}$ (h)	$AUC_{0\rightarrow t}$ (ng*h/ml)	CLp (ml/min/kg)	Vdss (L/kg)	VdB (L/kg)	MRT (h)
NCs PGA	13.3	69.3	224.8	230.4	258.7	17.08
NCs PGA-PEG	18.4	84.2	166.5	240.8	265.8	24.11
NE cationica	1.6	25	507.5	159.2	197.9	5.23
NE PEG	2.3	29.9	462.8	110.1	168.5	3.97

En el segundo ensayo se valoró el tiempo de permanencia plasmática del fármaco encapsulado en las nanocápsulas de PGA-PEG HP y disuelto en una formulación de referencia (Cremophor® EL/Etanol/Agua, 15/15/70 p/p/p) habitualmente empleada para administrar fármacos anticancerosos hidrófobos. En la Figura 5 se observa como la plitidepsina en las nanocápsulas PGA-PEG HP permanece más tiempo en circulación que cuando se encuentra disuelta en la formulación de referencia, modificando considerablemente el perfil farmacocinético de la misma. De hecho los niveles de plitidepsina en la formulación de referencia cayeron por debajo del límite de detección del método analítico (HPLC-MS) después de 8 h.

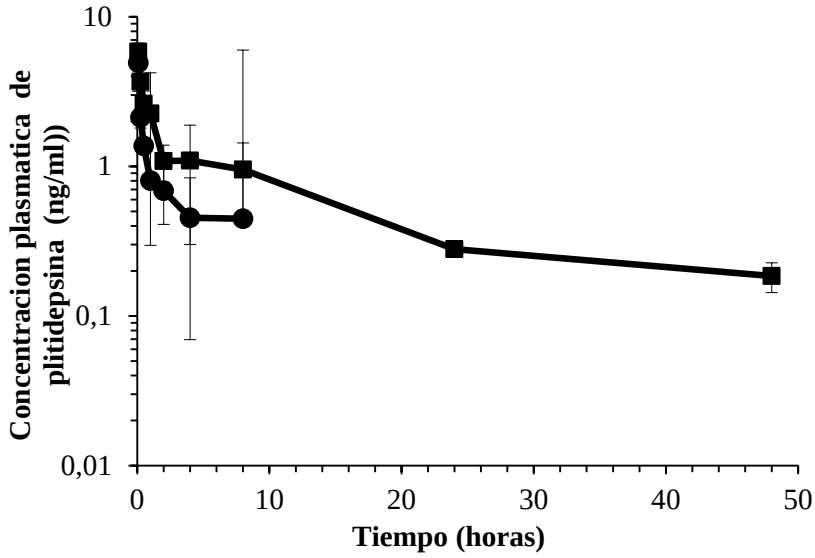

Figura 5: Evolución de la concentración de plitidepsina a lo largo del tiempo tras la administración IV en ratones de las nanocápsulas de PGA-PEG HP (■) o la formulación de referenci, Cremophor® EL (●). Cada punto representa la media ± D.E. n= 3

Comparando las nanocápsulas de PGA-PEG HP cargadas con plitidepsina con la formulación de referencia se puede destacar que las nanocápsulas poseen un tiempo de vida media y un tiempo de residencia medio (MRT) hasta dos veces superior. Además, el AUC de las nanocápsulas fue hasta 5 veces superior al de la formulación de referencia. Los valores de aclaramiento y el volumen de distribución se redujeron también de forma notable en las nanocápsulas respecto a la formulación de referencia.

Tabla 6: Parámetros farmacocinéticos de las nanocápsulas de PGA-PEG HP cargadas con apldina tras administración IV en ratones. Los datos han sido comparados con la plitidepsina en la formulación de referencia (Cremophor® EL/ etanol/ agua).

Formulación	$t_{1/2}$ (h)	$AUC_{0\rightarrow t}$ (ng*h/ml)	CLp (ml/min/kg)	Vdss (L/kg)	Vd_B (L/kg)	MRT (h)
Cremophor® EL	8.19	57.9	157.08	98.10	106.94	10.9
NCs PGA-PEG HP	17.02	274.7	52.71	65.91	77.70	22.1

Aunque no son estrictamente comparables por haberse realizado en ensayos distintos, estos resultados también parecen poner de manifiesto el mayor tiempo de residencia plasmática obtenido con las nanocápsulas recubiertas de PGA-PEG HP en comparación con las nanocápsulas de PGA-PEG. Creemos que estas divergencias no son debidas tanto a la estructura del copolímero -"grafted" o dibloque- si no a la diferente cantidad de PEG en estos sistemas. De hecho, el PEG representa sólo un 24% p/p en el copolímero "grafted", mientras que es de un 57% p/p en el copolímero dibloque. Como se ha comentado anteriormente, se ha calculado que la distancia entre cadenas de PEG en el sistema "dibloque" es inferior a 3 nm, mientras que en el sistema "grafted" es de 5 nm. Así, es posible que tan sólo el PEG en las nanocápsulas de PGA-PEG HP esté en una conformación óptima para ejercer su función de repulsión de las opsoninas.

En términos generales, estos resultados son acordes con una amplia literatura que ha empleado la PEGilación como modificación para aumentar el tiempo de circulación plasmática de los nanosistemas. Así, por ejemplo, Yang y col.[94] lograron triplicar la vida media plasmática del paclitaxel, de 5.05 h a 17.8 h, al modificar la superficie de liposomas con

[94] **Yang, T., F.-D. Cui, et al.** (2007). "Enhanced solubility and stability of PEGylated liposomal paclitaxel: In vitro and *in vivo* evaluation." Int. J. Pharm. **338**(1–2): 317-326.

PEG. En el estudio de Huynh y colaboradores[95] se pudo observar que el incremento del tamaño de las cadenas de PEG en la superficie de nanocápsulas lipídicas da lugar a un aumento en el tiempo de residencia plasmática de las mismas, y que esto incide positivamente en la posibilidad de alcanzar el tejido tumoral vía efecto EPR. Khalid y colaboradores[96] también comprobaron que la PEGilación de nanocápsulas mejora la farmacocinética de los fármacos encapsulados, y por esto provoca un aumento en la acumulación del fármaco en el tumor a través de efectos de vectorización pasiva.

En definitiva, la modificación de las nanocápsulas mediante la utilización de un copolímero con alta PEGilación (el PGA-PEG HP) ha permitido obtener sistemas estabilizados estéricamente que no son reconocidos por las opsoninas del sistema del complemento, y que por ello pueden permanecer circulación sanguínea por más tiempo.

Toxicidad de las nanocápsulas de PGA y de PGA-PEG cargadas con plitidepsina

La evaluación de la toxicidad de los sistemas de PGA-PEG con alto y bajo grado de PEGilación cargados con plitidepsina se llevó a cabo mediante los ensayos de dosis máxima tolerada (*Maximum Tolerated Dose* (MTD)) tras una administración IV en ratones sanos CD-1. Como valor comparativo, también se evaluó la toxicidad de la plitidepsina disuelta en la formulación de referencia.

El valor de MTD de las nanocápsulas de PGA fue superior al valor máximo de dosis administrada (1 mg/kg); las nanocapsulas de PGA-PEG tuvieron un MTD de 0.9 mg/kg; finalmente, las nanocápsulas de

95 **Huynh, N., M. Morille, et al.** (2011). "Treatment of 9L Gliosarcoma in Rats by Ferrociphenol-Loaded Lipid Nanocapsules Based on a Passive Targeting Strategy via the EPR Effect." Pharmaceutical Research: 1-10.

96 **Khalid, M., P. Simard, et al.** (2006). "Long Circulating Poly(Ethylene Glycol)-Decorated Lipid Nanocapsules Deliver Docetaxel to Solid Tumors." Ibid. **23**(4): 752-758.

PGA-PEG HP mostraron un MTD de 0.75 mg/kg. Ambas formulaciones muestran una toxicidad como mínimo 2.5 veces inferior a la plitidepsina en la formulación de referencia: 0.3 mg/kg. Además, estos estudios mostraron que la formulación de nanocápsulas de PGA sin plitidepsina no daba lugar a ningún indicio de toxicidad a ninguna de las dosis ensayadas. Estos resultados sugieren que las nanocápsulas de PGA o PGA-PEG presentan un perfil de toxicidad mucho más adecuado que la formulación de referencia. Así, basándose en estos datos, parece posible administrar plitidepsina en nanocápsulas bien a dosis similares pero con menos efectos secundarios o a dosis más altas de las que se pueden emplear en la formulación convencional. Es importante señalar, que otro estudio mostró que tampoco se registraban efectos tóxicos visibles tras la administración repetida de plitidepsina (0.15 mg/kg, durante 20 días) en nanocápsulas de PGA-PG HP.

La reducción de toxicidad observada con ambas formulaciones debe de ser atribuida fundamentalmente al empleo de biomateriales con un perfil de compatibilidad muy superior a los de la formulación de referencia. Este resultado es relevante, ya que dicha formulación es similar a la utilizada a nivel comercial para otros antitumorales como el paclitaxel (ej. Taxotere®), siendo los efectos tóxicos del vehículo de dichas formulaciones una fuente importante de complicaciones en el tratamiento[97]

Estudios del efecto antitumoral de las nanocápsulas de PGA-PEG HP cargadas con plitidepsina en el modelo tumoral xenograft MRI-H-121

La evaluación de la eficacia antitumoral del sistema desarrollado se realizó en el modelo de cáncer renal xenograft MRI-H-121. Para ello, se administró la formulación de nanocápsulas de PGA-PEG HP por vía IV

[97] **Gelderblom, H., J. Verweij, et al.** (2001). "Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation." European Journal of Cancer **37**(13): 1590-1598.

a ratones en una pauta de 0.15 mg/kg de plitidepsina durante 20 días. Como controles se administró serum salino (control negativo) o plitidepsina en la formulación de referencia (Cremophor EL®/etanol/agua 15/15/75 p/p/p) a 0.3 mg/kg durante 5 días, dejando intervalos de 4 días libres de administración, ciclo que se repitió por dos veces. En ambos grupos con plitidepsina, la dosis total de plitidepsina para ambas formulaciones fue la misma. Para la optimización de la pauta de dosificación se tuvieron en cuenta estudios de MTD y MTD en dosis múltiples previamente analizados por PharmaMar S.A.

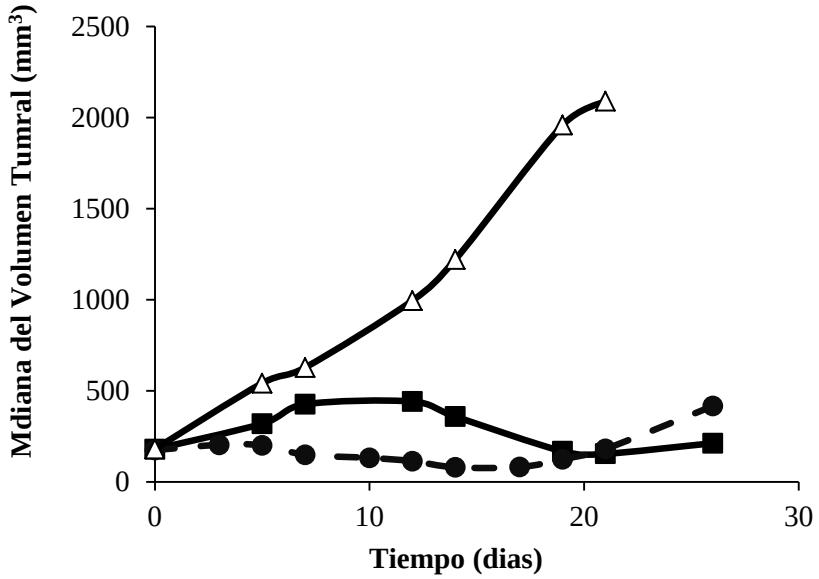

Figura 6: Evolución del tamaño del tumor a lo largo del tiempo tras la administración de plitidepsina formulada en las nanocápsulas de PGA-PEG HP (■) y en la formulación de referencia (●).Los experimentos se realizaron en modelos tumorales Xenograft MRI-H-121 implantados en ratones. El serum salino (Δ) se ha utilizado como control. Los datos representan la mediana del volumen tumoral frente al tiempo.

Los resultados presentados en la Figura 6 indican que ambas formulaciones de plitidepsina, tanto las nanocápsulas de PGA-PEG HP como la formulación de referencia, reducen eficazmente el crecimiento

del tumor en comparación al grupo control. En particular en el día 14 y 21 se observa una reducción significativa del volumen tumoral (Tabla 7). Asimismo en la Tabla 7 se indican el valor numérico y rango intercuartil de los volúmenes tumorales de cada grupo, y los análisis estadísticos de los resultados. Dicho análisis permite comprobar la clara eficacia de ambas formulaciones de plitidepsina respecto al control.

La formulación de referencia parece tener un efecto más marcado durante las primeras semanas de tratamiento, reduciendo la velocidad de desarrollo tumoral desde el día 7 de manera significativa ($p<0.0001$) con respecto al control. Sin embargo este efecto se hace menos importante en los días finales del tratamiento. Las nanocápsulas en la primera semana no causan reducción significativa del volumen tumoral, mientras que a partir del día 14 comienzan a producir efectos antitumorales evidentes. Ambas formulaciones ocasionan estasis tumoral en a día 25 del estudio (Tabla 8). La diferencia en términos del día de inicio de la acción puede estar relacionada con las diferentes pautas de dosificación de ambas formulaciones.

Tabla 7: Volumen tumoral (V.T.) obtenido en los ratones (modelo de cáncer renal xenograft MRI-H-121.) tras la administración de plitidepsina encapsulada en nanocápsulas de PGA-PEG HP y la apli plitidepsina dina la formulación de referencia.

Formulación	Día	V.T. mm^3 (IQR)	[a]P	[b]P	Mortalidad
Formulación Referencia	7	149 (133.9-268.6)	0.0001		0/10
	14	80.1 (52.7-127.7)	< 0.0001		0/10
	21	181.3 (144.0-264.9)	< 0.0001		0/10
NCs PGA- PEG HP	7	426.1 (376-5561.6)	NS		0/10
	14	358.8 (275.8-486.0)	< 0.0001		0/10
	21	154.5 (80.5-236.8)	< 0.0001	NS	0/10

Los datos están representados rangos entre cuartiles

[a]P valor de Mann –Whitney U test (grupo control frente a las otras formulaciones)

[b]P valor de Mann –Whitney U test (La formulación de referencia frente a las nanocápsulas),NS no es estadísticamente significativo.

Tabla 8: Efecto antitumoral y grado de actividad de la plitidepsina cargada en las nanocápsulas de PGA-PEG HP y en la formulación de referencia tras su administración en ratones.

Formulación	Dosis (mg/kg)	ΔT/ΔC %			Grado de actividad		
		Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Formulacion Referencia	0.30	-5.8	-9.1	0.3	++	+++	++
NCs PGA-PEG HP	0.15	55.0	17.2	-1.3	-	+	++

$\Delta T/\Delta C\ \% = (T_D\text{-}T_0/C_0\text{-}C_D)*100$

Grado de actividad:

$\Delta T/\Delta C > 50\%$ inactivo (-) | $\Delta T/\Delta C > 25\text{-}50\%$ inhibición tumoral (+) | $\Delta T/\Delta C < 25\%$ $T_X\text{-}T_0 > 75\text{-}125\%$ estasis tumoral (++)

$T_X\text{-}T_0 > 10\text{-}75\%$ regresión partial (+++) | TX-T0 Volumen tumoral del grupo tratado en el día X y en el día 0

Estudios del efecto antitumoral de las nanocápsulas de PGA-PEG HP cargadas con docetaxel en el modelo tumoral U87MG

La eficacia antitumoral de las nanocápsulas de PGA-PEG HP cargadas con docetaxel (DCX) se evaluó en un modelo de ortotópico U87MG implantado en ratones. En este estudio se comparó la formulación de nanocápsulas de PGA-PEG HP con DCX contra la formulación comercial (Taxotere®). Para ello se dividieron los ratones en 3 grupos de acuerdo al tratamiento recibido. Cada tratamiento con DCX consistió en una única dosis de 2 mg de docetaxel/kg de peso del animal administrada mediante la inyección IV de 150 µl de formulación por vena de cola. Al grupo control se le administró el mismo volumen de suero salino fisiológico.

Los resultados recogidos en la Figura 7 muestran que la eficacia del DCX en cualquiera de las formulaciones ensayadas respecto al control negativo (ratones tratados con suero salino). Por otro lado, las nanocápsulas de PGA-PEG HP con DCX obtuvieron valores de inhibición de crecimiento tumoral similares a los conseguidos con la formulación comercial Taxotere®. En los tiempos finales (día 18 y 21), los grupos tratados con nanocápsulas o Taxotere® presentaban tumores significativamente más reducidos (-60%) que el grupo control ($p<0.01$).

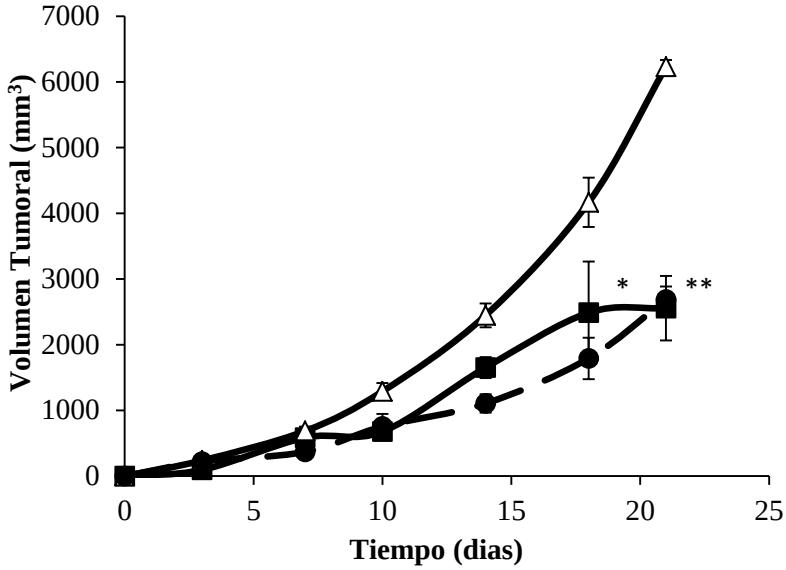

Figura 7: Evolución del tamaño del tumor a lo largo del tiempo tras la administración de nanocapsulas de PGA-PEG HP (■), Taxotere® (●) y el control negativo con serum salino (Δ). Modelo tumoral subcutáneo de U87MG en ratones. El análisis estadístico evidencia las diferencias significativas en el tamaño del tumor el día 18 y el día 21 en los animales tratados con las formulaciones de nanocápsulas y el Taxotere® en comparación con el control. *$p < 0.05$ **$p < 0.01$, según ANOVA.

Al mismo tiempo, los datos de supervivencia de los animales tratados (Figura 8 y Tabla 9) sugieren la superioridad de la formulación de nanocápsulas PGA-PEG HP frente al Taxotere®. Esta última puede considerarse un tratamiento más agresivo debido a la toxicidad del vehículo, pero también menos efectivo a raíz de los resultados de supervivencia obtenidos. Así, la mortalidad de los ratones tratados con el control negativo de suero salino comenzó al día 7, obteniéndose 100% de mortalidad para el día 17. El tiempo de supervivencia media fue de aproximadamente 11 días. El Taxotere® consiguió mejorar la supervivencia, consiguiéndose la supervivencia de un 20% de los ratones al final del experimento (día 21). La supervivencia media estimada de los ratones tratados con Taxotere® fue de aproximadamente 17 días, pero dicho incremento no fue estadísticamente significativo respecto al control

negativo. Por otro lado, hasta un 70% de los ratones tratados con nanocápsulas de PGA-PEG HP sobrevivieron hasta el final del experimento, resultando en un valor de supervivencia media estimada de aproximadamente 19 días, estadísticamente significativo respecto al control negativo.

Respecto a la discrepancia entre los resultados de crecimiento del tumor y supervivencia es importante señalar que tan sólo se puede medir el tamaño tumoral a los individuos supervivientes. Así, el proceso de mortalidad observado fundamentalmente en los grupos de control negativo y el Taxotere® producen un sesgo favorable en los datos de tamaño tumoral frente a los del grupo de nanocápsulas de PGA-PEG HP.

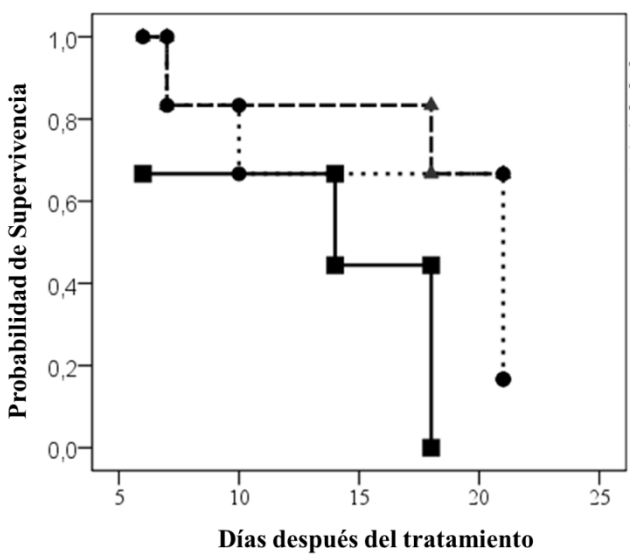

Figura 8: Curvas de supervivencia de Kaplan-Meier de los animales tratados con las diferentes formulaciones: nanocápsulas de PGA-PEG HP cargadas con docetaxel (▲), Taxotere ® (●) y control negativo de suero salino (■).

Tabla 9: Tiempo de supervivencia media de los animales tratados con las diferentes formulaciones: nanocápsulas de PGA-PEG HP cargadas con docetaxel, Taxotere® y el control, suero salino. IST= Incremento de tiempo de supervivencia media respecto al control negativo.

