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Nanocápsulas de quitosano: nuevos vehículos para el transporte de péptidos a través de la mucosa nasal e intestinal

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CERTIFICAN:

Que la presente memoria titulada "**Nanocápsulas de quitosano: nuevos vehículos para el transporte de péptidos a través de la mucosa nasal e intestinal**" ha sido elaborada bajo su dirección por la Licenciada en Farmacia **Dña. Cecilia Prego Rodríguez** 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.

Y para que así conste, expiden y firman la presente certificación en Santiago de Compostela a 11 de Mayo de 2005.

Fdo. M.J. Alonso Fernández

Fdo.: D. Torres López

Resumen

El objetivo global de la presente memoria experimental ha sido investigar el potencial de las nanocápsulas de quitosano como nuevos vehículos para la administración de péptidos a través de la mucosa nasal e intestinal. Estas nanoestrucutras, constituidas por un núcleo oleoso y una cubierta de quitosano con distintas propiedades (peso molecular, tipo de sal y grado de peguilación del polímero), se prepararon mediante la técnica de desplazamiento del disolvente. Todas las formulaciones presentaron un tamaño de partícula en rango nanométrico, una carga superficial positiva y la capacidad para encapsular eficazmente el péptido modelo calcitonina. Las nanocápsulas de quitosano mostraron una estabilidad adecuada en fluido gástrico. Asimismo, la peguilación del quitosano permitió obtener un sistema estable en los fluidos gastrointestinales. Los estudios llevados a cabo en cultivo celular Caco-2 revelaron que las distintas nanocápsulas de quitosano presentan una baja citotoxicidad, la cual se redujo como consecuencia de la peguilación del quitosano. Además, tras evaluar la interacción de las nanocápsulas de quitosano con un co-cultivo constituido por células Caco-2 y células secretoras de mucus, se observó una asociación muy importante del sistema a las células secretoras de mucus, resultado que constata las propiedades mucoadhesivas de dichas nanoestructuras. Finalmente, la evaluación *in vivo* de las nanocápsulas de quitosano permitió observar un importante efecto farmacológico tanto tras su administración por vía nasal como por vía oral. Además se observó que modulando el grado de peguilación del quitosano es posible obtener un sistema estable en los fluidos gastrointestinales, de toxicidad muy reducida y con capacidad para promover la absorción oral de péptidos. En definitiva, el trabajo en su conjunto permite pronosticar el interés de las nanocápsulas de quitosano para mejorar la absorción nasal e intestinal de péptidos.

Abstract

The main goal of the present work has been to investigate the potential of chitosan nanocapsules as new carriers for nasal and oral administration of peptides. These nanostructures, composed of an oily core and a chitosan coating with different properties (molecular weight, type of salt and pegylation degree of chitosan), were prepared by the solvent displacement technique. All the formulations presented a particle size in nanometer range, a positive zeta potential and the ability to encapsulate salmon calcitonin as a model peptide. The nanocapsules were stable in the gastric fluid. In addition, the pegylation of chitosan improved the stability of the nanocapsules in the gastrointestinal fluids. The studies performed in Caco-2 cells revealed that chitosan nanocapsules have a low citotoxicity, which could be further reduced as a consequence of the chitosan pegylation. Moreover, the evaluation of the interaction of chitosan nanocapsules with the co-culture Caco-2:HT29-M6 (enterocytes and mucus-secreting cells), evidenced an important association of the system with mucus-secreting cells, thus corroborating the mucoadhesive properties of these nanocapsules. Finally, the studies performed *in vivo* indicated that chitosan nanocapsules are able to elicit a pharmacological effect after nasal or oral administration to rats. Additionally, it was observed that modulating the pegylation degree of chitosan it was possible to achieve a stable carrier with a very low toxicity and the ability to promote the oral absorption of peptides. Briefly, altogether these results allowed us to suggest the interest of chitosan nanocapsules for improving the nasal and intestinal absorption of peptides.

Índice

INTRODUCCIÓN

Introducción

Los avances logrados en el campo de la biotecnología y de la ingeniería genética han permitido el creciente desarrollo de macromoléculas activas de naturaleza peptídica y proteica. A pesar del interesante potencial terapéutico de estas macromoléculas, su aplicación en la práctica clínica se ve notablemente restringida debido a la necesidad de ser administradas por vía parenteral. Esta necesidad deriva de la alta susceptibilidad de estas macromoléculas frente a la degradación y a su dificultad para atravesar barreras biológicas. Como consecuencia, en la actualidad, uno de los principales retos en el campo de los nuevos sistemas de liberación de fármacos, lo constituye el diseño de estrategias dirigidas a la administración de estas macromoléculas por vías alternativas a la parenteral, como son la vía nasal y la vía oral.

Interés de la vía nasal para la administración de péptidos

Tradicionalmente, la vía nasal se ha utilizado para el tratamiento local de distintas enfermedades como son la congestión nasal, infecciones o alergias nasales.

Sin embargo, el progreso en el conocimiento de las características fisiológicas cavidad nasal, ha hecho posible la explotación de esta vía para la administración de fármacos con fines sistémicos. Así, fármacos polares de pequeño peso molecular, como son los antimigrañosos, y distintos péptidos, entre ellos, la calcitonina[1], la oxitocina[1], la desmopresina[2], la buserelina[3] y la nafarelina[4], se administra actualmente por vía nasal. Además de estos fármacos, en estos momentos varias formulaciones destinadas a la administración nasal de insulina, hormona de crecimiento, paratohormona y leuprolide, entre otros, que se encuentran en distintas fases de investigación clínica[5]. No obstante, a pesar de los grandes avances realizados en la administración de estos fármacos por vía nasal, en ocasiones la biodisponibilidad es baja y muy variable debido principalmente a tres factores: (a) la escasa superficie para la absorción que representa el epitelio respiratorio, (b) el aclaramiento mucociliar que lleva consigo una regeneración del mucus que recubre el epitelio nasal cada 15-20 minutos[6, 7] y (c) la degradación proteolítica ocasionada por los enzimas presentes en la cavidad nasal[8]. Estas limitaciones han estimulado la búsqueda de nuevos vehículos con el fin de mejorar la biodisponibilidad nasal de dichas macromoléculas. Entre las estrategias orientadas a tal fin, destacan fundamentalmente: la administración junto con el fármaco de promotores de la absorción[9, 10, 11], así como, la asociación del fármaco a un transportador.

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[1] www.novartis.com

[2] www.ferring.com

[3] www.aventis.com

[4] http://home.intekom.com/pharm/searle/synarel.html

[5] Davis S.S., Illum L. Absorption enhancers for nasal drug delivery. Clinical Pharmacokinetics (2003) 42: 1107-1128.

[6] Gizurarson S. Animal models for intranasal drug delivery studies. Acta. Pharm. Nord. (1990) 2: 105-122.

[7] Marttin E., Schipper N.G.M., Verhoef J.C., Merkus F.W.H.M. Nasal mucociliary clearance as a factor in nasal drug delivery. Adv. Drug Deliv. Rev. (1998) 29: 13-38.

[8] Lee V.H. Enzymatic barriers to peptide and protein absorption. Crit. Rev. Ther. Drug Carrier Systems (1988) 5: 69-97.

[9] Merkus F.W.H.M., Verhoef J.C., Marttin E., Romeijn S.G., van der Kuy P.H.M., Hermens W.A.J.J., Schipper N.G.M. Cyclodextrins in nasal drug delivery. Adv. Drug Deliv. Rev. (1999) 36: 41-57.

Lamentablemente, utilización de promotores ha resultado en la mayoría de los casos inviable debido al hecho de que inducen daños histológicos o inhiben drásticamente el movimiento de los cilios[12]. Sin embargo, el diseño de transportadores coloidales se revela en la actualidad como una opción altamente prometedora. Los logros más destacados conseguidos con estos nanosistemas se describen a continuación.