Tratamiento	*n*	Tiempo de supervivencia (días)		Incremento del tiempo de supervivencia (%)	
		Rango	Media ± SD	IST $_{media}$	*p*-valor vs suero
NCs de PGA-PEG HP	6	14-21	18.7 ± 2	66.96	0.036
Taxotere®	6	7-21	16.8 ± 2	50	0.147
Suero salino	6	7-17	11.2 ± 2	-	

Diversos autores han demostrado que los recubrimentos con características "furtivas" mejoran la acumulación de los nanosistemas en el tejido tumoral además de conducir a incrementos en la supervivencia. Heraux y colaboradores publicaron un estudio en el que demostraron como las nanocápsulas PEGiladas mejoran la eficacia terapéutica del docetaxel encapsulado además de ser mejor toleradas. De esta forma fue posible administrar el fármaco sin necesidad de pre-medicación. Igualmente, Yuk y colaboradores llegaron a las mismas conclusiones utilizando nanopartículas PEGiladas. Estos sistemas resultaron ser más eficaces que la misma formulación comercial del docetaxel, Taxotere®, debido a las características de larga circulación y a su cubierta altamente hidrofílica que permite incrementar su presencia en el torrente circulatorio alcanzando el tejido tumoral mediante el efecto EPR .

En nuestro caso, el sistema de nanocápsulas de PGA-PEG HP aúna excelentes propiedades de encapsulación de fármacos anticancerígenos hidrófobos, una adecuada estabilidad en almacenamiento, una muy reducida toxicidad *in vivo* que permite reducir la citotoxicidad intrínseca de los tratamientos, y características "furtivas"

o de largo tiempo de residencia plasmática imbuidas por el recubrimiento polimérico con PGA-PEG HP. Estas características originan a una mejora en el ratio eficacia/toxicidad frente a las formulaciones de referencia. Globalmente, los resultados observados sugieren el gran potencial de esta formulación para aplicaciones en terapia del cáncer.

Desarrollo de nanocápsulas de poliarginina (PARG) para la administración oral de fármacos antitumorales

Como previamente se ha descrito, las nanocápsulas han demostrado ser un vehículo prometedor en la vehiculización de moléculas activas a través de las mucosas[98]. Las ventajas que ofrecen dichos sistemas residen en la capacidad del núcleo oleoso para proteger la molécula encapsulada frente a la degradación enzimática y al mismo tiempo promover la absorción del fármaco at través del epitelio[99]. Asimismo el recubrimiento polimérico elegido, en este caso la poliarginina (PARG), ofrece interesante ventajas para facilitar la absorción oral.

Trabajos previos han demostrado la capacidad de la PARG para mejorar la interacción de los sistemas coloidales con las mucosas (oral y nasal)[100]. De hecho, los oligopéptidos de arginina son parte descrita de los péptidos de penetración celular (CPP, *Cell Penetrating Peptides*), moléculas que pueden interaccionar con las membranas epiteliales mediante atracciones iónicas entre la carga positiva del poliaminoácido y la carga negativa de la membrana plasmática y, de esta manera, facilitar el transporte del fármaco asociado a través de ellos[101,102].

Di Colo y colaboradores [103] han descrito la posibilidad de utilizar nanosistemas de PARG para aumentar la absorción de la FD-4, una macromolécula hidrofílica, a través del epitelio nasal. Esta capacidad de

98 **Prego, C., M. García, et al.** (2005). "Transmucosal macromolecular drug delivery." Journal of Controlled Release **101**(1-3): 151-162.

99 **Plapied, L., N. Duhem, et al.** (2011). "Fate of polymeric nanocarriers for oral drug delivery." Curr. Opin. Colloid Interface Sci. **16**(3): 228-237.

100 **Di Colo, G., Y. Zambito, et al.** (2008). "Polymeric enhancers of mucosal epithelia permeability: Synthesis, transepithelial penetration-enhancing properties, mechanism of action, safety issues." Journal of Pharmaceutical Sciences **97**(5): 1652-1680.

101 **Morishita, M. and N. A. Peppas** (2006). "Is the oral route possible for peptide and protein drug delivery." Drug Discov. Today **11**(19-20): 905-910.

102 **Brasseur, R. and G. Divita** (2010). "Happy birthday cell penetrating peptides:
." Biochimica et Biophysica Acta (BBA) - Biomembranes **1798**(12): 2177-2181.

103 **Di Colo, G., Y. Zambito, et al.** (2008). "Polymeric enhancers of mucosal epithelia permeability: Synthesis, transepithelial penetration-enhancing properties, mechanism of action, safety issues." Journal of Pharmaceutical Sciences **97**(5): 1652-1680.

internalización también ha sido utilizada en terapia génica en la modificación de liposomas para la vehiculización de siRNA obteniéndose elevados niveles de transfección[104]. Todas estas referencias ponen de manifiesto el interés en la capacidad promotora de la absorción de la PARG a través de las mucosas. En función de la experiencia previa, el objetivo de este trabajo ha sido el diseño de un sistema de nanocápsulas recubiertas de PARG y su evaluación como posible plataforma para la administración oral de fármacos antitumorales.

Preparación y caracterización de las nanocápsulas de PARG

La elaboración de las nanocápsulas de PARG se llevó a cabo mediante la técnica del desplazamiento del disolvente previamente descrita[105]. Las nanocápsulas fueron cargadas con el principio activo PM2734, provisto por PharmaMar S.A. Además para su visualización por microscopía confocal de fluorescencia o por IVIS, las nanocápsulas de PARG fueron cargadas alternativamente con las sondas fluorescentes fluoresceína-DHPE y DiD. Como control se preparó una nanoemulsión aniónica con la misma composición que las nanocápsulas de PARG pero sin el recubrimiento polimérico.

El análisis del tamaño de las partículas blancas y cargadas muestra que los sistemas de nanocápsulas de PARG se encuentran en el rango nanométrico, y por debajo de los 200 nm. Se observa además que el tamaño se incrementa ligeramente al incorporarse el principio activo PM02734. La presencia de la cubierta de PARG pudo ser corroborada por la inversión de la carga superficial, que pasa de -50 mV para la nanoemulsión a +47 mV para las nanocápsulas de PARG.

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[104] **Zhang, C., N. Tang, et al.** (2006). "siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene." Journal of Controlled Release **112**(2): 229-239.

[105] **Lozano M. V., Lollo G., et al.** (Submitted). "Polyarginine nanocapsules: a new platform for intracellular drug delivery."

Tabla 10: Propiedades físico químicas de la nanoemulsión aniónica y de las nanocápsulas de PARG blancas y cargadas con el PM02734, las sondas fluorescentes fluoresceína-DHPE y DiD. (Media ±D.E.; n=6); NE: Nanoemulsión, NCs: Nanocápsulas, [a] Índice de Polidispersión; F/M: Farmaco o marcaje.

Formulación	F/M	[F] mg/ml	Tamaño nm	IP	ζ mV	E.E. %
NE aniónica		-	189 ± 6	0.1	-50 ± 7	-
NCs PARG		-	138 ± 9	0.1	+47 ± 8	-
NCs PARG	PM02734	0.25	186 ± 7	0.1	+ 55 ± 6	54.1 ± 3
NCs PARG	PM02734	1.6	178 ± 15	0.1	+30 ± 11	45.7 ± 6
NCs PARG	DiD	-	129 ± 2	0.1	+25 ± 1	75.0 ± 5
NCs PARG	Fluoresceína	-	140 ± 1	0.1	+52 ± 1	79.2 ± 1

Estabilidad de las nanocápsulas de PARG en los fluidos gastrointestinales

Teniendo en cuenta que los sistemas coloidales son susceptibles a la agregación en los fluidos biológicos, en el presente trabajo se investigó el comportamiento de las nanocápsulas de PARG tras su incubación en medios gástrico e intestinal simulados. La evaluación de la estabilidad de estos sistemas se realizó monitorizando la evolución del tamaño de partícula tras su incubación en dichos fluidos durante periodos de tiempo de 2 h, y a 37°C.

Los resultados mostraron que en el fluido gástrico simulado las nanocápsulas se mantienen estables durante el tiempo de duración del experimento (2 h). Por otro lado, se observa un gradual aumento de tamaño cuando las nanocápsulas de PARG son incubadas en fluido intestinal simulado. De cualquier modo, el tamaño del sistema se mantiene siempre dentro del rango nanométrico, por lo que creemos que la

estabilidad de estas nanocápsulas es suficiente para un sistema en el cual se espera un tránsito rápido por el tracto gastrointestinal.

La explicación sobre la aceptable estabilidad de las nanocápsulas está relacionada con las propiedades químicas de la superficie generadas por la PARG. Debido a que el pKa de la PARG es de 12.5, los grupos amínicos de la poliarginina se mantienen protonados en todo el rango fisiológico de pH[106], resultando en una elevada carga catiónica superficial en los sistemas que no se ve condicionada por el medio fisiológico de suspensión.

Estudios en cultivos celulares:

Interacción de las nanocápsulas de PARG con células Caco-2

Un objetivo importante del presente trabajo fue elucidar el mecanismo de interacción de las nanocápsulas de PARG con los epitelios y las mucosas. Para ello se seleccionó el cultivo celular Caco-2, línea proveniente de adenocarcinoma de colon, que presenta gran similitud morfológica y bioquímica con los enterocitos. Por esta similitud, las monocapas de Caco-2 son ampliamente utilizadas en la evaluación de la permeabilidad transepitelial de fármacos. Los estudios realizados en este modelo celular incluyeron; (i) determinación de la citotoxicidad, (ii) medida de resistencia eléctrica transepitelial y (iii) el análisis de la asociación de los nanosistemas a la monocapa.

Estudios de citotoxicidad en células Caco-2

Se evaluó la viabilidad celular en células Caco-2 tras su exposición a diferentes dosis de nanocápsulas de PARGy de la

[106] **Oyarzun-Ampuero, F. A., F. M. Goycoolea, et al.** (2011). "A new drug nanocarrier consisting of polyarginine and hyaluronic acid." European Journal of Pharmaceutics and Biopharmaceutics **79**(1): 54-57.

nanoemulsión aniónica. Como control positivo se utilizó medio celular sin nanosistemas. La viablidad celular fue estimada a través de un ensayo colorimétrico que mide la actividad mitocondrial de las células expuestas (MTT). Los valores absolutos de lectura del ensayo MTT fueron corregidos respecto al control positivo (100%).

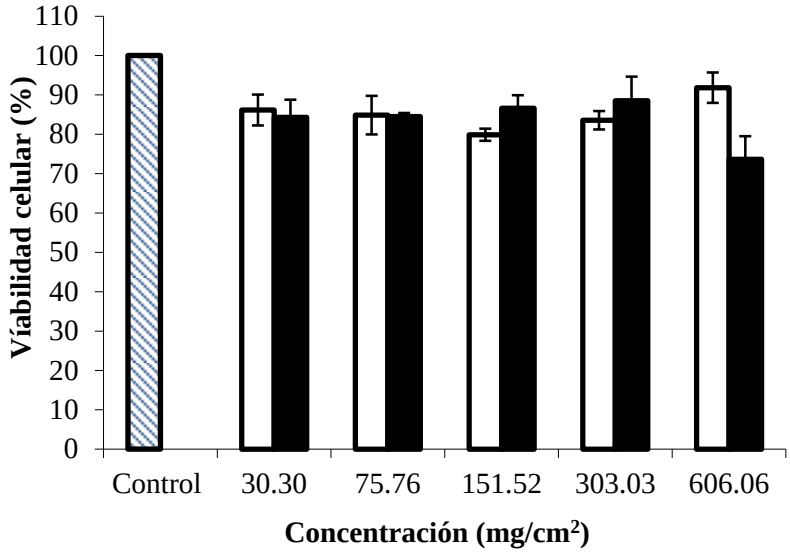


Figura 9: Viabilidad celular de Caco-2 expuestas a nanocápsulas de PARG (■) y la nanoemulsión (□) (Mean ± SD, n=2).

Este estudio, recogido en la Figura 9, mostró que tanto las nanocápsulas de PARG como las nanoemulsiones presentan una citotoxicidad muy baja, con viabilidades celulares superiores al 80% para todas las dosis ensayadas, excepto para las nanocápsulas a la dosis más alta (606 μg/cm^2). Incluso a esta dosis elevada, las nanocápsulas de PARG presentaron valores de viabilidad celular cercanos al 70%, y en todo caso muy superior a la dosis letal 50[107]. La comparación de estos valores con resultados anteriores extraídos de la literaturas sugieren que las

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

nanocápsulas recubiertas de PARG tienen una menor citotoxicidad que las nanocápsulas de otros polímeros muy estudiados como el quitosano[108] o las nanocápsulas de poli(L-lisina)[109]

Estudios de interacción con células Caco-2: medida de la resistencia eléctrica transepitelial

La capacidad de las nanocápsulas de PARG para alterar las uniones estrechas existentes entre las células de la monocapa de enterocitos se puede evaluar mediante la determinación de cambios en la resistencia eléctrica transepitelial (TEER). Con este objetivo, se incubó la monocapa Caco-2 con: nanocápsulas de PARG, nanoemulsión, y medio de cultivo (control negativo). Para poder realizar las lecturas, la monocapa Caco-2 debe de ser cultivada en un inserto especial con dos cámaras separadas por una membrana permeable a los iones (Transwell, Costar, USA). Estas cámaras permiten la medición del TEER mediante la inmersión de unos electrodos que miden la resistencia entre las cámaras apical y basolateral.

Los resultados obtenidos muestran que ni el medio de cultivo sin nanosistemas, ni la nanoemulsión dio lugar a variaciones en el TEER. Por otro lado, las nanocápsulas de PARG provocan un descenso en los valores de dicho parámetro. En concreto, la reducción ha resultado ser de un 30% a la concentración de nanocápsulas de 320 $\mu g/cm^2$, resultado que indica las propiedades permeabilizantes de los epitelios de las nanocápsulas PARG. Sin embargo, cuando el nanosistema era retirado de la cámara apical y sustituido por medio fresco, el TEER se recuperaba al cabo de 24 h. La capacidad de producir reducciones transitorias en la TEER junto con la elevada viabilidad celular observada tras la exposición a las

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

[109] **Brasseur, R. and G. Divita** (2010). "Happy birthday cell penetrating peptides" Biochimica et Biophysica Acta (BBA) - Biomembranes **1798**(12): 2177-2181.

nanocápsulas de PARG indica un buen perfil de seguridad para su potencial administración *in vivo*.

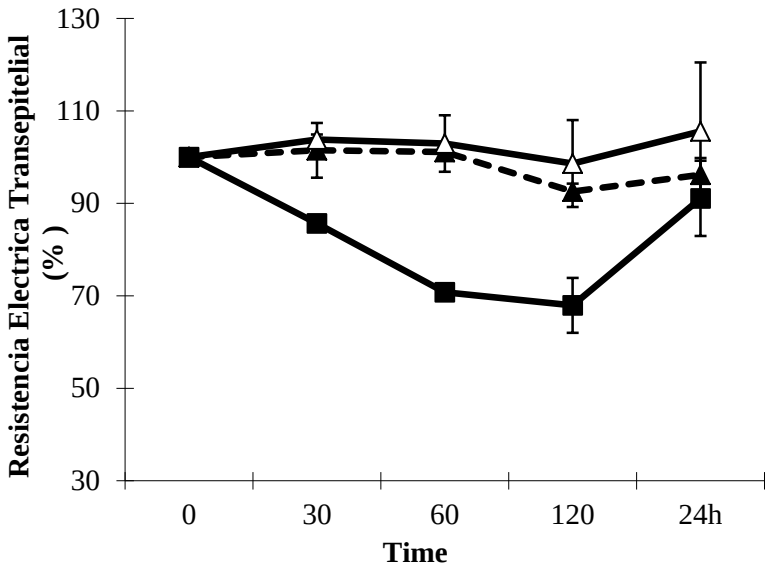


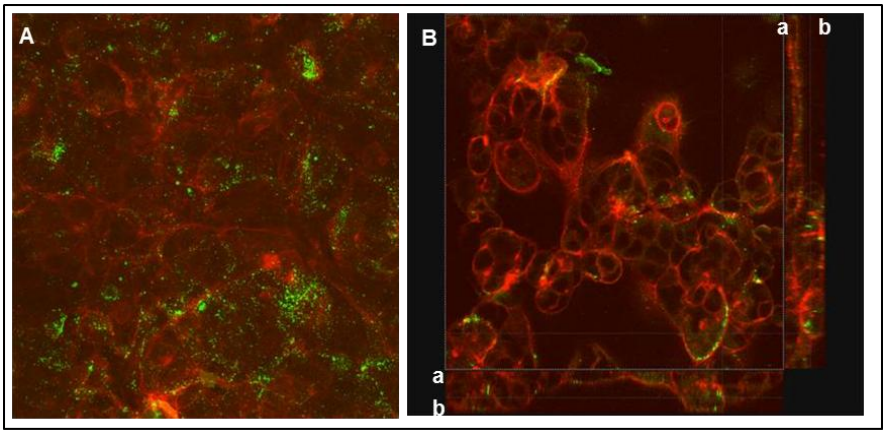
Figura 10: Medida de la resistencia eléctrica transepitelial (TEER) en monocapas Caco-2 tras su incubación con las (■) nanocápsulas de PARG y la (Δ) nanoemulsión a la concentración de 320μg/cm^2. Como control (▲) se ha utilizado una solución de DMEM.

Estudios de interacción con células Caco-2 mediante análisis cualitativo

La posible internalización o asociación íntima a la superficie celular de las nanocápsulas de PARG fue estudiada por microscopía confocal utilizando la formulación marcada con floresceina-DHPE. El análisis de las células por microscopía confocal confirma la presencia de las nanocápsulas de PARG dentro de la monocapa de Caco-2, evidenciándose su capacidad de penetración intracelular. Las nanocápsulas aparecen también localizadas en las uniones intercelulares y en la superficie celular. La penetración al interior de las células de la monocapa, sin embargo, no se aprecia con claridad en el caso de la

nanoemulsión. Este resultado sugiere que la presencia del PARG favorece el transporte de los nanosistemas a través de la membrana plasmática. Las imágenes también muestran una cierta acumulación de las partículas en las uniones entre las células, lo que plantea la potencial importancia de los mecanismos paracelulares en el transporte de fármacos a través de la monocapa.

(1)



(2)

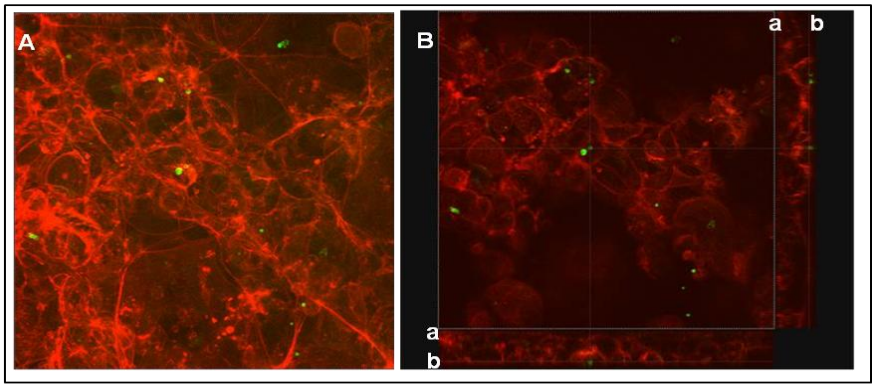


Figura 11: Imágenes de microscopía confocal de las células Caco-2 tras 2h de incubacion a 37°C con la con las nanocápsulas de PARG cargadas con fluoresceína (1) y con nanoemulsión cargadas con fluoresceína (1). Ejes-xy (A) con proyección xz- e yz- (B).Verde: nanosistemas marcados con fluoresceína-DHPE; rojo: Citoesqueleto marcado con faloidina fluorescente

Evaluación de la fluorescencia *in vivo* de las nanocápsulas de PARG cargadas con DiD

Además de la realización de estudios en cultivos celulares, se pretendió estudiar el comportamiento de las nanocápsulas de PARG *in vivo* tras su administración oral. Para ello, las nanocápsulas de PARG marcadas con el fluorocromo DiD fueron administradas oralmente a ratas, y su evolución en el tracto gastrointestinal fue estudiada mediante técnicas de imagen *in vivo*, a los tiempos preestablecidos de 1 h, 3 h, y 24 h. Para el estudio, se tomaron imágenes representativas de la biodistribución de este sistema en el organismo del animal.

Como se muestra en la Figura 12, se observó una señal de fluorescencia atribuible a las nanocápsulas de PARG en la región abdominal de los animales desde la primera hora tras la administración. La señal permaneció en dicha región hasta las 24 horas tras la administración. Para estudiar en más detalle la evolución de los nanovehículos, los animales fueron sacrificados a cada tiempo y se estudió el patrón de fluorescencia tras la necropsia. Se observó que las nanocápsulas de PARG son rápidamente eliminadas del esófago y del estómago, y que se encuentran principalmente en el intestino delgado hasta las 3 horas después de la administración (Figuras 13). Ademas, dentro de las limitaciones de la técnica para el análisis cuantitativo, las imágenes a las 3 h parecen sugerir una mayor fluorescencia en el intestino de las ratas tratadas con nanocápsulas de PARG que de las tratadas con nanoemulsiones.

La capacidad de los nanosistemas de asociarse íntimamente a la barrera mucosa es una característica crucial para la vehiculización oral de fármacos. Estos resultados hacen suponer que las nanocápsulas de PARG consiguen adherirse al epitelio intestinal debido a los grupos guanidinos que constituyen el polímero, y posteriormente, gracias al bien descrito comportamiento como CPP de la PARG, internalizarse en la monocapa.

Los estudios mecanísticos descritos sugieren el posible interés de las nanocásulas de PARG como vehículos orales de fármacos antitumorales.