Interés de los sistemas coloidales para la administración nasal de péptidos

El empleo de sistemas coloidales para la administración de péptidos por vía nasal resulta una alternativa muy atractiva debido a la capacidad de estos vehículos para interaccionar íntimamente con la mucosa y, de esta manera, facilitar el transporte del fármaco asociado a través del epitelio nasal. Asimismo, estos sistemas ofrecen la posibilidad de proteger al fármaco frente a la degradación en los fluidos biológicos.

Entre los distintos sistemas coloidales empleados para mejorar la administración nasal de péptidos se encuentran los liposomas[13], las emulsiones[14], las micelas[15] y las nanopartículas[16, 17].

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[10] Ugwoke M.I., Verbeke N., Kinget R. The biopharmaceutical aspects of nasal mucoadhesive drug delivery. J. Pharm. Pharmacol. (2001) 53: 3-22.

[11] Davis S. S., Illum L. Absorption enhancers for nasal drug delivery. Clin. Pharmacokinet. (2003) 42: 1107-1128.

[12] Quadir M., Zia H., Needham T.E. Toxicological implications of nasal formulations. Drug Delivery (1999) 6: 227-242.

[13] Law S.L., Shih C.L. Characterization of calcitonin-containing liposome formulations for intranasal delivery. J. Microencapsulation. (2001) 18: 201-211.

[14] Mitra R., Pezron I., Chuw A., Mitra A.K. Lipid emulsions as vehicles for enhanced nasal delivery of insulin. Int. J. Pharm. (2000) 205: 127-134.

[15] Tengamnuay P., Mitra A.K. Bile salt-fatty acid mixed micelles as nasal absorption promoters of peptides. II. In vivo nasal absorption of insulin in rats and effects of mixed micelles on the morphological integrity of the nasal mucosa. Pharm. Res. (1990) 7: 370-375.

[16] Fernández-Urrusuno R., Calvo P., Remuñán-López C., Vila-Jato J.L., Alonso M.J. Enhancement of nasal absorption of insulin using chitosan nanoparticles. Pharm. Res. (1999) 16: 1576-1581.

[17] Tobío M., Gref R,. Sánchez A., Langer R., Alonso M.J. Stealth PLA-PEG nanoparticles as protein carriers for nasal administration. Pharm. Res. (1998) 15: 270-275.

Además del tamaño coloidal, obviamente la composición del sistema juega un papel muy importante. Así pues, en los vehículos de naturaleza lipídica, como son las emulsiones o/w, se ha observado que el aceite juega un papel crítico en la mejora de la absorción de péptidos, como la insulina, por vía nasal[18]. Por otro lado, en los sistemas poliméricos, el tipo de polímero y su interacción con las superficies mucosas es también decisivo. Así por ejemplo, el quitosano, debido a sus características mucoadhesivas, entre otras, ha resultado ser una alternativa muy interesante para la administración nasal de fármacos. Concretamente, en nuestro laboratorio se ha observado que, tras la administración por vía nasal de quitosano en forma de nanopartículas, se consigue aumentar la absorción de insulina en mayor extensión que cuando el polímero se encuentra en solución[19, 16] tal y como se refleja en la figura 1.

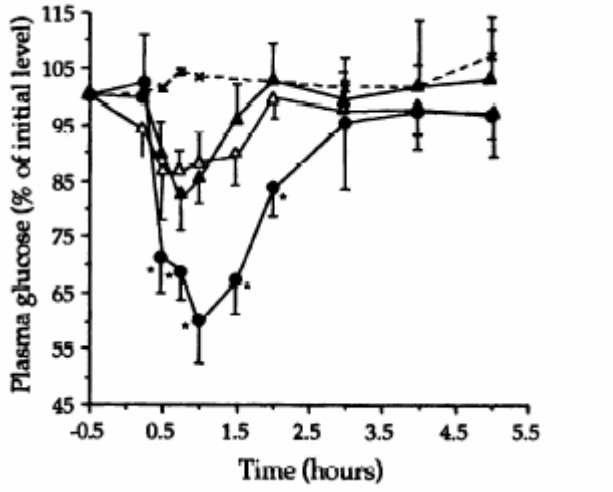

Fig. 1: Niveles de glucosa tras la administración nasal a conejos de: tampón acetato (x), solución de insulina en tampón acetato (Δ), solución de quitosano conteniendo insulina en tampón acetato (▲), insulina encapsulada en nanopartículas de quitosano (●). Media±SD; n=6. *Diferencias estadísticamente significativas con respecto a la solución de insulina ($p<0.05$).

[18] Mitra R., Pezron I., Chuw A., Mitra A.K. Lipid emulsions as vehicles for enhanced nasal delivery of insulin. Int. J. Pharm. (2000) 205: 127-134.

[19] Fernández-Urrusuno y col; 2000. *ibid.* pag 5.

Interés de la vía oral para la administración de péptidos

Aún conociendo las posibilidades y el amplio mercado que ofrece la administración nasal de fármacos de acción sistémica, sin duda la vía de administración que ofrece una mayor comodidad para el paciente y que, por tanto, lleva asociado un mejor cumplimiento del régimen posológico es la vía oral. A pesar de las grandes ventajas que ofrece esta vía, la administración oral de péptidos y proteínas todavía no es factible debido a las condiciones fisiológicas presentes a lo largo del tracto gastrointestinal[20, 21]. Ciertamente, las condiciones drásticas del tracto gastrointestinal (pH variable, contenido enzimático) y la baja permeabilidad del epitelio ocasionan una rápida degradación y una baja absorción de dichas macromoléculas tras ser administradas por esta vía. Como consecuencia, la biodisponibilidad oral de fármacos peptídicos es prácticamente despreciable.

En la última década, el esfuerzo realizado en el desarrollo de nuevas tecnologías que permitan la administración de péptidos y proteínas por vía oral ha dado lugar a resultados muy prometedores. De hecho, en estos momentos existen formulaciones comercializadas que permiten la administración oral de péptidos como la ciclosporina y la desmopresina. Asimismo, en este momento se encuentran en ensayos clínicos diversas formulaciones destinadas a la administración de otros péptidos. Estas formulaciones se basan en la coadministración de moléculas transportadoras[22], en la conjugación del péptido con PEG a través de un grupo alquil[23] o en la combinación del fármaco peptídico con inhibidores enzimáticos, promotores de la absorción y un recubrimiento entérico[24]. Por otro lado, los

[20] Lee V.H.L., Kashi S.D. Grass G.M., Rubas W. Oral route of peptide and protein drug delivery. En: Peptide and protein drug delivery. Lee V.H.L. (Ed.) Marcel Dekker, Inc. New York, (1991) 16: 691-738.

[21] TenHoor C.N., Dressman J.B. Oral absorption of peptides and proteins. S.T.P. Pharma Sciences (1992) 2:(4) 301-312.

[22] Singh B.N., Majuru S. Oral delivery of therapeutic macromolecules: a perspective using the eligen ^{TM} technology, Drug Del. Technol. (2003) 3: 58-62.

[23] Clement S., Dandona P., Still J.G., Kosutic G. Oral modified insulin (HIM2) in patients with type 1 diabetes mellitus: results from a phase I/II clinical trial. Metabolism (2004) 53: 54-58.

[24] Mehta N.M. Oral delivery and recombinant production of peptide hormones. Part I: Making oral delivery possible. BioPharm Int. (2004) 17: 38-43.

extraordinarios avances en el conocimiento del comportamiento biológico de los nanosistemas permiten vislumbrar una nueva era en cuanto a su aplicación para la administración oral de macromoléculas. Los resultados obtenidos para péptidos asociados a nanosistemas son de interés intrínseco al desarrollo del trabajo experimental que se presenta, por lo que se describen a continuación en detalle.