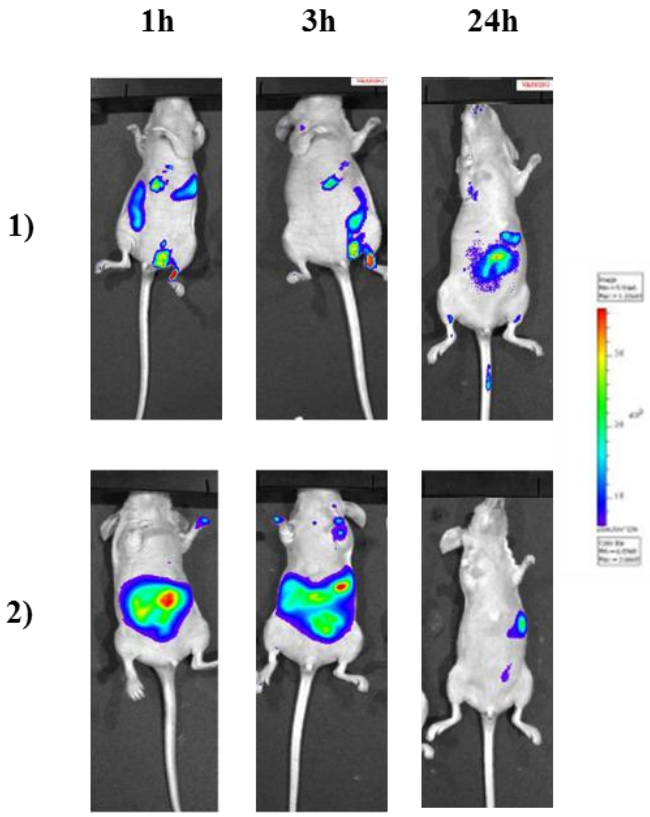
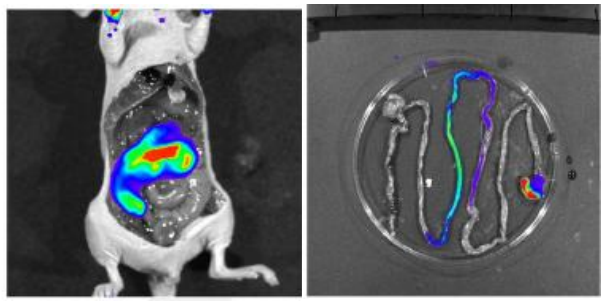


Figura 12: Imágenes de fluorescencia de los ratones a distintos tiempos (1h, 3h y 24h) administración oral de nanoemulsiones (1) y de las nanocápsulas de PARG (2).

(1)



(2)

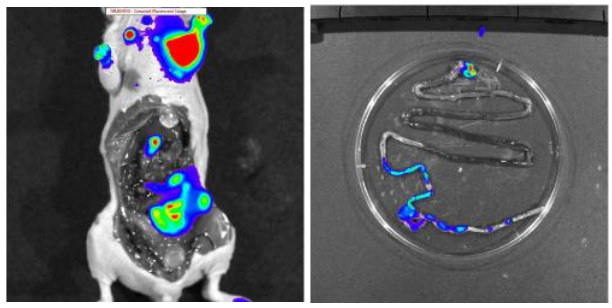


Figura 13: Imágenes de fluorescencia de los animales sacrificados y del tracto gastrointestinal tras administracion de las nanocápsulas de PARG cargadas con DiD: (1) 1 hora tras su administración; (2) 3 horas tras su administración.

CONCLUSIONES

Conclusiones

El trabajo experimental recogido en la presente memoria, se ha dirigido al diseño de nuevas formulaciones nanocapsulares para la vehiculización de fármacos antitumorales. Los resultados han permitido extraer las siguientes conclusiones:

Parte I:

Desarrollo de nanocápsulas de PGA y de PGA-PEG como nuevas plataformas en la terapia del cáncer:

1. Se han elaborado nanocápsulas constituidas por un núcleo oleoso y una cubierta polimérica a base de PGA o PGA-PEG. Para la elaboración de estos sistemas se han empleado dos polímeros con diferente grado de PEGilación y diferente estructura, un copolímero PGA-PEG grafted con un porcentaje del 24 % p/p de PEG y otro dibloque con un alto porcentaje PEG, 57 % p/p. Las nanocápsulas resultantes tienen un tamaño nanométrico de alrededor de 200 nm y una carga superficial altamente negativa para las nanocápsulas de PGA (-40mV) y menos marcada para los sistemas pegilados (-20 mV).

2. La evaluación *in vivo* de la cinética plasmática de las nanocápsulas de PGA y las nanocápsulas de PGA-PEG altamente pegiladas cargadas con la sonda fluorescente DiD ha evidenciado un incremento de los parámetros farmacocinéticos al ser comparados con la nanoemulsión, utilizada como control. Asimismo, las nanocápsulas de PGA-PEG altamente pegiladas tienen una vida media, un AUC y un MRT más alto con respecto a los sistemas sin PEGilar.

3. El fármaco antitumoral plitidepsina ha podido ser eficazmente encapsulado en las nanocápsulas de PGA y PGA-PEG con diferente grado de PEGilación, sin modificaciones en las propiedades físicoquímicas de los sistemas. Los estudios realizados *in vivo* para evaluar la farmacocinética de estos sistemas han evidenciado como la alta pegilacion de las nanocápsulas permite incrementar los parámetros farmacocinéticos, en términos de AUC y MRT. Asimismo se ha podido observar una reducción del aclaramiento y del volumen de distribución en comparación al fármaco disuelto en una solución de Cremophor® EL, utilizada como referencia. Además todos los nanosistemas ensayados han demostrado tener una menor toxicidad, un valor de MTD hasta 3 veces menor, con respecto a la formulación de referencia.

4. Tras su administración IV en ratones con un tumor renal modelo xenograft (MRI-H-121), las nanocápsulas de PGA-PEG con alto grado de PEGilación fueron altamente eficaces en la reducción del crecimiento tumoral, siendo su efecto comparable con la formulación de referencia y no evidenciándose ningún efecto toxico.

5. Las nanocápsulas de PGA-PEG con alto grado de PEGilación han podido encapsular el fármaco anticancerígeno docetaxel. Su evaluación *in vivo* en el modelo de glioma U87 en ratones ha evidenciado como estos sistemas son capaces de inhibir el crecimiento tumoral de modo comparable a la formulación comercial de docetaxel, Taxotere®. Además, los resultados de supervivencia de los animales tratados indican la superioridad de la formulación nanocápsular frente al Taxotere®.

Parte II:

Desarrollo de las nanocápsulas de PARG y evaluación de su potencial en la administración oral de fármacos antitumorales.

1. Mediante la técnica de desplazamiento del solvente se han podido elaborar nanocápsulas de PARG. Los sistemas resultantes tienen un tamaño de alrededor de 130 nm y un potencial zeta altamente positivo (+40 mV). El fármaco anticancerígeno PM02734 ha podido ser encapsulado en los sistemas desarrollados.

2. Los estudios de citotoxicidad llevados a cabo en cultivos celulares de Caco-2 han reflejado la tolerabilidad de estos sistemas, siendo la viabilidad celular de alrededor a un 80% en los ensayos realizados con las concentraciones más elevadas (606 μg/ml).

3. La facultad de las nanocápsulas de PARG para interaccionar con los cultivos celulares de Caco-2, determinada mediante la medida de la resistencia eléctrica transepitelial, ha mostrado la capacidad permeabilizante de estos sistemas. La asociación de los mismos a la monocapa obtenida mediante imágenes de microscopía confocal de fluorescencia ha permitido localizar los sistemas tanto en las uniones intercelulares como en el interior de las células. Estos resultados evidencian la posibilidad de un transporte paracelular de dichos sistema.

4. Los estudios de imagen de fluorescencia *in vivo* permiten visualizar las nanocápsulas de PARG durante su paso a través del tracto gastrointestinal, así como comprobar su permanencia en el intestino durante 3 horas.

CONCLUSIONS

Conclusions

The experimental work enclosed in this manuscript was aimed to design new nanocapsules as novel drug delivery systems for antitumor drugs.

The results allowed the following conclusions:

Part I:

Development of PGA and PGA-PEG as novel platform in oncology:

1. Novel nanocapsules constituted by an oil core and a polymeric shell made of PGA and PGA-PEG were developed. The polymers employed to obtain these systems have a different pegylation degree and a different structure: a grafted copolymer PGA-PEG with a PEG percentage of 24 % w/w and a dibloque copolymer PGA-PEG with a high PEG percentage, 57%. The resulting nanocapsules have a nanometric size, around 200 nm with a negative charge, around -40 mV for PGA nanocapsules and -20 for the pegylated systems.

2. The *in vivo* blood kinetic studies of PGA nanocapsules and PGA-PEG nanocapsules, with a high pegylation degree, loaded with the fluorescent probe DiD, evidence an increase of the pharmacokinetic parameters compared with the nanoemulsion, used as control. Moreover, PGA-PEG nanocapsules have a higher half-life, AUC and MRT in comparison to the no pegylated systems.

3. The cytostatic drug plitidepsin has been efficiently encapsulated into PGA and PGA-PEG nancapsules with high and low pegylation

degree. The *in vivo* pharmacokinetic studies show that nanocapsules with a high PEG content increase the pharmacokinetic parameters in terms of AUC and MRT. Furthermore, it could be observed a great reduction of clearance and distribution volume of the encapsulated drug in comparison to plitidepsin dissolved in a Cremophor® EL solution, used as a reference formulation. All the systems tested present a lower toxicity, the MTD is increased 3 times in comparison with the reference formulation.

4. After IV administration in mice with a renal xenograft tumor model, PGA-PEG nanocapsules with a high pegylation degree efficiently hindered tumor growth, being this effect comparable to the reference formulation.

5. PGA-PEG nanocapsules with a high pegylation degree efficiently encapsulate the antitumor drug docetaxel. The antitumor efficacy of docetaxel loaded PGA-PEG nanocapsules assed in the *in vivo* U87 glioma mice model showed that nanocapsules decrease tumor growth in a comparable manner with the commercial docetaxel formulation, Taxotere®. Besides, these systems increase the survival rate highlighting the potential and superiority of the nanocapsules formulation in comparison with Taxotere®.

Part II

Development of PARG nanocapsules and evaluation of their potential for the oral administration of antitumor drugs.

1. The solvent displacement has enabled the formation of PArg nanocapsules. The systems obtained showed a nanometric size of around 130 nm and a positive zeta potential of +40 mV. The anticancer drug PM02734 could be encapsulated into the developed system.

2. The citotoxicity studies performed in Caco-2 cell culture showed the high tolerability of these systems, being the cellular viability of around 80% at the highest concentration texted.

3. The ability of PARG nanocapsules to interact with Caco-2 cell monolayer carried out monitoring the modification of TERR, showed the permealization property of the systems. The interaction of the nanocapsules with the monolayer observed thought confocal microscopy illustrate that nanocapsules are located in both surface and interior of the cells.

4. The *in vivo* fluorescent studies enable to visualize PARG nanocapsules during their pass thought the gastrointestinal tract and confirm their permanence during 3 hours.

ANEXOS

Polyarginine nanocapsules: a new platform for intracellular drug delivery

Lozano M.V.[1], Lollo G.[1], Brea J.[2], Vidal A.[3], Torres D.[1] and Alonso, M.J.[1*]

[1] Department of Pharmaceutical Technology. Faculty of Pharmacy. University of Santiago de Compostela (USC). Campus Sur, 15782. Santiago de Compostela, Spain.

[2] Department of Pharmacology. Faculty of Pharmacy. University of Santiago de Compostela (USC). Campus Sur, 15782. Santiago de Compostela, Spain.

[3] Department of Physiology. Center for Molecular Medicine and Chronic Diseases (CIMUS), University of Santiago de Compostela (USC). Avda. Barcelona s/n, 15782. Santiago de Compostela, Spain

*Corresponding author

E-mail address: mariajalonso@usc.es (M.J. Alonso).

Abstract

This report describes the development of a new nanocarrier, named as polyarginine (PARG) nanocapsules, specifically designed for overcoming cellular barriers. The formation of these nanocapsules, consisting of oil cores surrounded by PARG, was achieved using the solvent displacement technique. Hybrid PARG/PEG nanocapsules could also be obtained by introducing PEG-stearate in the nanocapsules formation process. The nanocapsules had an average size in the range of 120-160 nm, and a positive surface charge between +56 and +28 mV depending on the presence of PEG-stearate in their composition. They could accommodate significant amounts of lipophilic drugs, i.e. docetaxel, in their core, and also highly polar negatively charged molecules, i.e. plasmid DNA, on their coating. As a preliminary proof-of-principle, we explored the ability of these nanocarriers to enter cancer cells and to inhibit proliferation in the non-small cell lung cancer NCI-H460 cell line. The results indicated that PARG nanocapsules are rapidly and massively accumulated into the NCI-H460 cells and that the PArg shell play a critical role in the internalization process. Moreover, upon incubation with docetaxel-loaded nanocapsules we observed an enhanced inhibition of cancer cells proliferation, as compared to the free drug. Overall, the evidence of the potential of this new nanocarrier for intracellular drug delivery discloses a new technological approach for overcoming cellular as well as epithelial barriers.

Keywords: Polyarginine, nanocapsules, nanocarrier, intracellular delivery, docetaxel, gene delivery.

Introduction

One of the main focuses of nanomedicine is the targeting and delivery of therapeutic molecules[1]. It is currently known that the incorporation of drugs into nanocarriers increases the efficacy of the treatments, reduces drug-associated side-effects and improves the quality of life of patients[2]. Overall, cancer, genetic or metabolic disorders are among the diseases that can significantly benefit from this extensive research[3].

Over the last decade a focus of our research group has been the design of nanocarriers for intracellular delivery of anticancer drugs and also gene molecules[4, 5]. For example, we have reported the potential of polysaccharide-based nanostructures for the intracellular delivery of antitumour drugs[6, 7] as well as the first proof-of-principle of polysaccharide nanostructures for ocular gene therapy[8]. As an advanced step towards this goal we have attempted to develop a new intracellular drug delivery platform based upon the use of polyaminoacids[9]. Polyaminoacids have raised great expectancy in the development of drug delivery systems due to their interesting physicochemical properties as well as to their promising safety profile[10, 11]. Indeed, polyaminoacids are hydrosoluble polymers that exhibit a structural resemblance to polypeptides, thus making their degradation by human enzymes possible[12]. Moreover, because of their chemical structure, they are particularly attractive polymers for the chemical attachment of PEG or other molecules[13].

Among these polyaminoacids, polyarginine (PARG), a cationic polymer belonging to the family of the protein transduction domains, has shown an ability to translocate through the mammalian cell membranes and facilitate the uptake of the molecules attached[14]. These cell penetrating properties of PARG have been attributed to the presence of the guanidine

moiety in its side chain. The guanidine group is known to form bidentate hydrogen bonds with the anionic groups of the surface of the cells and, subsequently, facilitates the polymer cellular internalization[15].

This interesting feature has been the rationale of its use for intracellular drug delivery of low molecular weight anticancer drugs[16, 17], proteins[18] and nucleic acid-based molecules[19]. For example, Kim et al. have reported the synthesis and use of a cholesteryloligo-D-arginine conjugate as an siRNA delivery vehicle for the silencing of the vascular endothelial growth factor (VEGF), an angiogenic growth factor involved in the vascularization of solid tumours [20]. Similarly, other authors have shown the potential of oligoarginines for enhancing the transfection efficiency of DNA/protamine in mice bearing HeLa tumour xenografts[21].

Additionally, there is significant evidence of the benefits of arginine oligomers and arginine homopolymers of higher molecular weight (PARG) for the delivery of peptides and also vaccines. In fact, Yang et al. produced a pro-apoptotic Smac-peptide/oligoarginine conjugate, which was shown to selectively reverse the apoptosis resistance of NCI-H460 cells, thus increasing cell death induced by chemotherapy[22]. Likewise, the conjugation of the cardioprotective peptide ψεRACK with a heptamer of arginine led to an increase in its intracellular uptake and, consequently, to an improvement of the protection of myocardium from ischemic episodes[23]. More importantly, PArg is being evaluated in Phase II clinical trials as an adjuvant for a hepatitis C vaccine. Indeed, the incorporation of PARG to the antigen formulation has resulted in a significant T cell immunostimulation, in the absence of specific polymer responses[24].

Finally, it is worth mentioning some preliminary data showing the role of PARG as transmucosal penetration enhancer. Namely, PARG has been shown to enhance the transcorneal penetration of hydrophilic molecules such as FITC-labelled dextran and pyridoxamine[25]. Moreover, repeated dosing of the formulation was shown to be harmless to the cornea, as no alterations of the corneal thickness, infiltration of neutrofils or production of TNF-α were observed. On the other hand, Zaki et al. have also reported the ability of PARG to enhance the nasal absorption of drugs such as metoclopramide, without evident signs of toxicity[26].

Based on this background information, the purpose of this work was to develop an original nanocarrier named as PARG nanocapsules, consisting of an oil nano-container surrounded by a PARG shell and to provide the first proof-of-principle of its utility for intracellular delivery. Besides the obvious selection of the coating, the oil core was conceived in order to facilitate the accommodation of hydrophobic molecules. Alternatively, we have also explored the possibility of modulating the surface properties of the nanocapsules by introducing PEG-stearate on the shell composition.

Materials and Methods

Chemicals

PARG (MW 5-15 kDa), docetaxel, Trizma® base, agarose, xylene cyanole and bromophenol blue were purchased from Sigma-Aldrich (Spain). Miglyol 812®, neutral oil formed by esters of caprylic and capric fatty acids and glycerol, was a kind gift from Sasol Germany GmbH (Germany), and the surfactant Epikuron 145V, a phosphatidylcholine enriched fraction of soybean lecithin was donated by Cargill (Spain). The products SYBR® Green I Nucleic Acid Gel Stain and N-(fluorescein-5-

thiocarbamoyl)-1.2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (fluorescein-DHPE) were obtained from Molecular Probes. Plasmid DNA (pDNA) encoding green fluorescent protein (pEGFP-C1) driven by a CMV promoter was purchased from Elim. Biopharmaceutical (USA).

Preparation of PARG nanocapsules

Blank PARG nanocapsules were obtained by a modification of the solvent displacement technique based on a polymer ionic interaction after solvents diffusion[27]. Briefly, an organic phase was formed by dissolving 20 mg of Epikuron 145V in 0.25 mL ethanol, followed by 62 µL of Miglyol® 812 and 4.7 mL acetone. This organic phase was immediately poured over a solution of PARG (0.01, 0.025, 0.05, 0.25, 0.3% w/v) obtaining the immediate formation of PARG nanocapsules. Finally, solvents were eliminated from the suspension to constant volume and under reduced pressure yielding a nanocapsule concentration of 17 mg/mL.

PEG-surface modified PARG nanocapsules were formed following the same method described previously, but additionally including 48 or 96 mg of PEG stearate to the organic phase in order to obtain the PEG incorporation onto the surface of the nanocapsules, yielding the 1 or 2% w/v PARG -PEG nanocapsules.

The encapsulation of the hydrophobic molecules docetaxel or the fluorescent probe fluorescein-DHPE in PARG nanocapsules was achieved by adding aliquots of the drug stock solutions in ethanol to the organic phase previous to the formation of the nanocapsules.

Fluorescent nanoemulsion and fluorescent dispersion controls used for the cell uptake studies were obtained by the method previously described. Unlike nanocapsules, nanoemulsion is only formed by the hydrophobic cores without the polymer cover. With respect to the fluorescent dispersion, an aliquot of the dye was diluted in the ethanol/acetone mixture of the organic phase following the same process.

Physicochemical characterization of PARG nanocapsules

The different PARG nanocapsules formulations were characterized with regard to size, zeta potential and morphology as follows.

Particle size and polydispersion index were determined by photon correlation spectroscopy (PCS). Samples were diluted to the appropriate concentration with filtered water. Each analysis was carried out at 25ºC with an angle detection of 90º. The zeta potential values were calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry (LDA). Samples were diluted with KCl 1 mM and placed in the electrophoretic cell where a potential of ±150 mV was established. PCS and LDA analysis were performed in triplicate using a NanoZS® (Malvern Instruments, Malvern, UK).

Nanocapsules were isolated in order to assess the adhesion strength of the PArg layer to the droplet surface. Therefore, a 5 mL aliquot was ultracentrifuged at 85000 g for 1 hour and the remaining nanocapsule-rich fraction was collected and diluted with ultrapure water. Isolated PARG nanocapsules were also characterized according to particle size and zeta potential.

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 copper grids with Formvar® films for analysis.

pDNA association to PARG nanocapsules

Plasmid DNA (pDNA) encoding green fluorescent protein was adsorbed on the surface of PArg nanocapsules at 3% and 10% loadings, defined as the percentage between the mass of pDNA and the total mass of the formulation. For the adsorption procedure, a pDNA solution (50 µL) was added to an isolated PARG nanocapsules suspension (200 µL) and subsequently vortexed for 30 seconds. Formulations were left at room temperature for 1 hour to achieve an optimal interaction of the pDNA with the nanocapsules. Different weight ratios were obtained by modifying the concentration of pDNA, meanwhile the concentration of PARG nanocapsules was maintained constant at 1.4 mg/mL.

pDNA-associated nanocapsules were characterized according to size and zeta potential as detailed previously. Additionally, the association of pDNA to the nanocapsules was studied by a conventional agarose gel electrophoresis assay. In order to displace the pDNA adsorbed to the nanocapsules, a far excess of heparin (15 mg/mL) was added to the suspension and the mixture was incubated for 2 hours. pDNA was stained with SYBR® Green I by adding 3 µL of the reagent diluted 1:10000 in TAE buffer, to 25 µL of the pDNA-associated nanocapsules, and left for 15 minutes to interact. Then, the samples and the control of free pDNA were loaded in 1% agarose gel and ran for 90 minutes at 60 V in TAE buffer (Sub-Cell GT 96/192, Bio-Rad Laboratories Ltd., England).

The potential of pDNA-associated nanocapsules as delivery vehicles of pDNA was assessed by comparison with the pDNA/ PARG complexes

previously described in the literature[28]. For that purpose, two formulations of complexes 1:3 and 1:1 pDNA/PARG weight ratios were prepared as respective comparison to 3 and 10% pDNA/PARG nanocapsules. The preparation method consisted on adding equal volumes of pDNA and PARG aqueous solutions (final volume of 100 µL), vortexing the mixture for 30 minutes at room temperature and leaving for 1 hour to stabilize. Then, the complexes were characterized with respect to size and zeta potential. Additionally, the different formulations of nanocapsules and complexes were studied according to their stability in phosphate buffer by diluting the samples with the buffer to a final ionic strength of 75 mM and measuring their size values after an incubation of 30 minutes. The premature release of the pDNA from the pDNA-associated nanocapsules and pDNA/PARG complexes in phosphate buffer was determined by agarose gel electrophoresis as already exposed.

Long term stability

The long term stability of PARG nanocapsules was assessed by analyzing the particle size values of the nanocarrier after the storage of the nanocapsule suspension without previous dilution of the system at 4 and 37ºC. The procedure for particle size measurement was already described in the previous sections. Additionally, we have also determined the feasibility of the system to be lyophilized. For that purpose, the effects of different conditions, such as the concentration of nanocapsules (0.25, 0.5 and 1% w/v), and the concentration of the cryoprotective agent trehalose (5 and 10% w/v) were evaluated. Nanocapsules concentrations were prepared by dilution with ultrapure water, subsequent addition of trehalose solutions and freezing of the suspension by immersion in liquid nitrogen (-196ºC). Samples were placed in the freeze-drier (Labconco Corp., USA)

for primary drying at -34ºC for 24 hours followed by the secondary drying for 12 hours up to a gradual increase of the temperature to 20ºC. Lyophilizates were reconstituted with ultrapure water by shaking in a vortex for 5 minutes and afterwards the size and polydispersity index were determined as explained before.

Docetaxel encapsulation in PARG nanocapsules

The incorporation of the anticancer drug docetaxel in PARG nanocapsules was achieved by adding 50 µL of a drug solution in ethanol to the organic phase in order to obtain a final drug concentration of 10 µg/mL and the process was continued as described previously. Docetaxel encapsulation efficiency in PARG 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 after ultracentrifugation. Therefore, the total amount of drug was estimated by dissolving an aliquot of non-isolated docetaxel-loaded PARG nanocapsules with acetonitrile. This sample was centrifuged for 20 minutes at 4000 xg 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 PARG nanocapsules from the aqueous medium by ultracentrifugation.

Docetaxel was assayed by a slightly modified version of the method proposed by Lee et al.[29]. 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 U.S.A.). 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/minute. The standard calibration curves of docetaxel were linear (r2> 0.999) in the range of concentrations between 0.3-2 μg/mL.

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

E.E. % = [(A-B)/A] x 100

where A is the experimental total drug concentration (mg/mL), and B is the drug concentration measured in the external aqueous medium, corresponding to unloaded drug (mg/mL).

The release studies of docetaxel from PARG nanocapsules were performed by incubating a sample of the formulation with acetate buffer (pH=5) at an appropriate 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 by using Amicon Ultra® devices (Millipore, Spain) at different time intervals (1, 3, 6, 24 and 48 hours). The docetaxel released was calculated indirectly by determining the amount of drug remaining in the system by processing the isolated PArg nanocapsules with acetonitrile before HPLC analysis.

Tumour cells growth inhibition

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 37ºC in a humidified atmosphere containing 5% CO2. Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Acros Organics) was used for mitochondrial activity evaluation. Briefly, cells were plated onto 96-well plates, with a seeding density of 15x103 cells/well in 100 μL culture medium. After 24 hours, the medium was removed and dilutions of

docetaxel solution, docetaxel-loaded PArg nanocapsules and blank PArg nanocapsules in medium were added to the wells. Finally, after 48 hours of incubation of the formulations, cell survival was measured by the MTT assay[30]. 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 4 hours.

Afterwards, the 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, using Prism 2.1 software (GraphPad, San Diego, CA).

PARG nanocapsules cellular internalization

Cells were seeded in multiwell-12 plates (Falcon) at 16x104 cells/well in supplemented medium for 24 hours. Afterwards, the medium was removed and dilutions of the fluorescent dispersion, fluorescent nanoemulsion and fluorescent PARG nanocapsules were added to the wells. After 2 hours of incubation, cells were washed with acidic phosphate saline buffer (PBS, Sigma), trypsinized and resuspended in PBS supplemented with 3% v/v of FBS.

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

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 and cell uptake results was performed by an ANOVA test followed by a multiple comparison analysis (SigmaStat Program, Jandel Scientific, version 3.5). Differences were considered to be significant at level of 0.05. IC50 values were compared by means of a "t" test for independent samples using SPSS v 15.0 (SPSS Inc.).

Results and Discussion

This article describes for the first time a new drug nanocarrier consisting of an oil core surrounded by a PARG shell (Figure 1). The rationale behind the design of this nanocarrier, named PArg nanocapsules, was as follows: the oil core is intended to allocate significant amounts of lipophilic active ingredients, whereas the external polymer shell is expected to have three differentiated roles: (i) to facilitate the interaction and internalization of the nanocarrier within the cells, (ii) to provide the nanocarrier with adequate stability in biological media and during storage and (iii) to associate negatively charged molecules, i.e. nucleic acid molecules. These expected properties are justified by the positive charge of PArg and, also, by the capacity of this polymer to work as a cellular penetration enhancer, as reported in the introduction[14, 15, 25]. An additional interesting property of PARG nanocapsules relies on the possibility to PEGylate them, simply by the addition of a hydrophobic

derivative of PEG into the nanocapsules formation medium. This modification enlarges the utility frame of these nanocapsules, thus making them useful not only for mucosal or regional delivery but also for parenteral administration. In fact, PEGylation is known to improve the stability of the nanosystems in biological media and to avoid the clearance by the mononuclear phagocyte system (MPS)[31].

In the next paragraphs we present and discuss the results of this work in what concerns: (i) The construction and characterization of the nanocarrier, (ii) the evaluation of its ability to associate lipophilic drugs (docetaxel) and polar molecules (pDNA), (iii) the study of the stability of the nanocarrier in simulated biological media and (iv) the evaluation of its capacity to enter cells and to deliver an associated biologically-active compound inside the cell. This was verified using docetaxel as a model compound.

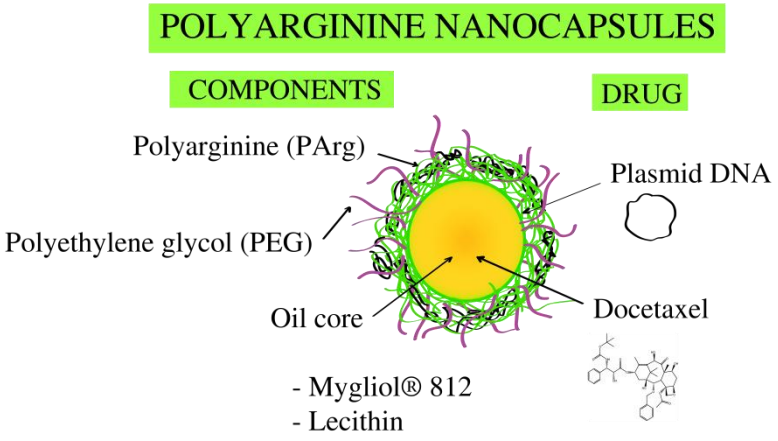


Figure 1: Illustration of PARG nanocapsules showing the components that constitute the system and the drugs that can be co-encapsulated.

The construction and characterization of the nanocarrier

The key elements for the construction of the nanocarrier have been the polymer shell (PARG), an oil (Miglyol® 812), and a surfactant that facilitates the formation of nanodroplets as well as the attachment of the PArg shell (lecithin). These three elements were combined according to the solvent displacement technique, that we have previously applied to the formation of other types of nanocapsules[32]. This procedure is based on the controlled nanodispersion of lipid components in an aqueous solution followed by electrostatic interaction between the negatively charged lecithin and a cationic polymer. Using this experimental approach we could obtain a monodispersed population of PARG nanocapsules with a mean size lower than 200 nm. As expected, these nanocarriers exhibited a high positive net charge due to the PARG layer disposed over the hydrophobic core, formed by lecithin and the oil Miglyol® 812, showing the typical charge inversion. Additionally we investigated the effect of PARG concentration (from 0.01 to 0.3%) on the physicochemical properties of the nanocapsules.

Table 1: Physicochemical properties of polyarginine nanocapsules (PARG NCs) and PARG -PEG NCs.

Formulation	[PArg] (% w/v)	Size (nm)	PDI	Zeta potential (mV)
PArg NCs	0.01	166 ± 1	0.01	+35.5 ± 1.2
PArg-PEG 1% w/v NCs	0.01	150 ± 2	0.09	+24.7 ± 1.1
PArg-PEG 2% w/v NCs	0.01	117 ± 1	0.12	+27.1 ± 1.5
PArg NCs	0.025	137 ± 11	0.13	+49.8 ± 5.9
PArg NCs	0.05	145 ± 13	0.10	+52.7 ± 5.6
PArg-PEG 1% w/v NCs	0.05	115 ± 4	0.15	+28.4 ± 2.6
PArg-PEG 2% w/v NCs	0.05	107 ± 5	0.17	+26.9 ± 4.3
PArg NCs	0.25	120 ± 2	0.11	+60.9 ± 1.2
PArg NC	0.3	199 ± 1	0.54	+55.6 ± 2.4
PArg-PEG 1% w/v NC	0.3	122 ± 4	0.37	+43.0 ± 2.4
PArg-PEG 2% w/v NC	0.3	111 ± 2	0.11	+41.5 ± 2.9

The results (Table 1) indicate that as PARG concentration is higher, the positive charge of the nanocapsules increases reaching a maximum value of +60 mV. This increase in the zeta potential was accompanied of an initial reduction in the particle size (from 166 nm to 120 nm) followed by a subsequent increase (up to 190 nm). The initial size reduction was attributed to the stabilizing role of PARG, whereas the size increase observed for high PARG concentrations could be related to the thickness of the PARG coating. Based on these results we selected the concentration of PARG 0.05% for further experiments.

Interestingly, PARG nanocapsules could be separated from the suspension medium and resuspended in water without altering their original properties. This possibility to manipulate them in order to obtain

the desired concentration in the adequate suspension medium is very important from a pharmaceutical standpoint.

The morphological appearance of the nanocapsules was observed by transmission electron microscopy. The micrograph presented in Figure 2 indicates that PARG nanocapsules have a round shape and size of less than 200 nm, similar to the values obtained by photonic correlation spectroscopy. In addition, the micrograph illustrates the presence of a polymer corona covering the nanodroplets.

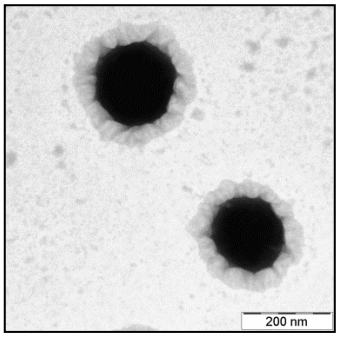


Figure 2: Transmission electron micrograph of PARG nanocapsules.

As indicated above, in an additional set of experiments, we attempted to produce a PEGylated nanocarrier. For this, we incorporated PEG-stearate in the oil phase during the nanocapsules formation process. The resulting PEG-surface modified PARG nanocapsules were slightly smaller than those made without PEG-stearate, most probably due to the stabilizing properties of PEG-stearate to the nanoemulsion previous to the coating with PARG (Table 1). As expected, these nanocapsules exhibited a significantly reduced positive charge due to the presence of the PEG coating[33]. Both effects, reduction in particle size and zeta potential were more remarkable as the concentration of PEG-sterate increased.

Association of pDNA to PARG nanocapsules

As presented in the introduction, the use of PArg for the intracellular delivery of gene material has already been reported [20, 21, 34]. In these previous studies the polymer has been directly complexed to the polynucleotide molecules, whereas the strategy presented here involves the adsorption of gene material onto the preformed PArg nanocapsules. The hypothesis behind this strategy was that the surface disposition of the PARG would favor its interaction with the pDNA molecules due to the great surface area while the oily core would allow us the co-administration of auxiliary lipophilic ingredients, which might further enhance the efficacy of the nanocarrier.

Given the negative charge of the pDNA molecules and the positive charge of the PARG and PARG-PEG nanocapsules we have assumed that the interaction between them would be driven by electrostatic forces.

Table 2: Physicochemical properties of plasmid DNA (pDNA)-associated and docetaxel (DCX)-loaded polyarginine nanocapsules (PARG NC) and PARG -PEG NC. PDI: polydispersity index. (Mean ± s.d.; n = 3)

Formulation	Size (nm)	PDI	Zeta potential (mV)
3% pDNA-PARG NCs	129 ± 4	0.22	+46.8 ± 1.9
10% pDNA- PARG NCs	136 ± 9	0.16	+31.3 ± 6.1
3% pDNA- PARG -PEG NCs	126 ± 6	0.18	+23.7 ± 3.9
10% pDNA- PARG -PEG NCs	135 ± 3	0.16	+19.1 ± 5.4
DCX-loaded PARG NCs	170 ± 10	0.12	+55.6 ± 6.1

As expected, the results presented in Table 2 indicate that it is possible to associate significant amounts of pDNA with loadings of 3 and 10% w/w of pDNA/nanocapsules, equivalent to 1:3 and 1:1 of pDNA/PARG

ratios respectively. In addition, the results of the particle size analysis indicated that the association of pDNA did not affect the mean size and polydispersity index of the nanocapsules (Table 2). On the other hand, as expected, we observed a significant reduction in the zeta potential values, a result that was attributed to the shielding of the positive charge due to the interaction of PARG with the negatively charged pDNA molecules. Accordingly, the zeta potential reduction was related to the amount of pDNA associated (more remarkable for the 10% than for the 3% pDNA loading) (Table 2). Despite the surface charge reduction, pDNA-associated nanocapsules exhibited a positive charge (above +20 mV), a result that evidences the prevalence of PARG at the shell of the nanocapsules.

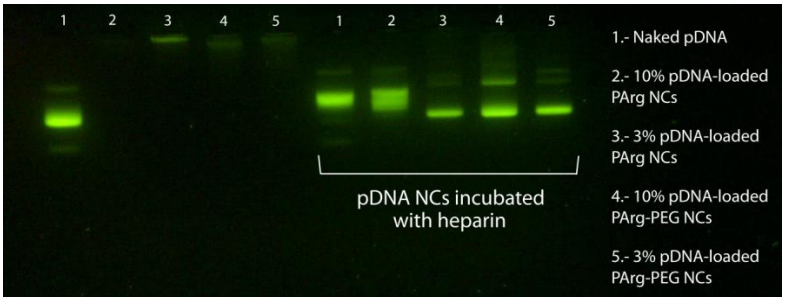


Figure 3: Gel electrophoresis of naked pDNA, pDNA-loaded PARG nanocapsules and pDNA-loaded PARG-PEG nanocapsules at 3% and 10% loading pDNA/nanocapsules, without and after treatment with heparin.

The degree of association of pDNA onto PARG and PArg-PEG nanocapsules was measured by agarose gel electrophoresis. As shown in Figure 3, no migration of free pDNA was observed for the theoretical loadings of 3% and 10% w/w of pDNA/nanocapsules, thus illustrating the important association of pDNA onto the nanocapsules surface. Then, in order to verify the reversibility of the pDNA association to the nanocapsules we evaluated the displacement of pDNA molecules by

challenging with competitive anions such as heparin. For this, we incubated the 3% and 10% pDNA-associated PARG and PArg-PEG nanocapsules with a far excess of heparin. The appearance of the bands after heparin treatment illustrated the detachment of pDNA molecules from the PARG shell of the nanocapsules. Therefore, based on the previous results we selected the 3% loading formulations for the rest of the studies.

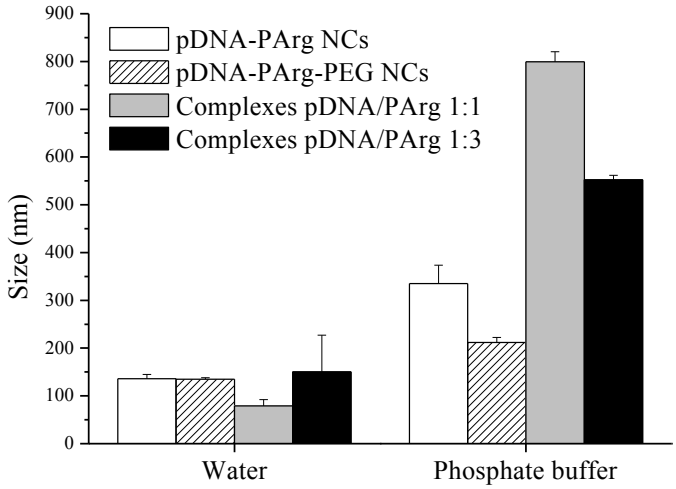


Figure 4: Size values of pDNA-loaded PARG nanocapsules, pDNA-loaded PARG-PEG nanocapsules and pDNA/ PARG complexes in water and phosphate buffer. (Mean ± s.d.; n=3).

In order to assess the possible advantages of the nanocapsules in comparison to the polymer-polynucleotide complexes, we have compared the physicochemical and stability properties of both types of nanocarriers.

Table 3: Physicochemical properties of plasmid DNA-associated polyarginine nanocapsules (pDNA- PARG NCs) and (pDNA-PARG) complexes. PDI: polydispersity index. (Mean ± s.d.; n = 3).

Formulation	Size (nm)	PI	Zeta Potential (mV)
3% pDNA-PARG NCs	129 ± 4	0.22	+46.8 ± 1.9
1:1 pDNA-PARG complexes	79 ± 13	0.28	+19.6 ± 3.2
10% pDNA-PARG NCs	136 ± 9	0.16	+31.3 ± 6.1
1:3 pDNA-PARG complexes	150 ± 77	0.34	+14.3 ± 1.7

The results presented in Table 3, indicate that the size of complexes and nanocapsules are in similar ranges. However, the reproducibility of the average particle size values is more important and the particle size distribution narrower for the nanocapsules than for the complexes. It can also be noted that the reduction in the zeta potential values upon association of DNA molecules is more important for the complexes than for the nanocapsules. This could be attributed to the different polymer-polynucleotide nanostructural organization. In the case of the nanocapsules, the interaction polymer-polynucleotide takes place on their surface, whereas in the case of the complexes, the polymer has an extended conformation in solution and adopts a particulated conformation upon complexation with DNA. On the other hand, we observed and additional drawback of pDNA/PARG complexes which was related to their poor stability in simulated biological fluids. In fact, the complexes suffered an immediate aggregation in the presence of phosphate buffer (Figure 4) leading to a 3 to 5-fold increase in their size whereas pDNA-loaded.

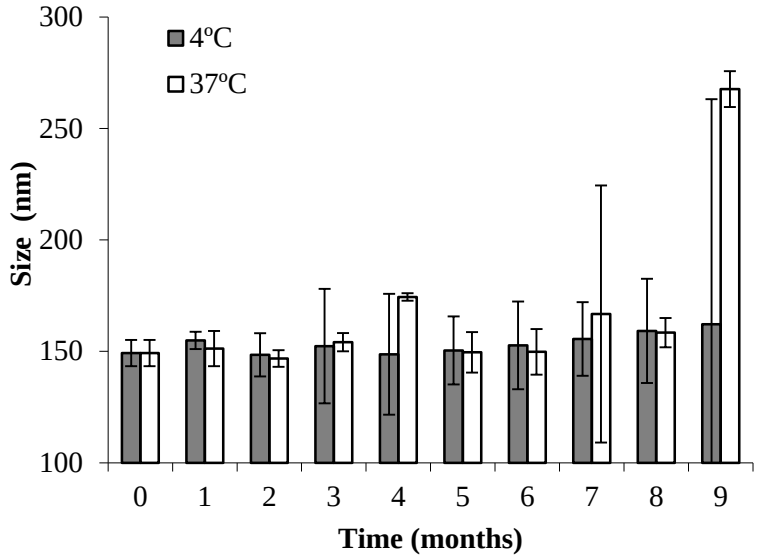


Figure 5: Size evolution of PARG nanocapsules after storage of the suspension at 4 and 37ºC.

PArg nanocapsules exhibited a 2-fold size increase and pDNA-loaded PARG-PEG nanocapsules remained totally stable.

Long term stability

The long term stability of PARG nanocapsules was monitored following storage at 4 and 37ºC and also upon freeze-drying. The high positive charge of PARG nanocapsules could be responsible of the prolonged colloidal stability in suspension observed for up to 9 months at 4ºC. Interestingly, a remarkable stability was also observed upon storage at 37ºC for up to 7 months (Figure 5).

On the other hand, the information obtained from the freeze drying study indicated that the concentrations of cryoprotectant and nanocapsules have a crucial effect on the recovery of the initial properties of the system. Overall the conclusion was that using 10% w/v trehalose as a

cryoprotectant it was possible to adequately reconstitute the freeze-dried nanocapsules irrespective of their concentration. The most satisfactory results were however obtained for nanocapsules concentrations of 0.25 and 0.5% (Figure 6).