Nanosistemas destinados a la administración oral de péptidos

El empleo de sistemas coloidales para la administración oral de macromoléculas ha adquirido un gran interés en los últimos años como consecuencia de su capacidad para interaccionar íntimamente con superficies biológicas. Estos sistemas han sido diseñados con el fin de proteger a la molécula encapsulada del ataque enzimático y de promover la absorción del fármaco a través del epitelio. En este sentido, juega un papel muy importante, no sólo el tamaño del sistema, sino también la composición del mismo. Los distintos nanosistemas y polímeros empleados para mejorar la absorción de los péptidos administrados por vía oral aparecen reflejados en la tabla 1. En dicha tabla se han omitido los trabajos realizados utilizando el quitosano para mejorar la absorción oral de péptidos ya que dicha revisión aparece reflejada en el artículo 1 de la presente tesis doctoral, titulado "The potential of chitosan of oral drug delivery".

Los estudios encaminados a aumentar la biodisponibilidad de la ciclosporina por vía oral o disminuir sus efectos adversos se centran en la elaboración de nanopartículas o nanocápsulas. Además del potencial de las nanopartículas de quitosano para mejorar la absorción oral de la ciclosporina[25] (discutido en el artículo 1) es destacable el interés que para dicho péptido ofrecen las nanocápsulas de poli-ε-caprolactona (PECL)[26]. Sin embargo, los resultados

[25] El-Shabouri M.H. Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A. Int. J. Pharm. (2002) 249: 101-108.

[26] Varela M.C., Guzman M., Molpeceres J., Aberturas M.R., Rodriguez-Puyol D., Rodriguez-Puyol M. Cyclosporine-loaded polycaprolactone nanoparticles: immunosuppression and nephrotoxicity in rats. Eur. J. Pharm. Sci. (2001) 12: 471-478.

obtenidos hasta el momento para las nanopartículas lipídicas[27, 28] no permiten concluir su eficacia como vehículos para la administración oral de ciclosporina. Por otro lado, tras la evaluación del potencial de las nanopartículas constituidos por polímeros pH-sensibles, se observó que la mejora de la biodisponibilidad oral de la ciclosporina era dependiente del tipo de polímero utilizado. Así pues, mientras las nanopartículas de Eudragit® S 100[29] permiten incrementar la absorción oral del péptido, las constituídas de Eudragit® RS 100 y RL 100 y ácidos grasos[30], así como las de ftalato de hidroxipropil metilcelulosa (HPMCP)[31], no dieron lugar a una mejora de la biodisponibilidad oral de la ciclosporina. Estas diferencias en el comportamiento de los distintos nanosistemas podrían estar en relación con sus características de liberación y en su capacidad de interacción con la mucosa intestinal.

En el caso concreto de la insulina, los trabajos más significativos realizados hasta el momento se basan en el desarrollo de nanocápsulas de cianoacrilatos, nanopartículas de polianhídridos e hidrogeles formados a partir de derivados metacrílicos. Así por ejemplo, tras la administración oral de insulina encapsulada en nanocápsulas de poli(isobutilcianoacrilato) (PIBCA), se observó un descenso marcado del nivel de glucosa en ratas diabéticas a partir del segundo día tras la administración y dicho efecto se prolongó hasta 20 días[32]. Dicho efecto biológico prolongado fue atribuido a la capacidad de las nanocápsulas de ser internalizadas en

[27] Zhang Q., Yie G., Li Y., Yang Q., Nagai T. Studies on the cyclosporin A loaded stearic acid nanoparticles. Int. J. Pharm. (2000) 200: 153-159.

[28] Bekerman T., Golenser J., Domb A. Cyclosporin nanoparticulate lipospheres for oral administration. J. Pharm. Sci. (2004) 93: 1264-1270.

[29] Dai J., Nagai T., Wang X., Zhang T., Meng M., Zhang Q. pH-sensitive nanoparticles for improving the oral bioavailability of cyclosporine A. Int. J. Pharm. (2004) 280: 229-240.