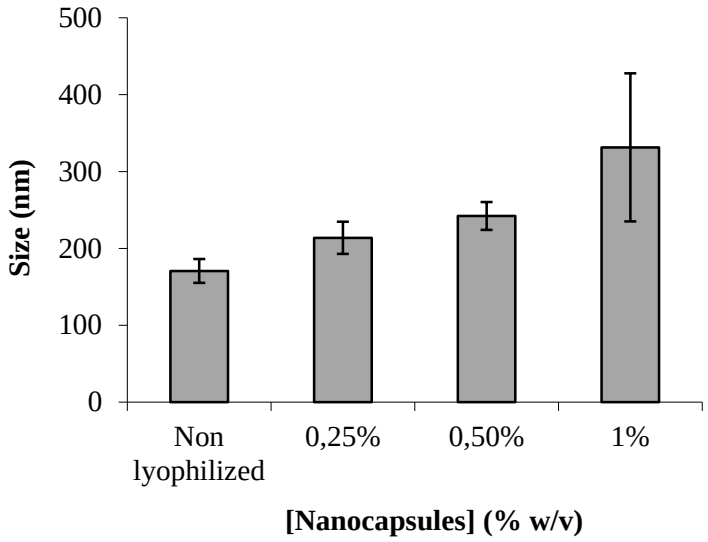


Figure 6: Size values of PARG nanocapsules before and after freeze-drying with 10% trehalose and further reconstitution with water (Mean ± s.d.; n=3).

Encapsulation of docetaxel into the nanocapsules

An advantage of nanocapsules relies on their versatility for the association of different molecules [35]. In order to validate their potential for the encapsulation of hydrophobic drugs we chose the cytotoxic drug docetaxel as a model compound. As expected, the results showed that docetaxel could be efficiently encapsulated within the core of PARG nanocapsules (encapsulation efficiency of 74%) without changing the original size and zeta-potential values of the nanocapsules (Table 2).

In a second step we evaluated the release pattern of the encapsulated docetaxel upon incubation of highly diluted nanocapsules in simulated biological media (sink conditions). The results presented in Figure 7 indicate that docetaxel is released from PARG nanocapsules according to a biphasic profile, characterized by a rapid initial release (40% of the encapsulated drug) followed by a second phase in which no further drug release was observed. The initial release phase, typically observed in these reservoir systems [7, 36], is related to the important dilution of the nanocapsules in the incubation medium and the subsequent partition of the drug between the oil core and the external aqueous phase. Once this partition occurred, no further release was observed. The release of docetaxel from a control nanoemulsion followed a very similar profile, thus indicating that the polymer shell does not modify the release pattern of the drug.

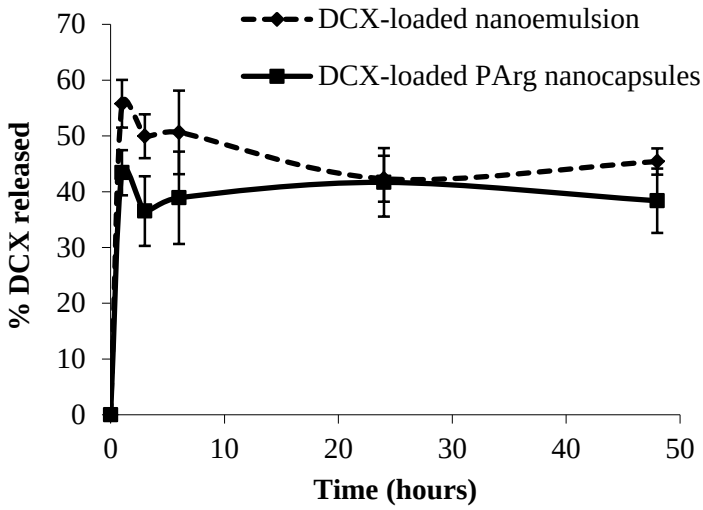


Figure 7: *in vitro* docetaxel release from docetaxel-loaded PARGnanocapsules (solid line) vs docetaxel-loaded nanoemulsion (dashed line). (Mean ± s.d.; n=3).

The efficacy of PARG nanocapsules for the intracellular delivery of docetaxel

In order to assess the efficacy of PARG nanocapsules for the intracellular delivery of docetaxel, we evaluated the cytotoxicity of docetaxel-loaded nanocapsules in the non-small cell lung cancer NCI-H460 cell line. Figure 8 shows the cell viability profile observed after a-48 hour exposure time to docetaxel-loaded PARG nanocapsules, in comparison with those corresponding to a docetaxel solution or to the unloaded-PArg nanocapsules.

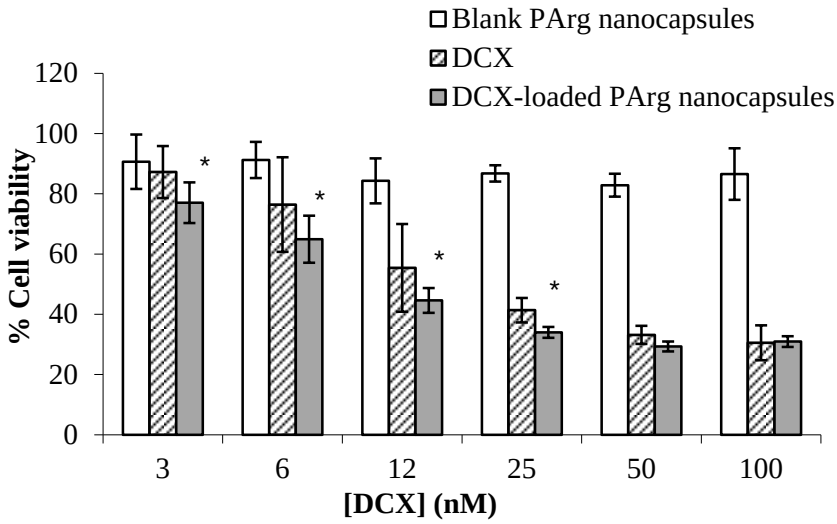


Figure 8: Cell viability profiles of blank PARG nanocapsules, docetaxel and docetaxel-loaded PArg nanocapsules on NCI-H460 cell line. (Mean ± s.d.; n=3). * Shows statistical differences between DCX-loaded PARG nanocapsules and the two control groups.

The results indicated that the encapsulated drug decreased the cell viability in a concentration dependent manner, reaching a 70% reduction at the highest concentrations tested (100 nM). This reduction was significantly higher for the encapsulated drug compared to the free

docetaxel. In fact, the IC50 value for docetaxel-loaded PARG nanocapsules was close to four times lower (3.11 nM) than that of the docetaxel solution (11.8 nM). We hypothesize that the enhanced antiproliferative effect of docetaxel could be related to the faster uptake of the nanocarriers by the cells and the more efficient intracellular delivery of the drug. Similar studies performed with another human non-small cell lung cancer cell line, A549, showed comparable IC50 values for docetaxel (12.8 nM) and docetaxel-loaded chitosan nanocapsules (4.5 nM) [7]. Another interesting finding of this study was the lack of toxicity of blank PARG nanocapsules under the range of concentrations investigated. This is in agreement with previous studies that provided evidence of the low cell toxicity of PARG-based nanosystems such as PARG-polyleucine polymeric vesicles and PARG-heparin complexes [37].

Internalization of PARG nanocapsules in cancer cells

In order to test the hypothesis of the enhanced intracellular delivery, we studied the internalization of fluorescently labelled nanocarriers (172 nm, +42 mV) into the NCI-H460 cells by flow cytometry. For the correct evaluation of the contribution of the PARG coating to the uptake of the nanocarriers, we used the uncoated nanoemulsion and a dispersion of the fluorescent probe fluorescein-DHPE as controls. As shown in Figure 9 the uptake of PARG nanocapsules exhibited a dose-dependent fluorescent pattern both in percentage of fluorescent cells and in the mean fluorescent signal of the cell population. First, the percentage of fluorescent cells went from 60% up to 90% when the nanocapsule concentration was increased ten times (Figure 9A). Second, an increase of 3-fold in the mean intensity clearly confirmed the presence of a stronger positive cell population after incubation with PARG nanocapsules (Figure 9B). The situation was

drastically different upon incubation of the cells with the control formulations showing, at both doses and by both measured parameters, a clearly reduced fluorescent signal. Thus, these results clearly show that PARG has a critical effect on the delivery of the fluorescent label to the cells. By flow cytometry we cannot discriminate the adhesion vs. the internalization of the nanocapsules, however this information together with the increased efficacy of docetaxel-loaded nanocapsules allow us to speculate on the favorable uptake of the nanocapsules as compared to the emulsion.

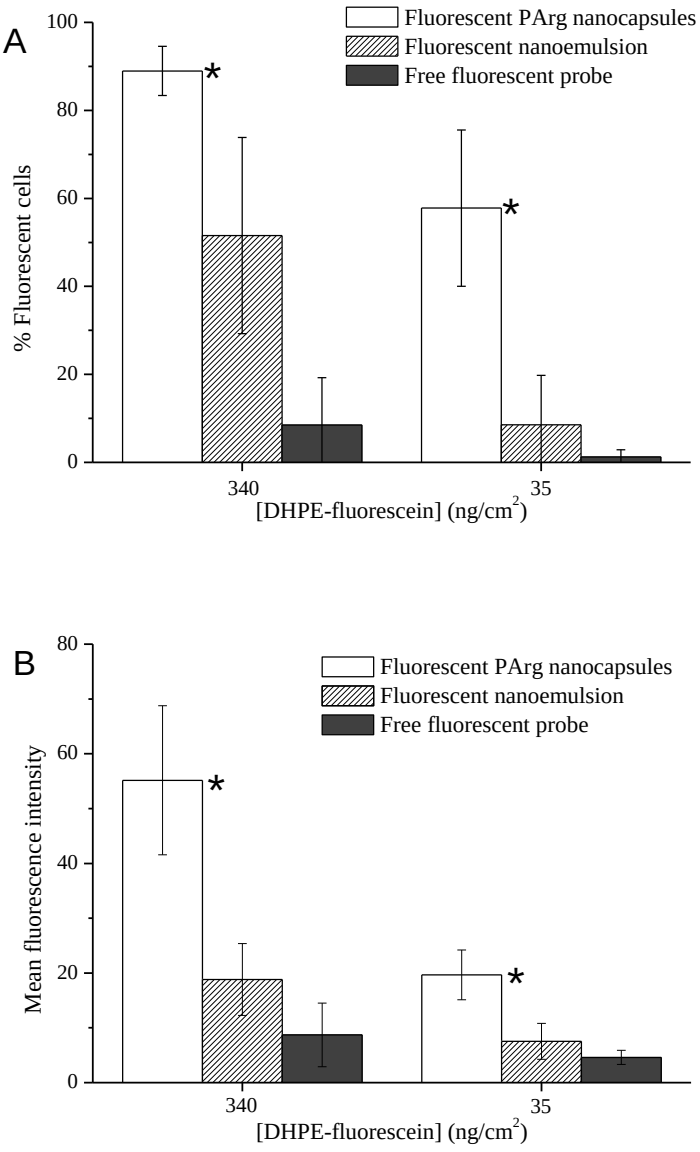


Figure 9: Percentage of fluorescent cells (Figure 9A) and mean fluorescence intensity of the cell population (Figure 9B) measured in NCI-H460 cells upon incubation with fluorescent PARG nanocapsules (white bars), fluorescent nanoemulsion (dashed bars) or free fluorescent probe (grey bars). (Mean ± s.d.; n=3). * Shows statistical differences ($p<0.05$) between fluorescent PArg nanocapsules and the two control groups

The effect of PARG on the intracellular accumulation has already been shown for different types of nanostructures. For example, it has been reported that PAG-modified liposomes as well as PArg-polyleucine vesicles showed more efficient cell uptake compared to the control systems, which did not incorporate PARG [37, 38]. Despite this evidence, the precise mechanism of cellular entry of cell penetrating peptides (CPPs), and in particular, that of PARG, is a matter of controversy. Several authors have claimed that the internalization process is based on a transduction mechanism rather than on endocytic receptor- energy- or temperature-dependent mechanisms [39]. However, nowadays it is accepted that alternative mechanisms, which remain to be elucidated (clathrin mediated, caveolin mediated, clathrin and caveolin independent endocytosis or macropinocytosis), might be involved in the internalization process[40, 41]. Moreover, it is important to take into account that the internalization pathway could probably be influenced by parameters such as the PARG length or the PARG surface density [42].

Conclusions

In this work we report a new drug delivery platform consisting of a hydrophobic oil core surrounded by a shell made of PARG or PARG and PEG. From a pharmaceutical standpoint these nanocapsules display a number of advantages including (i) their versatility for the association of lipophilic drugs as well as hydrosoluble macromolecules, i.e. pDNA, (ii) their stability upon dilution of buffers and during storage for prolonged periods of time (iii) their capacity to overcome cellular barriers and deliver the associated drugs inside the cells and, iv) their potential to increase the pharmacological effect of drugs with intracellular targets i.e. docetaxel. In conclusion, this platform may represent a new strategy for

intracellular drug delivery. Current *in vivo* experiments will allow us to fully assess their potential.

Acknowledgements

This work has been supported by the Ministry of Sciences and Innovation

(MICINN) Consolider Program SD2006-00012, Euronanomed ERA-NET Program, Lymphotarg PI09/2670 and the Xunta de Galicia, Competitive Reference Groups 2010/18 (FEDER Funds). Victoria Lozano and Giovanna Lollo acknowledge the fellowship received from the Spanish Government.

References

1. Farokhzad, O.C. and R. Langer, Impact of nanotechnology on drug delivery. ACS Nano, 2009. 3(1): p. 16-20.

2. Byrne, J.D., T. Betancourt, and L. Brannon-Peppas, Active targeting schemes for nanoparticle systems in cancer therapeutics. Adv. Drug Delivery Rev., 2008. 60(15): p. 1615-1626.

3. Sakamoto, J.H., et al., Enabling individualized therapy through nanotechnology. Pharmacol. Res., 2010. 62(2): p. 57-89.

4. Hervella, P., et al., Nanomedicine: New Challenges and Opportunities in Cancer Therapy. J. Biomed. Nanotechnol., 2008. 4: p. 276-292.

5. de la Fuente, M., et al., Nanoparticles as protein and gene carriers to mucosal surfaces. Nanomedicine, 2008. 3(6): p. 845-857.

6. Janes, K.A., et al., Chitosan nanoparticles as delivery systems for doxorubicin. J. Controlled Release, 2001. 73(2-3): p. 255-267.

7. 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.

8. de la Fuente, M., B. Seijo, and M.J. Alonso, Bioadhesive hyaluronan-chitosan nanoparticles can transport genes across the ocular mucosa and transfect ocular tissue. Gene Ther., 2008. 15(9): p. 668-676.

9. Oyarzun-Ampuero, F.A., et al., A new drug nanocarrier consisting of polyarginine and hyaluronic acid. Eur. J. Pharm. Biopharm., 2011. 79: p. 54-57.

10. Bae, Y. and K. Kataoka, Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers. Adv. Drug Delivery Rev., 2009. 61(10): p. 768-784.

11. González-Aramundiz, J.V., et al., Polypeptides and polyaminoacids in drug delivery. Expert Opin. Drug Delivery, 2012. 9(2): p. 183-201.

12. Romberg, B., et al., Poly(amino acid)s: promising enzymatically degradable stealth coatings for liposomes. Int. J. Pharm., 2007. 331(2): p. 186-189.

13. Kim, H.K., et al., Enhanced siRNA delivery using cationic liposomes with new polyarginine-conjugated PEG-lipid. Int. J. Pharm., 2010. 392(1-2): p. 141-147.

14. Lundberg, M., S. Wikström, and M. Johansson, Cell Surface Adherence and Endocytosis of Protein Transduction Domains. Mol. Ther., 2003. 8(1): p. 143-150.

15. Patel, L.N., J.L. Zaro, and W.C. Shen, Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. Pharm. Res., 2007. 24(11): p. 1977-1992.

16. Kirschberg, T.A., et al., Arginine-Based Molecular Transporters: The Synthesis and Chemical Evaluation of Releasable Taxol-Transporter Conjugates. Org. Lett., 2003. 5(19): p. 3459-3462.

17. Miklán, Z., et al., New daunomycin-oligoarginine conjugates: synthesis, characterization, and effect on human leukemia and human hepatoma cells. Biopolymers, 2009. 92(6): p. 489-501.

18. Lingnau, K., K. Riedl, and A. Von Gabain, IC31® and IC30, novel types of vaccine adjuvant based on peptide delivery systems. Expert Rev. Vaccines, 2007. 6(5): p. 741-746.

19. Torchilin, V.P., Tat peptide-mediated intracellular delivery of pharmaceutical nanocarriers. Adv. Drug Delivery Rev., 2008. 60(4-5): p. 548-558.

20. Kim, W.J., et al., Cholesteryl Oligoarginine Delivering Vascular Endothelial Growth Factor siRNA Effectively Inhibits Tumor Growth in Colon Adenocarcinoma. Mol. Ther., 2006. 14(3): p. 343-350.

21. Fujita, T., et al., High gene delivery in tumor by intratumoral injection of tetraarginine-PEG lipid-coated protamine/DNA. J. Controlled Release, 2008. 129(2): p. 124-127.

22. Yang, L., et al., Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide. Cancer Res., 2003. 63(4): p. 831-837.

23. Chen, L., et al., Molecular transporters for peptides: Delivery of a cardioprotective εPKC agonist peptide into cells and intact ischemic heart using a transport system, R7. Chem. Biol., 2001. 8(12): p. 1123-1129.

24. Klade, C.S., et al., Therapeutic Vaccination of Chronic Hepatitis C Nonresponder Patients With the Peptide Vaccine IC41. Gastroenterology, 2008. 134(5): p. 1385-1395.

25. Nemoto, E., et al., Ability of poly-L-arginine to enhance drug absorption into aqueous humor and vitreous body after instillation in rabbits. Biol. Pharm. Bull., 2007. 30(9): p. 1768-1772.

26. Zaki, N.M., et al., Rapid-onset intranasal delivery of metoclopramide hydrochloride. Part II: Safety of various absorption enhancers and pharmacokinetic evaluation. Int. J. Pharm., 2006. 327(1-2): p. 97-103.

27. Calvo, P., J.L. Vila-Jato, and M.J. Alonso, Evaluation of cationic polymer-coated nanocapsules as ocular drug carriers. International Journal of Pharmaceutics, 1997. 153(1): p. 41-50.

28. Rudolph, C., et al., Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells. J. Biol. Chem., 2003. 278(13): p. 11411-11418.

29. Lee, S.H., S.D. Yoo, and K.H. Lee, Rapid and sensitive determination of paclitaxel in mouse plasma by high-performance

liquid chromatography. J. Chromatogr., B: Biomed. Sci. Appl., 1999. 724(2): p. 357-363.

30. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, 1983. 65(1-2): p. 55-63.

31. Wang, M. and M. Thanou, Targeting nanoparticles to cancer. Pharmacological Research, 2010. 62(2): p. 90-99.

32. Prego, C., D. Torres, and M.J. Alonso, Chitosan nanocapsules: a new carrier for nasal peptide delivery. J. Drug Delivery Sci. Technol., 2006. 16(5): p. 331-337.

33. Garcia-Fuentes, M., D. Torres, and M.J. Alonso, Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules. Colloids Surf., B 2003. 27(3/2): p. 159-168.

34. Theodossiou, T.A., et al., Guanidinylated dendritic molecular transporters: Prospective drug delivery systems and application in cell transfection. ChemMedChem., 2008. 3(11): p. 1635-1643.

35. Hervella, P., et al., Nanocapsules as Carriers for the Transport and Targeted Delivery of Bioactive Molecules, in Nanocomposite particles for bio-aplications: Materials and bio-interfaces, T. Trindade and A.L. Daniel, Editors. 2011, Pan Stanford: Singapur. p. 45-67.

36. Calvo, P., J.L. Vila-Jato, and M.J. Alonso, Evaluation of cationic polymer-coated nanocapsules as ocular drug carriers. Int. J. Pharm., 1997. 153(1): p. 41-50.

37. Holowka, E.P., et al., Polyarginine segments in block copolypeptides drive both vesicular assembly and intracellular delivery. Nat. Mater., 2007. 6(1): p. 52-57.

38. Zhang, C., et al., siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. J. Controlled Release, 2006. 112(2): p. 229-239.

39. Nagahara, H., et al., Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27(Kip1) induces cell migration. Nat. Med, 1998. 4(12): p. 1449-1452.

40. Richard, J.P., et al., Cell-penetrating peptides: A reevaluation of the mechanism of cellular uptake. J. Biol. Chem., 2003. 278(1): p. 585-590.

41. Kaplan, I.M., J.S. Wadia, and S.F. Dowdy, Cationic TAT peptide transduction domain enters cells by macropinocytosis. J. Controlled Release, 2005. 102(1): p. 247-253.

42. Furuhata, M., et al., Decaarginine-PEG-liposome enhanced transfection efficiency and function of arginine length and PEG. Int. J. Pharm., 2009. 371: p. 40-46.

Nanocapsules as carries for the transport and targeted delicery os bioactive molecules

P. Hervella, G. Lollo, F. Oyarz´un-Ampuero, G. Rivera,
D. Torres and M. J. Alonso

Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Santiago de Compostela (USC) 15782 Santiago de Compostela, Spain

E-mail: mariaj.alonso@usc.es

Introduccion

Nanocapsules, first developed by Couvreur et al. (Couvreur, Tulkens et al. 1977), offer unique opportunities with the poupose of improving the biological profile of drugs in terms of transport across biological barriers, biodistribution and cellular uptake. They have a vesicular organization whose internal reservoir can be composed of aqueous or oily components, and they are surrounded by a polymeric coating (Legrand, Barratt et al. 1999; Couvreur, Barratt et al. 2002). This reservoir system offers the possibility of great loadings of either lipophilic or hydrophilic drugs, depending on the nature of the liquid core (Figure 1). Additionally, the core has the role of protecting the drug from the physiological environment. Finally, the liquid nature of nanocapsules and, thus, their elasticity, may facilitate the contact of the nanostructures with the ephithelia and further internalization.

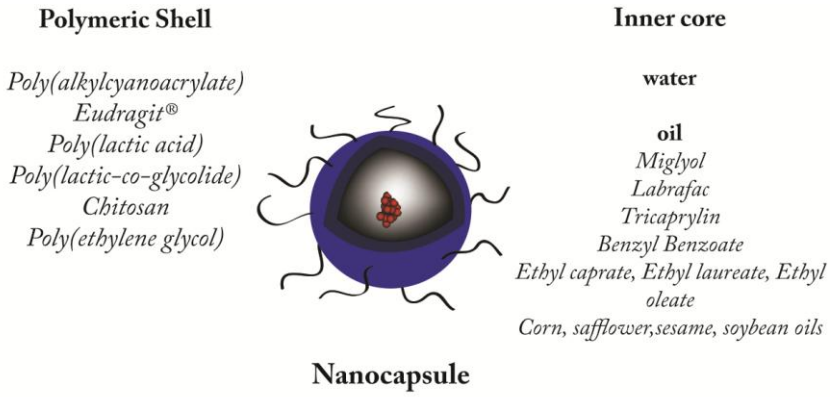


Figure 1: Schematic diagram of nanocapsules containing an aqueous or oily core.

Polymeric Nanocapsules: Production and Characterization

Several methods have been developed to date for the production of nanocapsules and the encapsulation of drugs. These methods are based on different physicochemical principles including (i) interfacial polymerization(Couvreur, Tulkens et al. 1977; Couvreur, Kante et al. 1979), (ii) interfacial deposition or solvent displacement (Fessi, Puisieux et al. 1989), (iii) phase inversion temperature(Heurtault, Saulnier et al. 2002) and (iv) polymer adsorption onto a preformed emulsion (Prego, Torres et al. 2006; Prego, Torres et al. 2006; Lozano, Torrecilla et al. 2008)The choice of the most appropriate materials and methods for the preparation of the nanocapsules is critical. The specific details of the different approaches and formulations will be described in detail in the next section.

A deep characterization of the nanocapsules is essential as there are specific parameters, such as size, morphology and stability, which can significantly affect their biopharmaceutical behavior (Desai, Labhasetwar et al. 1996; Vila, Gill et al. 2004; Yin Win and Feng 2005).

Most of the techniques employed to characterize the morphology of nanocapsules are based on microscopy, such as scanning electron microscopy (SEM), atomic force microscopy (AFM) or transmission electron microscopy (TEM). These techniques have been widely used to elucidate not only the shape but also the size and wall thickness of nanocapsules structure. These techniques can also be combined with other methods like freeze-fracture, cryogenic techniques or negatively stained preparations in order to obtain deep information on the structural organization of the different components of the nanocapsules (Quintanar-Guerrero, Alle?mann et al. 1998; Couvreur, Barratt et al. 2002; Heurtault, Saulnier et al. 2002).

Regarding the size of the nanocapsules, several techniques can give accurate values of diameter and wall thickness. Scattering methods are the most recommended for obtaining accurate values of particle size distributions. The dynamic light scattering technique, also named as photon correlation spectroscopy, is a measurement of the dynamics of the Brownian motion of particles, this being related to their hydrodynamic diameter. This is a suitable method for particles with diameters between a few nanometers and a few microns(Moinard-Checot, Chevalier et al. 2008). Recently, small angle neutron scattering has proved to be a very powerful tool for calculating the size and the wall thickness of the nanocapsules.(Rube, Hause et al. 2005).

The characterization of the surface properties of the nanocapsules can be made through the measurement of zeta potential by laser doppler anemometry(Calvo, Vila-Jato et al. 1997). Nuclear Magnetic Resonance (NMR) can also be employed for a deeper characterization of the nanocapsule surface: the hydratation and the physical state of the shell forming polymer can be determined by cross polarization NMR (Guinebretiere, Briancon et al. 2002), while Pulsed Field Gradient NMR can be used to study the permeability, hydratation and the mobility of the nanocapsule shell(Wohlgemuth and Mayer 2003).

A brief description of nanocapsule drug delivery systems developed to date is presented in Table 1.

Table 1: Polymers used as wall materials in nanocapsules for the delivery of different therapeutics, using varied administration routes.

Polymer	Drug	Drug effect	Route	ref
PACA NC	Nucleic Acids	Antitumor		19,28,90
(water core)	Salmon calcitonin	Hypocalcemic	Oral	29
PACA NC	Insulin	Hypoglucemic	Oral	52-55
	Phtalocyanines	Imaging	i.t.	80
	siRNA, ODNs	Antitumor	i.t.	(Bouclier, Moine et al. 2008), 19
	Cyclosporin	Immunosuppresor	Ocular	68,66
	Pilocarpine	Antiglaucomatous		
Eudragit	Tacrolimus	Immunosuppresor	Oral	31,56,59
	Cyclosporine	Immunosuppresor		
PLA	Indometacin, Diclofenac	Antiinflamatory	Oral	57
PCL	Spironolactone	Diuretic	Oral	58
	Betaxolol, Carteolol, Metipranolol	Antiglaucomatous	Ocular	63-65
	Indomethacin	Antiinflamatory	Ocular	70,71
	Cyclosporine	Immunosuppresor	Ocular	67,70
Chitosan	Calcitonin	Hypocalcemic	Oral	38

	Calcitonin	Hypocalcemic	Nasal	[7]
	Docetaxel	Antitumor		[9]
LNC	Paclitaxel	Antitumor	Oral	[60]
	Paclitaxel	Antitumor	i.v.	[79]
	Docetaxel	Antitumor	i.v.	[45]

Nanocapsules made of synthetic polymers

Polyacrylate nanocapsules

The first generation of nanocapsules was developed by the group of Couvreur in the late 1970s employing poly(alkylcyanoacrylates) (PACA) as wall material(Couvreur, Tulkens et al. 1977; Couvreur, Kante et al. 1979). Since then, PACA nanocapsules have been widely used in drug delivery.

PACA nanocapsules can be prepared following two main methods: interfacial polymerization or interfacial deposition (Hillaireau, Le Doan et al. 2007; Vauthier and Bouchemal 2008) (Figure 2 and 3). In the first case, nanocapsules are formed due to the fast polymerization of the alkylcyanoacrylate monomers at the interface of o/w or w/o emulsions leading to the production of oil or water containing nanocapsules, respectively. Aprotic solvents and a suitable oil/solvent ratio are necessary to achieve an adequate yield of nanocapsules (Gallardo, Couarraze et al. 1993; Puglisi, Fresta et al. 1995). Oil-containing nanocapsules prepared by this method allow the efficient encapsulation of lipophilic drugs because of their solubility in the oily phase (Fresta, Cavallaro et al. 1996).

Water soluble molecules, i.e. insulin or calcitonin, can also be entrapped in the form of a suspension in the oily phase (Lowe and Temple 1994; Aboubakar, Puisieux et al. 1999) due to the instantaneous formation of the shell around the oily droplets. The mean diameter of the nanocapsules formed by interfacial polymerization is normally in the range 200-350 nm. However, recently, the possibility of reducing the size of the nanocapsules down to 100 nm was reported, thanks to the use of the appropriate combination of surfactants (Vauthier, Labarre et al. 2007).

Nanocapsules consisting of an aqueous core are of special interest for the encapsulation of water-soluble molecules such as peptides (Watnasirichaikul, Davies et al. 2000) and nucleic acids, including antisense oligonucleotides (Lambert, Fattal et al. 2000). In these cases, the nanocapsules are formed in an external oily phase and need to be isolated and resuspended in water prior to their use. Nanocapsules with aqueous core have mean diameters ranging from 50 to 350 nm depending on the type of surfactants used for their preparation(Vranckx, Demoustier et al. 1996; Hillaireau, Le Doan et al. 2007).

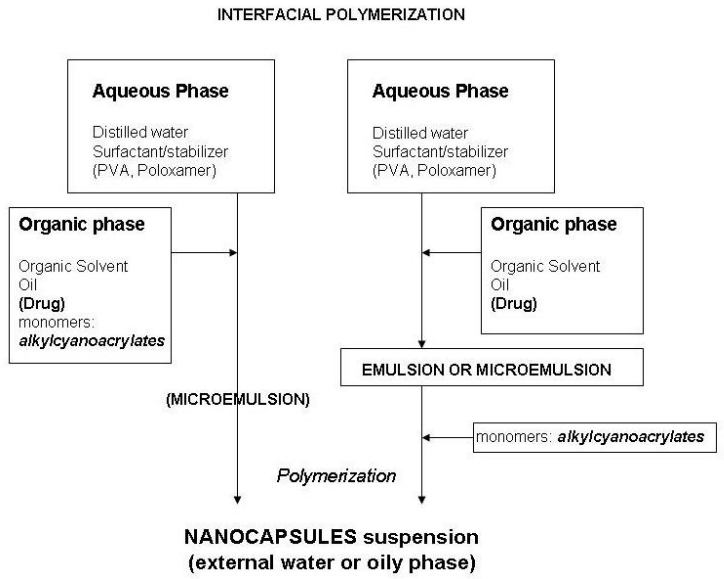


Figure 2: Preparation of nanocapsules by interfacial polymerization

PACA nanocapsules can also be obtained by interfacial deposition of a preformed polymer (Figure .3). This technique was first described by Fessi et al.(Fessi, Puisieux et al. 1989) and is based on the spontaneous emulsification of the oil due to the diffusion of a organic solvent, where the polymer and oil are dissolved, into water. The nanocapsules are formed due to the precipitation of the preformed polymer at the interface of the emulsion(Al Khouri Fallouh, Roblot-Treupel et al. 1986; Fessi, Puisieux et al. 1989).The size of the nanocapsules prepared by this method usually ranges from 150 to 300 nm.

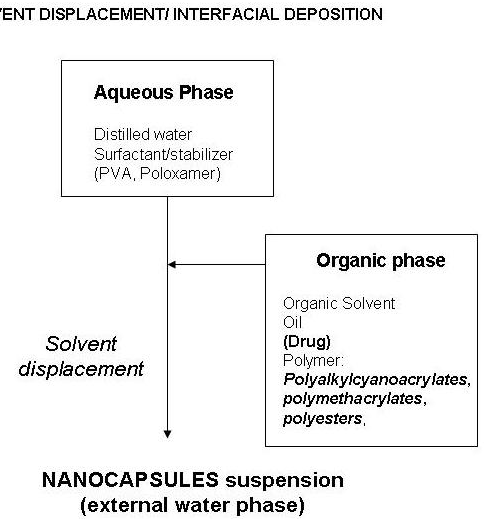


Figure 3: Preparation of nanocapsules by interfacial polymer deposition following solvent displacement

Other interesting polyacrylates, also used to prepare nanocapsules, are polymethacrylates (Eudragit®). These nanocapsules have been obtained by interfacial deposition of the preformed Eudragit®. The interest of these systems relies in their pH sensitive character that can be employed to improve the stability and bioavailability of therapeutic drugs after oral administration (Nassar, Rom et al. 2008).

Polyester nanocapsules

Polyesters such as poly-□-caprolactone (PCL), poly lactic acid (PLA) and its copolymer poly(lactic-co-glycolic) acid (PLGA) have also been used for the preparation of nanocapsules. To date, all polyester nanocapsules have been prepared by the interfacial deposition of a preformed polymer following solvent displacement (Fessi, Puisieux et al. 1989; Quintanar-Guerrero, Alle?mann et al. 1998; Moinard-Checot,

Chevalier et al. 2008). This effective and reproducible method allows the production of polyester nanocapsules with size ranges between 100-350 nm and wall thickness of 1 to 20 nm(Guinebretiere, Briançon et al. 2002; Cauchetier, Deniau et al. 2003; Rube, Hause et al. 2005).

The surface properties of polyester nanocapsules can be modified in order to reach the therapeutical purpose. For example, chitosan, a bioadhesive polymer, can be attached to the surface of polyester nanocapsules by incubation (Calvo, Vila-Jato et al. 1997). In addition, it is possible to obtain PEG-coated polyester nanocapsules by using the amphiphilic PEGylated copolymer, i.e. PEG-PCL, PEG-PLA or PEG-PLGA(Mosqueira, Legrand et al. 2001; Mosqueira, Legrand et al. 2001; De Campos, Sa?nchez et al. 2003; Bouclier, Moine et al. 2008). The polymer deposition technique leads to the orientation of the hydrophobic segment towards the oily phase whereas the PEG portion protrudes towards the external aqueous medium.

Nanocapsules made of natural polymers

Naturally occurring polymers such as polysaccharides have also been used for the formation of nanocapsules. Among these, chitosan has received increasing attention for a number of years as a biomaterial for transmucosal drug delivery. Our group described for the first time the preparation of chitosan nanocapsules according to an interfacial deposition method slightly modified when compared to that used for PACA or polyester nanocapsules described above (Figure 4). In this case, chitosan is incorporated into the external aqueous phase and its deposition at the oil/water interphase occurs because of its electrostatic interaction with the negatively charged phosphatidylcholine, which is used as a stabilizer of the nanodroplets. (De Campos, Sa?nchez et al. 2003; Prego,

Torres et al. 2006; Prego, Torres et al. 2006) We have also proposed an alternative method which involves first, the formation of a nanoemulsion and, the incubation of the nanoemulsion in an aqueous solution of chitosan (Prego, Torres et al. 2006; Prego, Torres et al. 2006; Lozano, Torrecilla et al. 2008). This method has also been employed for the formation of PEG-chitosan nanocapsules(Prego, Torres et al. 2006). In this case, the PEG molecule gets oriented towards the external phase due to the cationic nature of chitosan and its natural tendency to associate to the negatively charged nanodroplets.

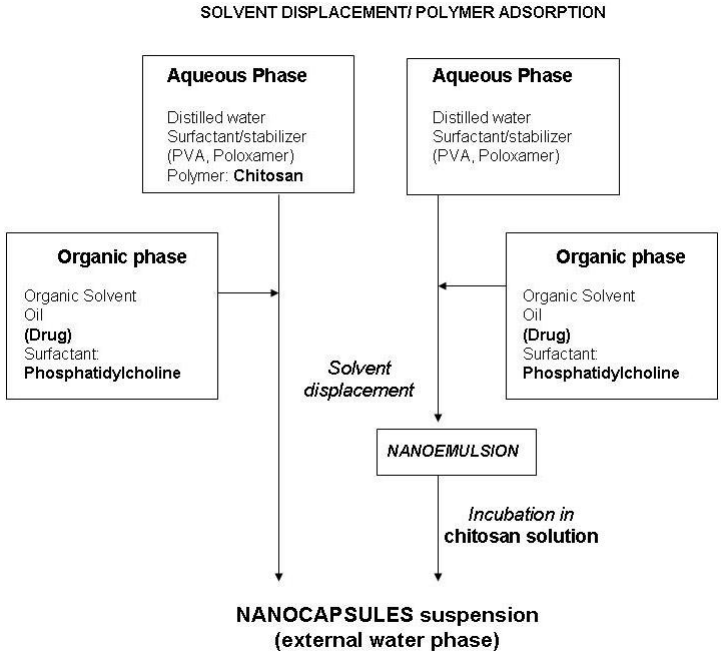


Figure 4: Preparation of nanocapsules by polymer adsorption following solvent displacement

The incubation approach has been recently proposed for the formation of nanocapsules with a double polysaccharic wall consisting of chitosan and lambda-carrageenan (Preetz, Ru?be et al. 2008). In this case, the nanoemulsion was formed by high pressure homogenization using a

modified starch as negatively charged stabilizer; then, it was incubated first in a chitosan solution and afterwards in a lambda-carrageenan solution.

Lipid nanocapsules

A new generation of nanocapsules, named lipid nanocapsules, were first prepared by the group of Benoit(Heurtault, Saulnier et al. 2002; Heurtault, Saulnier et al. 2003; Heurtault, Saulnier et al. 2003; Heurtault, Saulnier et al. 2003). These systems consists of an oil core surrounded by a thick polymeric shell, made of PEG-hydroxystearate and phosphatidylcholine. These nanocapsules can be prepared via a novedous, solvent-free, phase inversion process (Figure 5). In this process, all the components of the system are mixed together with the aqueous phase and, then, exposed to several cycles of heating and cooling (usually between temperatures around 65 and 85ºC). The size and polydispersity of the nanocapsules decrease as a function of the number and temperature cycles and a thick interfacial layer is created with this cycling process, since the surfactant is forced to overconcentrate at the interface of the oily droplets.(Anton, Gayet et al. 2007) Finally, the process is quenched at a temperature below the phase inversion temperature (o/w emulsion), followed by addition of cold water. This fast cooling-dilution process led to the formation of lipid nanocapsules with particle sizes between 20 and 100 nm(Heurtault, Saulnier et al. 2003). These nanocapsules showed a rigid shell surrounding the oily core and were physically stable for at least 18 months without fusion of the dispersed oily phase(Heurtault, Saulnier et al. 2003). These nanocapsules are very versatile as they can be produced using different types of oils and lipids, thus exhibiting high drug encapsulation efficiency values (Khalid, Simard et al. 2006; Babu Dhanikula, Mohamed Khalid et al. 2007).

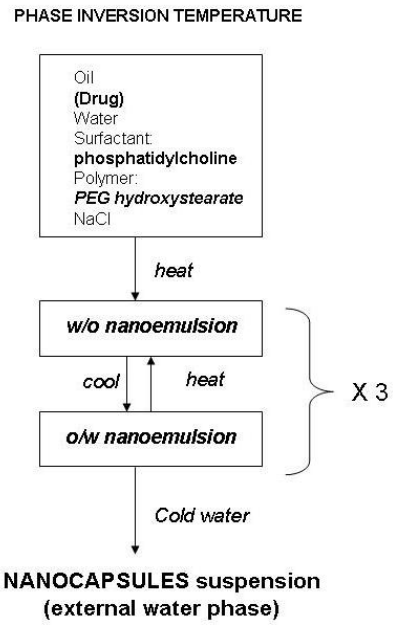


Figure 5: Preparation of nanocapsules by phase inversion temperature.

Therapeutical applications of nanocapsules

The use of nanocapsules has been reported as a promising strategy for improving the oral bioavailability of therapeutic molecules (Prego, Garci?a et al. 2005). It has been shown that due to their colloidal size, nanocapsules are able to interact favorably with the mucosal barrier and, simultaneously, protect the encapsulated drug from the harsh environment of the gastrointestinal tract (Prego, Garci?a et al. 2005; Csaba, Garcia-Fuentes et al. 2006). For these reasons, nanocapsules have been extensively studied as vehicles for improving the oral bioavailability of poorly absorbed drugs such as peptides or some lipophilic compounds, as well as for obtaining drug controlled release(Pinto Reis, Neufeld et al. 2006).

Nanocapsules for oral peptide delivery

The oral administration of peptides and proteins continue to be a challenge because of their susceptibility to the enzymatic degradation and their low permeability across the intestinal ephitelium. The encapsulation of these macromolecules into polymeric nanocapsules is nowadays considered a promising approach towards this ambitious goal(Alonso 2004; des Rieux, Fievez et al. 2006). An example is represented by the nanocapsules made of mucoadhesive polymers, such as chitosan, as described for transmucosal absorption of calcitonin (Prego, Fabre et al. 2006). Chitosan nanocapsules loaded with calcitonin were able to enhance and prolong the systemic absorption of the drug, thus leading to an improvement of the hypocalcemic effect (Figure 6). The *in vitro* studies performed in the Caco-2 cells cocultured with the a model of mucus-secreting cells (HT29-M6) suggested that chitosan nanocapsules do not cross the monolayer, but rather they remain at the apical side of the cells (Prego, Fabre et al. 2006).

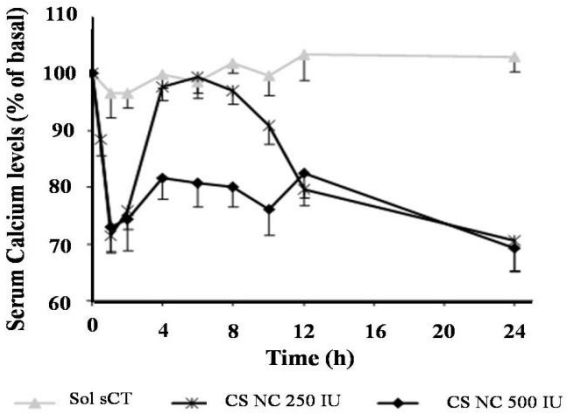


Figure 6: Serum calcium levels in rats after oral administration of salmon calcitonin in an aqueous solution (sCT Sol) or encapsulated in chitosan nanocapsules (CS NC) at two different doses (250 and 500 IU/Kg), (mean ± SE; n = 6).51 Reproduced by permission of Springer.

Promising results have also been obtained with poly (isobutylcyanoacrylate) (PBCA) nanocapsules containing calcitonin. The results of the *in vivo* studies performed on rats indicated that these nanocapsules allowed a great decrease of calcium levels to occur(Vranckx, Demoustier et al. 1996).

On the other hand, Damgé et al. investigated the potential of PACA nanocapsules for the oral administration of insulin(Damge, Michel et al. 1988; Damge, Vranckx et al. 1997). Following intragastric administration of insulin loaded nanocapsules (12.5, 25 and 50 IU of insulin per kg) to diabetic rats (diabetes induced by the administration of 65 mg/kg of spreptozocin), the authors observed that the nanocapsules remained intact in simulated gastric fluid, thus ensuring a good protection of the peptide. Moreover, the new formulation produced a significant reduction of the glycemia (50-60%), a response that was maintained for up to 20 days. The authors attributed this long-term effect to the adsorption of the nanocapsules across the intestinal epithelium and the subsequent release of the encapsulated peptide(Pinto-Alphandary, Aboubakar et al. 2003).

More recently, other authors studied the bioavailability of orally administered insulin loaded PBCA nanocapsules (50 IU of insulin per kg) in diabetic rats (diabetes induced by the administration of 65 mg/kg of spreptozocin (Cournarie, Auchere et al. 2002). These results showed that the oral administration of nanocapsules allows the delivery of noticeable levels of insulin into the bloodstream in diabetic rats, however decrease in glycemia could not be observed. The low reproducibility of the results in animal models hampered the comprehensive analysis of the results from the different studies.

Nanocapsules were also investigated for the delivery of hydrophobic peptides such as cyclosporine. The oral absorption of this peptide was

tested using nanocapsules made of an oily core consisting of Cremophor® or Maisine® and surrounded by Eudragit RL® or RS®. Unfortunately, the absolute bioavailability achieved with the nanocapsules ranged from 4 to 7.5 %, a result that is far below that observed with the marketed Neoral® premicroemulsion (about 22%) (Ubrich, Schmidt et al. 2005). The authors related the lower cyclosporine bioavailability to the size of the nanocapsules, more than to the constituents of the systems, however, the low bioadhesion of the polymers used to the intestinal epithelium could also play an important role in the absorption of the drug.

Nanocapsules for oral delivery of lipophilic low molecular weight drugs

Nanocapsules have also been used for oral delivery of low molecular weight compounds. Anti-inflammatory agents are known to exhibit important gastrointestinal side effects such as irritation and mucosal damage. Moreover, they are characterized by very low water solubility, a property which makes these good candidates for the encapsulation within oily core nanocapsules (Staniscuaski Guterres, Fessi et al. 1995). Nanocapsules made of PLA were investigated for their potential of improving the gastrointestinal tolerance to indometacin and diclofenac(Staniscuaski Guterres, Fessi et al. 1995). The encapsulation of these drugs into PLA nanocapsules led to a great reduction of the irritation of the gastrointestinal mucosa.

The diuretic drug, spironolactone, used in premature infants to reduce lung congestion, has also been efficiently encapsulated in PCL nanocapsules(Limayem Blouza, Charcosset et al. 2006). Nowadays there is no commercially available oral liquid preparation of spironolactone due to its poor water solubility and its dissolution rate. Its incorporation into

nanocapsules solved these problems, although further pharmacokinetics studies are needed in order to fully demonstrate their *in vivo* effectiveness.

The use of nanocapsules has also been proposed for the oral administration of drugs which suffer the efflux transport, mediated by P-glycoprotein (P-gp), across the apical membrane of the intestinal ephitelium. This transport is known to drastically reduce the absorption of antibiotics, antivirals, antitumorals and other drugs. Recently, it was shown that the encapsulation of tacrolimus, an immunosuppresor agent substrate of P-gp, into Eudragit® nanocapsules, protect the drug from the efflux transports and increase the concentration of the drug within the cell and therefore its bioavailability(Nassar, Rom et al. 2009). This evidence was observed in two animal models, rats and minipigs. In addition, in these studies it was also observed that the small lipophilic oil cores were able to enter the enterocytes and reach the lamina propria behind the P-gp. Similar results were obtained with the encapsulation of the antitumor drug, paclitaxel, into lipid nanocapsules. Due to the effect of P-gp and its low water solubility, paclitaxel is currently admistered intravenously. Following *in vivo* administration of lipidic nanocapsules containing placlitaxel to rats, an increase in its absorption when compared to that of the control was observed (Taxol®, paclitaxel dissolved in Cremophor® and ethanol). The positive role of the nanocapsules was attributed to two mechanisms: first, as could be expected, the presence of lipids in the formulation increased the intestinal lymphatic transport and, second, the entrapment of the molecule in the nanocapsules could reduce the P-gp-mediated transport of the drug. Nevertheless, these promising results should be taken into account cautiously due to the high interindividual variability and need to be confirmed by further experimentation(Peltier, Oger et al. 2006).

Overall, nanocapsules can be considered as potential vehicles for promoting the oral absorption of peptides and lipophilic low molecular weight drugs. Particularly noticeable is their capacity to overcome multidrug resistance (MDR) mechanisms, such as the P-gp efflux transport. Despite this evidence, the validation of the efficacy of these nanosystems in large-scale animals, in fed and fasted conditions will have to be proved in order to make sure of their potential for clinical use.

Nanocapsules as nasal drug carriers

The intranasal delivery is an attractive non-invasive route which offers several unique advantages for peptide drugs, such as the ease of administration, the looseness of the epithelium and the avoidance of the hepatic first-pass metabolism. Our group has explored the potential of chitosan nanocapsules for increasing the nasal absorption of the peptide salmon calcitonin(Prego, Torres et al. 2006). The results observed in the rat model indicated that, as expected, the response of this peptide could be significantly enhanced and prolonged following its association to the nanocapsules (Figure 7). These results highlight the critical role of the polymer in enhancing the transport of the associated peptide and consequently the potential of chitosan nanocapsules for nasal peptide delivery.

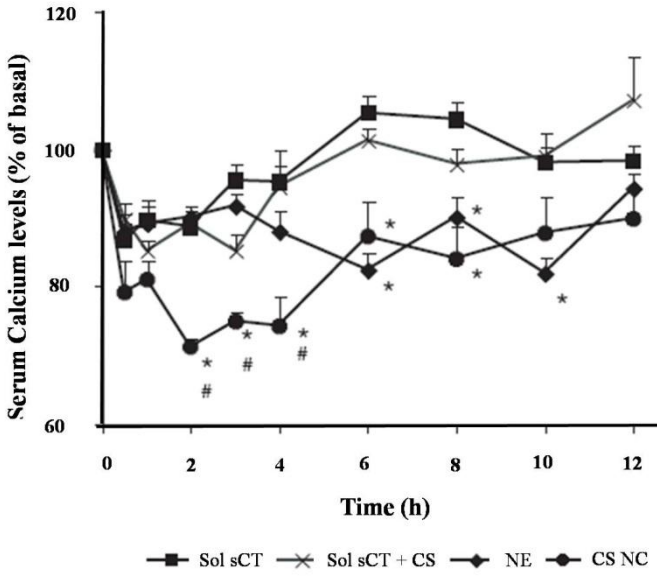


Figure 7: Serum calcium levels in rats after nasal administration of salmon calcitonin (sCT, dose: 15 IU/kg) in aqueous solution (with or without CS) or encapsulated in the control nanoemulsion (NE) or in chitosan nanocapsules (CS NC); (mean ± SE; n = 6). *Significantly different from salmon calcitonin solutions ($p < 0.05$). * Significantly different from nanoemulsion ($p < 0.05$).7 Reproduced by permission of Ed. Sante.

Nanocapsules as ocular drug carriers

The vast majority of intraocular diseases are treated by the instillation of aqueous solution eye-drops in the cul-de-sac. In order to penetrate into the eye, drugs must diffuse through different hurdles, such as the cornea, that acts as a barrier for hydrophilic and lipophilic drugs, limiting dramatically the intra-ocular penetration(Barar, Javadzadeh et al. 2008). Another impediment is represented by the lachrymal fluid which is continuously spread over the surface of the cornea and is quickly drained, together with the instilled drug, into the nasolachrymal ducts (Barar, Javadzadeh et al. 2008). In conclusion, less than 5% of the instilled drug is able to enter into

the eye (Lang 1995), therefore several instillations of the drug solution are required to obtain a sustained therapeutic effect. Importantly, drugs which are drained into the nasolachrymal ducts can be absorbed directly into the systemic circulation, thereby it could be possible to observe secondary effects (Marchal-Heussler, Fessi et al. 1992; Losa, Marchal-Heussler et al. 1993; Marchal-Heussler, Sirbat et al. 1993).

The use of nanocapsules has been proposed as a strategy to increase the penetration of lipophilic drugs into the eye by prolonging their precorneal residence time. The strategy has been explored for a number of β-blocking antiglaucomatous agents such as betaxolol, carteolol and metipranolol. In the case of betaxolol and carteolol it was found that their association to PCL nanocapsules led to a significant improvement of their pharmacological effect (intraocular pressure) (Marchal-Heussler, Fessi et al. 1992; Marchal-Heussler, Sirbat et al. 1993). Additionally, in all cases, the association of the drug to the nanocapsules resulted in a significant reduction of their side effects (Marchal-Heussler, Fessi et al. 1992; Losa, Marchal-Heussler et al. 1993; Marchal-Heussler, Sirbat et al. 1993). As an approach to further improving the efficacy of the nanocapsules, Desai et al. (Desai and Blanchard 2000) associated the antiglaucomatous drug pilocarpine to PCL nanocapsules that were dispersed in a Pluronic® F127 gel. This formulation was more effective than the nanocapsules without the gel or than the pilocarpine incorporated into the gel. The authors explained the positive effect of Pluronic® F127 in terms of the ability of the gel to increase the contact time of the nanocapsules within the ocular mucosa.

Another interesting study was conducted by Calvo et al. (Calvo, Sa?nchez et al. 1996) who demonstrated an improvement in ocular absorption of cyclosporine A by encapsulation into PCL nanocapsules. The corneal levels of this drug were 5-fold higher than the drug

formulated in oily solution, and significant differences in the concentration of cyclosporine A were even found for up three days. Le Bourlais et al. also studied formulations with cyclosporine A showing that the absorption of this drug was higher when the drug was included in PACA nanocapsules, poly(acrylic) gels, or a combination of both compared with the drug being dispersed in oil (Le Bourlais, Chevanne et al. 1997). Importantly, nanocapsules dispersed in gel did not show any toxic effect differing from the other carriers.

In an attempt to understand the mechanism of action of nanocapsules following topical ocular administration, our research group has conducted several studies. In an initial study we could demonstrate by confocal microscopy that PCL nanocapsules penetrate selectively into the corneal epithelium by endocityc process, without cause a disruption in the cells membrane (Calvo, Thomas et al. 1994). In addition, we identified that the size of the particles, but not the inner structure or the composition, was a critical factor for their effectiveness as drug carriers across the epithelial barrier. More specifically, following topical instillation of different carriers containing indomethacin: PCL nanoparticles, PCL nanocapsules, and a submicron emulsion (Figure 8). We found that all nanostructured formulations behaved significantly better than the commercial eye drops (Indocollyre®)(Calvo, Vila-Jato et al. 1996) in terms of increasing the corneal permeation of the associated drug, while PCL microparticles failed to produce this benefit in a different study(Calvo, Alonso et al. 1996).

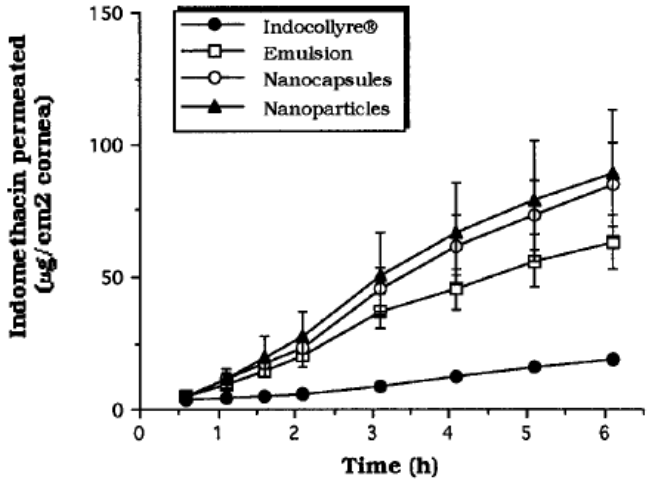


Figure 8: Permeation of indomethacin through isolated rabbit cornea: (□) PCL nanoparticles, (□) PCL nanocapsules, (•) submicron emulsion, and (□) commercial eye drops (Indocollyre®).70 Reproduced by permission of Wiley InterScience.

In addition to the influence of the particle size, we also studied the importance of the surface charge and composition of the nanocapsules in their ability to work as drug carriers. More specifically, we compared the behavior of indomethacin-loaded PCL nanocapsules with that of chitosan-coated and poly-L-lysine-coated PCL nanocapsules following topical instillation to rabbits. The results indicated that the chitosan-coated nanocapsules provide better corneal drug penetration than poly-L-lysine or uncoated nanocapsules(Calvo, Vila-Jato et al. 1997). Given the fact that chitosan and poly-L-lysine are both polycationic polymer, the positive behavior of the chitosan-coated nanocapsules could not be simply attributed to the positive surface charge but to the specific properties of chitosan, i.e. mucoadhesive and permeability-enhancing properties of this polymer (Alonso and SÃ¡nchez 2003).

In an attempt to explore further the effect of the surface polymer composition in the interaction of the nanostructures with the ocular barriers, we comparatively investigated chitosan- and PEG-coated PCL nanocapsules(De Campos, Sa?nchez et al. 2003). These studies were conducted using nanocapsules loaded with a fluorescent dye and their ocular distribution was observed by confocal microscopy. Two main conclusions were extracted from this study: (i) both formulations were internalized by the corneal epithelium; (ii) the chitosan formulations were favourably retained in the superficial layers while the PEG formulations were able to reach deep layers of the corneal epithelium.

All the above information indicates that nanocapsules are interesting tools for improving the drug ocular bioavailability and reducing the systemic side effects of drugs administered topically onto the eye. Additionally, these results underline the importance of the particle size and surface composition on the therapeutic efficacy of the nanocapsules.

Nanocapsules in cancer therapy

The main limitations in cancer therapies are related to their lack of specificity and subsequent toxicity. Moreover, in many cancers, there are specific biological barriers, such as the MDR mechanisms, which limit the efficacy of the treatments (Gottesman, Fojo et al. 2002; Ehdaie 2008). Finally, from the formulation point of view, most of the anticancer drugs suffer from poor water solubility and instability. In this context, nanocapsule technology emerges as an important approach for the formulation of anticancer drugs, as it offers the possibility of incorporating hydrophobic drugs and protecting them in the biological fluids(Couvreur, Barratt et al. 2002). The size and large surface-to-volume ratios (McNeil 2005) of the nanocapsules facilitate their accumulation in

the tumor by the well-known enhanced permeability and retention effect (EPR) (Iyer, Khaled et al. 2006; Greish 2007) and their capacity to be internalized by the tumor cells. Moreover, it has been shown that lipid nanocapsules behave as a MDR-inhibiting system.(Brigger, Dubernet et al. 2002; Garcion, Lamprecht et al. 2006).

There are a number of reports showing the advantages of nanocapsules for specific anticancer drugs. For example, Lenaerts et al. (Lenaerts, Labib et al. 1995) encapsulated phtalocyanines, important agents in photodynamic tumor therapy, in poloxamer surface modified-PACA nanocapsules. They found that the presence of some types of poloxamer significantly decreased the uptake of nanocapsules by organs rich in phagocytic cells and increased the accumulation of phtalocyanines in primary tumors. The concentration of photosensitizers in the tumor was maximal 12 h post-administration, these carriers allowing a 200-fold higher accumulation in the tumor.

In different reports it has been shown that lipid nanocapsules are adequate vehicles for the delivery of taxanes. More specifically, the encapsulation of paclitaxel into lipid nanocapsules led to a significant concentration increase in the tumoral tissue, and significantly reduced the tumor mass compared to the commercial product (Taxol ®) as we can see in Figure 9(Garcion, Lamprecht et al. 2006). Additionally, *in vivo* studies in rats have shown that lipid nanocapsules enhanced around 3-fold the oral bioavailability of the anticancer drug, in comparison with the commercial product (Peltier, Oger et al. 2006; Lacoeuille, Hindre et al. 2007). Docetaxel is another taxane that has been encapsulated into lipid nanocapsules; these nanocapsules showed an enhanced drug deposition in mice tumors which was characterized by a 5-fold increase in the area under the curve of the tumor (AUCtumor) when compared to the control formulation (Taxotere®)(Khalid, Simard et al. 2006).

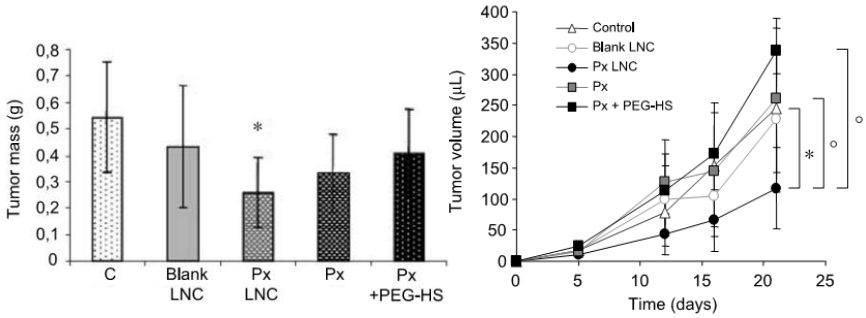


Figure 9: *in vivo* effects of paclitaxel-loaded lipid nanocapsules (LNC) treatment on the growth of F98 glioma cells implanted. (C, control; Px-LNC, paclitaxel-loaded LNC; Px, Taxol only; Px + PEG-HS, Taxol with Solutol HS15 solution) *, $P<0.05$ (Dunnett´s test). º, $P<0.05$ (Fisher´s test). Statistical analysis by pairs show significant diferences on day 21 between formulations.79 Reproduced by permission of American Association for Cancer Research, Inc.

Our group has also proposed an alternative carrier for the intracellular delivery of docetaxel consisting of oligomer chitosan nanocapsules (Lozano, Torrecilla et al. 2008). The results have shown that chitosan nanocapsules are able to facilitate the rapid internalization of the drug into the cancer cells, leading to a significant increase of the antiproliferative effect of the drug.

Overall, the results presented here indicate that nanocapsules represent an alternative for the intracellular delivery of hydrophobic anticancer drugs. This potential is related to their capacity to be internalized by the cells and inhibit the MDR mechanisms, thus maximizing the antitumoral drug effects.

Nanocapsules as carriers for gene therapy

The discovery of antisense oligodeoxynucleotides (ODNs) and more recently siRNA, has opened wide perspectives in therapeutics for the treatment of cancer, infectious and inflammatory diseases, or to block cell proliferation and diseases caused thereby. However, the clinical use of these molecules is limited by their poor stability in biological media and their important hydrophilic character, which strongly limit tissular, cellular and subcellular internalization(Li and Chan 1999; Fattal and Bochot 2008). Besides, another disadvantage of the ODNs and siRNA is the toxicity related with the cationic charge, and the poor activity of these naked molecules.

A few research groups have explored the potential of nanotechnology for the development of suitable carriers for gene delivery. Among the different options, nanocapsule technology has been shown to offer some specific advantages. Due to its hydrophilic character, siRNA and ODNs molecules are usually adsorbed onto the polymeric surface of nanoparticles or polymeric micelles(Schwab, Chavany et al. 1994; Nakada, Fattal et al. 1996), however, water containing nanocapsules can eficiently encapsulate these molecules within its aqueous core. An interesting method for the encapsulation of ODNs into PACA nanocapsules was that described by Lambert et al. 28. These aqueous core-containing nanocapsules improved the ODNs stability against enzymatic degradation and considerably increased their half-life in serum in comparison with the naked molecules or those adsorbed onto nanospheres(Lambert, Fattal et al. 2001). Moreover, the ODNs cell uptake was significantly improved when the molecule was included in the nanocapsules(Lambert, Bertrand et al. 2000).

The encapsulation into the aqueous core of PBCA nanocapsules of an antisense-siRNA (siRNA-AS) against a fusion oncogen (Fli1) overexpressed in Ewing sarcoma, resulted in an important inhibition of tumor growth tested in a murine model of Ewing sarcoma-related tumor (Figure 10)(Toub, Bertrand et al. 2006; Toub, Bertrand et al. 2006).

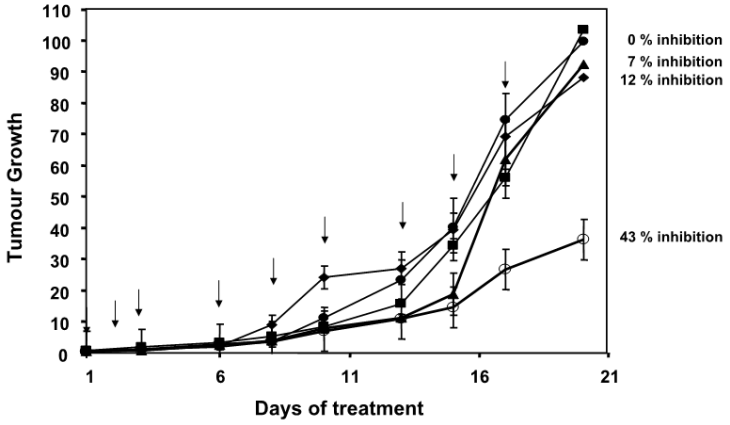


Figure 10: Inhibition of Erwing sarcoma fusion oncogen (EWSFli1)-expressing tumor growth in nude mice by: ○ siRNA-antisense (siRNA-AS) loaded NCs; ▲siRNA-control loaded NCs; ■, siRNA-AS naked; ♦, siRNA-control naked; ●, saline.89 Reproduced by permission of Springer.

Hillaireau et al.19 described the incorporation of ODNs to PBCA nanocapsules. They observed that the association could be significantly improved when ODN is associated first to a cationic polymer, such as chitosan or poly(ethylenimine), and afterwards this complex being encapsulated into a water containing nanocapsule. In a different work, Bouclier et al.(Bouclier, Moine et al. 2008) reported the encapsulation of a specific siRNA (target to estrogen receptor alfa [ERα-siRNA]) in three different systems: PBCA nanocapsules, PEG-PLGA nanoparticles and PEG-PCL-malic acid nanoparticles. The *in vitro* studies indicated that

PBCA nanocapsules showed a high efficiency in MCF-7 cancer cells, whereas the other systems showed no antiproliferative effect in the same cancer cell lines. In a preliminary *in vivo* study, these nanocapsules showed a slight decrease in tumor growth in comparison to scramble-siRNA loaded nanocapsules or the siRNA naked(Bouclier, Moine et al. 2008), showing the benefits of the nanocapsules over other nanosystems for the encapsulation of siRNA.

Conclusions

The liquid nature of nanocapsules and, thus, their fluidity and elasticity make them ideal nanovehicles able to facilitate the contact with the epithelia and target cells, as well as to enter intracellularly. They have unique properties as their simplicity and their capacity of obtaining great loadings of either lipophilic or hydrophilic drugs. Moreover, nanocapsules have shown to be capable of inhibiting multidrug resistance cellular mechanisms, specially important in cancer therapy. In conclusion, polymeric or lipid nanocapsules are a promising tool for transmucosal drug delivery as well as for cancer therapeutics, particularly for drugs which are water-insoluble and that, until recently, have required solvents to be formulated. Concerning gene therapy, nanocapsules emerge as an interesting approach, due to the high affinity of nucleic acids for their water core and to the possibility of adapting these systems to the requirements of this novel therapy.

In addition, the use of reservoir structures composed by inorganic nanoparticles (iron, silica or gold nanoparticles, quantum dots, carbon nanotubes, etc.) surronded by a polymer and, optionally, a targeting ligand, represents a promising and powerful tool to enhance the biocompatibility and the biodistribution of these nanostructures widely

used in the diagnostics and threatment of several diseases. This composite nanocapsules will be discussed widely in following chapters.

References

1. Couvreur, P., Tulkens, P., and Roland, M. (1977).Nanocapsules: a new type of lysosomotropic carrier. FEBS Lett.84.pp 323-326.

2. Legrand, P., Barratt, G., Mosqueira, V., Fessi, H., and Devissaguet, J.P. (1999).Polymeric nanocapsules as drug delivery systems: A review. STP Pharm. Sci.9.pp 411-418.

3. Couvreur, P., Barratt, G., Fattal, E., Legrand, P., and Vauthier, C. (2002).Nanocapsule technology: A review. Crit Rev Ther Drug Carrier Syst.19.pp 99-134.

4. Couvreur, P., Kante, B., and Roland, M. (1979).Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: Preparation, morphological and sorptive properties. J Pharm Pharmacol.31.pp 331-332.

5. Fessi, H., Puisieux, F., Devissaguet, J.P., Ammoury, N., and Benita, S. (1989).Nanocapsule formation by interfacial polymer deposition following solvent deplacement. Int J Pharm.55.pp 25-28.

6. Heurtault, B., Saulnier, P., Pech, B., Proust, J.E., and Benoit, J.P. (2002).A novel phase inversion-based process for the preparation of lipid nanocarriers. Pharm Res.19.pp 875-880.

7. Prego, C., Torres, D., and Alonso, M.J. (2006).Chitosan nanocapsules: A new carrier for nasal peptide delivery. JDDST.16.pp 331-337.

8. Prego, C., Torres, D., and Alonso, M.J. (2006).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. J Nanosci Nanotechnol.6.pp 2921-2928.

9. Lozano, M.V., Torrecilla, D., Torres, D., Vidal, A., Dominguez, F., and Alonso, M.J. (2008).Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers. Biomacromolecules.9.pp 2186-2193.

10. Desai, M.P., Labhasetwar, V., Amidon, G.L., and Levy, R.J. (1996).Gastrointestinal Uptake of Biodegradable Microparticles: Effect of Particle Size. Pharm Res.13.pp 1838-1845.

11. Vila, A., Gill, H., McCallion, O., and Alonso, M.J. (2004).Transport of PLA-PEG particles across the nasal mucosa: effect of particle size and PEG coating density. J Control Release.98.pp 231-244.

12. Yin Win, K. and Feng, S. (2005).Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials.26.pp 2713-2722.

13. Quintanar-Guerrero, D., Allemann, E., Doelker, E., and Fessi, H. (1998).Preparation and characterization of nanocapsnles from preformed polymers by a new process based on emulsification-diffusion technique. Pharm Res.15.pp 1056-1062.

14. Moinard-Checot, D., Chevalier, Y., Briancon, S., Beney, L., and Fessi, H. (2008).Mechanism of nanocapsules formation by the emulsion-diffusion process. J Colloid Interface Sci.317.pp 458-468.

15. Rube, A., Hause, G., Mader, K., and Kohlbrecher, J. (2005).Core-shell structure of Miglyol/poly(D,L-lactide)/Poloxamer nanocapsules

studied by small-angle neutron scattering. J Control Release.107.pp 244-252.

16. Calvo, P., Vila-Jato, J.L., and Alonso, M.J. (1997).Evaluation of cationic polymer-coated nanocapsules as ocular drug carriers. Int J Pharm.153.pp 41-50.

17. Guinebretiere, S., Briancon, S., Lieto, J., Mayer, C., and Fessi, H. (2002).Study of the emulsion-diffusion of solvent: Preparation and characterization of nanocapsules. Drug Dev Res.57.pp 18-33.

18. Wohlgemuth, M. and Mayer, C. (2003).Pulsed field gradient NMR on polybutylcyanoacrylate nanocapsules. J Colloid Interface Sci.260.pp 324-331.

19. Hillaireau, H., Le Doan, T., Chacun, H., Janin, J., and Couvreur, P. (2007).Encapsulation of mono- and oligo-nucleotides into aqueous-core nanocapsules in presence of various water-soluble polymers. Int J Pharm.331.pp 148-152.

20. Vauthier, C. and Bouchemal, K. (2008).Methods for the Preparation and Manufacture of Polymeric Nanoparticles. Pharm Res.1-34.

21. Gallardo, M., Couarraze, G., Denizot, B., Treupel, L., Couvreur, P., and Puisieux, F. (1993).Study of the mechanisms of formation of nanoparticles and nanocapsules of polyisobutyl-2-cyanoacrylate. Int J Pharm.100.pp 55-64.

22. Puglisi, G., Fresta, H.T.M., Giammona, G., and Ventura, C.A. (1995).Influence of the preparation conditions on poly(ethylcyanoacrylate) nanocapsule formation. Int J Pharm.125.pp 283-287.

23. Fresta, M., Cavallaro, G., Giammona, G., Wehrli, E., and Puglisi, G. (1996).Preparation and characterization of polyethyl-2-cyanoacrylate nanocapsules containing antiepileptic drugs. Biomaterials.17.pp 751-758.

24. Aboubakar, M., Puisieux, F., Couvreur, P., Deyme, M., and Vauthier, C. (1999).Study of the mechanism of insulin encapsulation in poly(isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization. J Biomed Mater Res.47.pp 568-576.

25. Lowe, P.J. and Temple, C.S. (1994).Calcitonin and insulin in isobutylcyanoacrylate nanocapsules: Protection against proteases and effect on intestinal absorption in rats. J Pharm Pharmacol.46.pp 547-552.

26. Vauthier, C., Labarre, D., and Ponchel, G. (2007).Design aspects of poly(alkylcyanoacrylate) nanoparticles for drug delivery. J Drug Target.15.pp 641-663.

27. Watnasirichaikul, S., Davies, N.M., Rades, T., and Tucker, I.G. (2000).Preparation of biodegradable insulin nanocapsules from biocompatible microemulsions. Pharm Res.17.pp 684-689.

28. Lambert, G., Fattal, E., Pinto-Alphandary, H., Gulik, A., and Couvreur, P. (2000).Polyisobutylcyanoacrylate nanocapsules containing an aqueous core as a novel colloidal carrier for the delivery of oligonucleotides. Pharm Res.17.pp 707-714.

29. Vranckx, H., Demoustier, M., and Deleers, M. (1996).A new nanocapsule formulation with hydrophilic core: Application to the oral administration of salmon calcitonin in rats. Eur J Pharm Biopharm.42.pp 345-347.

30. Al Khouri Fallouh, N., Roblot-Treupel, L., and Fessi, H. (1986).Development of a new process for the manufacture of polyisobutylcyanoacrylate nanocapsules. Int J Pharm.28.pp 125-132.

31. Nassar, T., Rom, A., Nyska, A., and Benita, S. (2008).A novel nanocapsule delivery system to overcome intestinal degradation and drug transport limited absorption of P-glycoprotein substrate drugs. Pharm Res.25.pp 2019-2029.

32. Guinebretiere, S., Briançon, S., Fessi, H., Teodorescu, V.S., and Blanchin, M.G. (2002).Nanocapsules of biodegradable polymers: Preparation and characterization by direct high resolution electron microscopy. Mater Sci Eng C.21.pp 137-142.

33. Cauchetier, E., Deniau, M., Fessi, H., Astier, A., and Paul, M. (2003).Atovaquone-loaded nanocapsules: Influence of the nature of the polymer on their in vitro characteristics. Int J Pharm.250.pp 273-281.

34. De Campos, A.M., Sanchez, A., Gref, R., Calvo, P., and Alonso, M.J. (2003).The effect of a PEG versus a chitosan coating on the interaction of drug colloidal carriers with the ocular mucosa. Eur J Pharm Sci.20.pp 73-81.

35. Mosqueira, V.C.F., Legrand, P., Gulik, A., Bourdon, O., Gref, R., Labarre, D., and Barratt, G. (2001).Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules. Biomaterials.22.pp 2967-2979.

36. Mosqueira, V.C.F., Legrand, P., Morgat, J.L., Vert, M., Mysiakine, E., Gref, R., Devissaguet, J.P., and Barratt, G. (2001).Biodistribution

of long-circulating PEG-grafted nanocapsules in mice: Effects of PEG chain length and density. Pharm Res.18.pp 1411-1419.

37. Bouclier, C.l., Moine, L., Hillaireau, H., Marsaud, V.r., Connault, E., Opolon, P., Couvreur, P., Fattal, E., and Renoir, J.-M. (2008).Physicochemical Characteristics and Preliminary *in Vivo* Biological Evaluation of Nanocapsules Loaded with siRNA Targeting Estrogen Receptor Alpha. Biomacromolecules.9.pp 2881-2890.

38. Prego, C., Torres, D., Fernandez-Megia, E., Novoa-Carballal, R., Quinoa, E., and Alonso, M.J. (2006).Chitosan-PEG nanocapsules as new carriers for oral peptide delivery: Effect of chitosan pegylation degree. J Control Release.111.pp 299-308.

39. Preetz, C., Rube, A., Reiche, I., Hause, G., and Mader, K. (2008).Preparation and characterization of biocompatible oil-loaded polyelectrolyte nanocapsules. Nanomedicine.4.pp 106-114.

40. Heurtault, B., Saulnier, P., Pech, B., Benoit,t, J.P., and Proust, J.E. (2003).Interfacial stability of lipid nanocapsules. Colloids Surf B Biointerfaces.30.pp 225-235.

41. Heurtault, B., Saulnier, P., Pech, B., Proust, J.E., and Benoit, J.P. (2003).Physico-chemical stability of colloidal lipid particles. Biomaterials.24.pp 4283-4300.

42. Heurtault, B., Saulnier, P., Pech, B., Venier-Julienne, M.C., Proust, J.E., Phan-Tan-Luu, R., and Benoit, J.P. (2003).The influence of lipid nanocapsule composition on their size distribution. Eur J Pharm Sci.18.pp 55-61.

43. Anton, N., Gayet, P., Benoit, J.P., and Saulnier, P. (2007).Nano-emulsions and nanocapsules by the PIT method: An investigation on the role of the temperature cycling on the emulsion phase inversion. Int J Pharm.344.pp 44-52.

44. Babu Dhanikula, A., Mohamed Khalid, N., Lee, S.D., Yeung, R., Risovic, V., Wasan, K.M., and Leroux, J.C. (2007).Long circulating lipid nanocapsules for drug detoxification. Biomaterials.28.pp 1248-1257.

45. Khalid, M.N., Simard, P., Hoarau, D., Dragomir, A., and Leroux, J.C. (2006).Long circulating poly(ethylene glycol)-decorated lipid nanocapsules deliver docetaxel to solid tumors. Pharm Res.23.pp 752-758.

46. Prego, C., Garcia, M., Torres, D., and Alonso, M.J. (2005).Transmucosal macromolecular drug delivery. J Control Release.101.pp 151-162.

47. Csaba, N., Garcia-Fuentes, M., and Alonso, M.J. (2006).The performance of nanocarriers for transmucosal drug delivery. Expert Opin Drug Deliv.3.pp 463-478.

48. Pinto Reis, C., Neufeld, R.J., Ribeiro, A.J., and Veiga, F. (2006).Nanoencapsulation II. Biomedical applications and current status of peptide and protein nanoparticulate delivery systems. Nanomedicine.2.pp 53-65.

49. Alonso, M.J. (2004).Nanomedicines for overcoming biological barriers. Biomed Pharmacother.58.pp 168-172.

50. des Rieux, A., Fievez, V., Garinot, M., Schneider, Y.J., and Preat, V. (2006).Nanoparticles as potential oral delivery systems of proteins

and vaccines: A mechanistic approach. J Control Release.116.pp 1-27.

51. Prego, C., Fabre, M., Torres, D., and Alonso, M.J. (2006).Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery. Pharm Res.23.pp 549-556.

52. Damge, C., Michel, C., Aprahamian, M., and Couvreur, P. (1988).New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. Diabetes.37.pp 246-251.

53. Damge, C., Vranckx, H., Balschmidt, P., and Couvreur, P. (1997).Poly(alkyl cyanoacrylate) nanospheres for oral administration of insulin. J Pharm Sci.86.pp 1403-1409.

54. Pinto-Alphandary, H., Aboubakar, M., Jaillard, D., Couvreur, P., and Vauthier, C. (2003).Visualization of insulin-loaded nanocapsules: In vitro and *in vivo* studies after oral administration to rats. Pharm Res.20.pp 1071-1084.

55. Cournarie, F., Auchere, D., Chevenne, D., Lacour, B., Seiller, M., and Vauthier, C. (2002).Absorption and efficiency of insulin after oral administration of insulin-loaded nanocapsules in diabetic rats. Int J Pharm.242.pp 325-328.

56. Ubrich, N., Schmidt, C., Bodmeier, R., Hoffman, M., and Maincent, P. (2005).Oral evaluation in rabbits of cyclosporin-loaded Eudragit RS or RL nanoparticles. Int J Pharm.288.pp 169-175.

57. Staniscuaski Guterres, S., Fessi, H., Barratt, G., Puisieux, F., and Devissaguet, J.P. (1995).Poly(D,L-Lactide) nanocapsules containing non-steroidal anti-inflammatory drugs: Gastrointestinal tolerance

following intravenous and oral administration. Pharm Res.12.pp 1545-1547.

58. Limayem Blouza, I., Charcosset, C., Sfar, S., and Fessi, H. (2006).Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use. Int J Pharm.325.pp 124-131.

59. Nassar, T., Rom, A., Nyska, A., and Benita, S. (2009).Novel double coated nanocapsules for intestinal delivery and enhanced oral bioavailability of tacrolimus, a P-gp substrate drug. J Control Release.133.pp 77-84.

60. Peltier, S., Oger, J.M., Lagarce, F., Couet, W., and Benoit, J.P. (2006).Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules. Pharm Res.23.pp 1243-1250.

61. Barar, J., Javadzadeh, A.R., and Omidi, Y. (2008).Ocular novel drug delivery: Impacts of membranes and barriers. Expert Opin Drug Deliv.5.pp 567-581.

62. Lang, J.C. (1995).Ocular drug delivery conventional ocular formulations. Adv Drug Deliv Rev.16.pp 39-43.

63. Losa, C., Marchal-Heussler, L., Orallo, F., Vila-Jato, J.L., and Alonso, M.J. (1993).Design of new formulations for topical ocular administration: Polymeric nanocapsules containing metipranolol. Pharm Res.10.pp 80-87.

64. Marchal-Heussler, L., Fessi, H., Devissaguet, J.P., Hoffman, M., and Maincent, P. (1992).Colloidal drug delivery systems for the eye. A comparison of the efficacy of three different polymers: Polyisobutylcyanoacrylate, polylactic-co-glycolic acid, poly-epsilon-caprolactone. STP Pharma Sciences.2.pp 98-104.

65. Marchal-Heussler, L., Sirbat, D., Hoffman, M., and Maincent, P. (1993).Poly(ε-caprolactone) nanocapsules in carteolol ophthalmic delivery. Pharm Res.10.pp 386-390.

66. Desai, S.D. and Blanchard, J. (2000).Pluronic® F127-based ocular delivery system containing biodegradable polyisobutylcyanoacrylate nanocapsules of pilocarpine. Drug Delivery: J Delivery and Targeting Therapeutic Agents.7.pp 201-207.

67. Calvo, P., Sanchez, A., Martinez, J., Lopez, M.I., Calonge, M., Pastor, J.C., and Alonso, M.J. (1996).Polyester nanocapsules as new topical ocular delivery systems for cyclosporin A. Pharm Res.13.pp 311-315.

68. Le Bourlais, C.A., Chevanne, F., Turlin, B., Acar, L., Zia, H., Sado, P.A., Needham, T.E., and Leverge, R. (1997).Effect of cyclosporine A formulations on bovine corneal absorption: Ex-vivo study. J Microencapsulation.14.pp 457-467.

69. Calvo, P., Thomas, C., Alonso, M.J., Vila-Jato, J.L., and Robinson, J.R. (1994).Study of the mechanism of interaction of poly(E-caprolactone) nanocapsules with the cornea by confocal laser scanning microscopy. Int J Pharm.103.pp 283-291.

70. Calvo, P., Vila-Jato, J.L., and Alonso, M.J. (1996).Comparative in vitro evaluation of several colloidal systems, nanoparticles, nanocapsules, and nanoemulsions, as ocular drug carriers. J Pharm Sci.85.pp 530-536.

71. Calvo, P., Alonso, M.J., Vila-Jato, J.L., and Robinson, J.R. (1996).Improved Ocular Bioavailability of Indomethacin by Novel Ocular Drug Carriers. J Pharm Pharmacol.48.pp 1147-1152.

72. Alonso, M.J. and Sanchez, A. (2003).The potential of chitosan in ocular drug delivery. J Pharm Pharmacol.55.pp 1451-1463.

73. Gottesman, M.M., Fojo, T., and Bates, S.E. (2002).Multidrug resistance in cancer: Role of ATP-dependent transporters. Nat Rev Cancer.2.pp 48-58.

74. Ehdaie, B. (2008).Application of Nanotechnology in Cancer Research: Review of Progress in the National Cancer Institute's Alliance for nanotechnology.

75. McNeil, S.E. (2005).Nanotechnology for the biologist. J Leukoc Biol.78.pp 585-594.

76. Iyer, A.K., Khaled, G., Fang, J., and Maeda, H. (2006).Exploiting the enhanced permeability and retention effect for tumor targeting. Drug Discov Today.11.pp 812-818.

77. Greish, K. (2007).Enhanced permeability and retention of macromolecular drugs in solid tumors: A royal gate for targeted anticancer nanomedicines. J Drug Target.15.pp 457-464.

78. Brigger, I., Dubernet, C., and Couvreur, P. (2002).Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev.54.pp 631-651.

79. Garcion, E., Lamprecht, A., Heurtault, B., Paillard, A., Aubert-Pouessel, A., Denizot, B., Menei, P., and Benoit, J.P. (2006).A new generation of anticancer, drug-loaded, colloidal vectors reverses multidrug resistance in glioma and reduces tumor progression in rats. Mol Cancer Ther.5.pp 1710-1722.

80. Lenaerts, V., Labib, A., Chouinard, F., Rousseau, J., Ali, H., and Van Lier, J. (1995).Nanocapsules with a reduced liver uptake: Targeting

of phthalocyanines to EMT-6 mouse mammary tumor *in vivo*. Eur J Pharm Biopharm.41.pp 38-43.

81. Lacoeuille, F., Hindre, F., Moal, F., Roux, J., Passirani, C., Couturier, O., Cales, P., Le Jeune, J.J., Lamprecht, A., and Benoit, J.P. (2007).*In vivo* evaluation of lipid nanocapsules as a promising colloidal carrier for paclitaxel. Int J Pharm.344.pp 143-149.

82. Fattal, E. and Bochot, A. (2008).State of the art and perspectives for the delivery of antisense oligonucleotides and siRNA by polymeric nanocarriers. Int J Pharm.364.pp 237-248.

83. Li, X. and Chan, W.K. (1999).Transport, metabolism and elimination mechanisms of anti-HIV agents. Adv Drug Deliv Rev.39.pp 81-103.

84. Nakada, Y., Fattal, E., Foulquier, M., and Couvreur, P. (1996).Pharmacokinetics and biodistribution of oligonucleotide adsorbed onto poly(isobutylcyanoacrylate) nanoparticles after intravenous administration in mice. Pharm Res.13.pp 38-43.

85. Schwab, G., Chavany, C., Duroux, I., Goubin, G., Lebeau, J., Helene, C., and Saison-Behmoaras, T. (1994).Antisense oligonucleotides adsorbed to polyalkylcyanoacrylate nanoparticles specifically inhibit mutated Ha-ras-mediated cell proliferation and tumorigenicity in nude mice. Proc Natl Acad Sci U S A.91.pp 10460-10464.

86. Lambert, G., Fattal, E., Pinto-Alphandary, H., Gulik, A., and Couvreur, P. (2001).Polyisobutylcyanoacrylate nanocapsules containing an aqueous core for the delivery of oligonucleotides. Int J Pharm.214.pp 13-16.

87. Lambert, G., Bertrand, J.R., Fattal, E., Subra, F., Pinto-Alphandary, H., Malvy, C., Auclair, C., and Couvreur, P. (2000).EWS Fli-1

antisense nanocapsules inhibits Ewing sarcoma-related tumor in mice. Biochem Biophys Res Commun.279.pp 401-406.

88. Toub, N., Bertrand, J.R., Malvy, C., Fattal, E., and Couvreur, P. (2006).Antisense oligonucleotide nanocapsules efficiently inhibit EWS-Fli1 expression in a Ewing's sarcoma model. Oligonucleotides.16.pp 158-168.

89. Toub, N., Bertrand, J.R., Tamaddon, A., Elhamess, H., Hillaireau, H., Maksimenko, A., Maccario, J., Malvy, C., Fattal, E., and Couvreur, P. (2006).Efficacy of siRNA nanocapsules targeted against the EWS-Fli1 oncogene in Ewing sarcoma. Pharm Res.23.pp 892-900.

90. Bouclier, C., Moine, L., Hillaireau, H., Marsaud, V., Connault, E., Opolon, P., Couvreur, P., Fattal, E., and Renoir, J.M. (2008).Physicochemical characteristics and preliminary *in vivo* biological evaluation of nanocapsules loaded with siRNA targeting estrogen receptor alpha. Biomacromolecules.9.pp 2881-2890