[30] Ubrich N., Schmidt C., Bodmeier R., Hoffman M., Maincent P. Oral evaluation in rabbits of cyclosporin-loaded Eudragit RS or RL nanoparticles. Int. J. Pharm. (2005) 288: 169-175.

[31] Wang X., Dai J., Chen Z., Zhang T., Xia G., Nagai T., Zhang Q. Bioavailability and pharmacokinetics of cyclosporine A-loaded pH-sensitive nanoparticles for oral administration. J. Control. Release (2004) 97: 421-429.

[32] Damge C., Michel C., Aprahamian M., Couvreur P. New approach for oral administration of insulin with poly(alkyl cyanoacrylate) nanocapsules as drug carrier. Diabetes (1988) 37: 246-251.

el epitelio intestinal y de liberar la insulina de forma sostenida[33]. Por otro lado, las nanocápsulas han mostrado poseer un efecto protector del péptido encapsulado frente a la degradación enzimática[33]. Estudios posteriores realizados en perros han permitido constatar el potencial de estas formulaciones, si bien los resultados del efecto hipoglucémico tanto en intensidad como en extensión han sido menos llamativos que los obtenidos en ratas[34].

Dentro de la gama de polímeros acrílicos utilizados para mejorar la absorción oral de insulina cabe destacar los interesantes resultados obtenidos para las nanopartículas elaboradas a partir de poli(ácido metacrílico-etilén glicol) P(MAA-g-EG)[35, 36, 37]. La eficacia de estos vehículos se debe a su capacidad para responder a cambios de pH, lo que permite proteger al péptido atrapado en la matriz del entorno gástrico y liberarlo en el fluido intestinal[35]. Además, se ha demostrado que estos vehículos poseen propiedades mucoadhesivas[38], así como también la facultad de inducir una reducción de la resistencia transepitelial en cultivos Caco-2, dando lugar a un aumento en el transporte de la insulina a través de las células[39].

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[33] Damge C., Michel C., Aprahamian M., Couvreur P., Devissaguet J.P. Nanocapsules as carriers for oral peptide delivery. J. Control. Release (1990) 13: 233-239.

[34] Damgé C., Hillaire-Buys D., Puech R., Hoeltzel A., Michel C., Ribes G.Effects of orally administered insulin nanocapsules in normal and diabetic dogs. Diabetes, Nutrition & Metabolism (1995) 8: 3-9.

[35] Lowman A.M., Morishita M., Kajita M., Nagai T., Peppas N.A. Oral delivery of insulin using pH-responsive complexation gels. J. Pharm. Sci. (1999) 88: 933-937.

[36] Nakamura K., Murray R.J., Joseph, J.I., Peppas N.A. Morishita, Mariko; Lowman, Anthony M. Oral insulin delivery using P(MAA-g-EG) hydrogels: effects of network morphology on insulin delivery characteristics. J. Control. Release (2004) 95: 589-599.

[37] Morishita M., Goto T., Peppas N.A., Joseph J.I., Torjman M.C., Munsick C., Nakamura K., Yamagata T., Takayama K., Lowman A.M. Mucosal insulin delivery systems based on complexation polymer hydrogels: effect of particle size on insulin enteral absorption. J. Control. Release (2004) 97: 115-124.

[38] Ascentiis A., deGrazia J.L., Bowman C.N., Colombo P., Peppas N.A. Mucoadhesion of poly(2-hydroxyethyl methacrylate) is improved when linear poly(ethylene oxide) chains are added to the polymer network. J. Control. Release (1995) 33: 197-201.

[39] Ichikawa H., Peppas N.A. Novel complexation hydrogels for oral peptide delivery: In vitro evaluation of their cytocompatibility and insulin-transport enhancing effects using Caco-2 cell monolayers. J. Biomed. Mat. Res. Part A (2003) 67: 609-617.

Las nanopartículas de poli(ácido láctico-glicólico) (PLGA) en asociación con polianhídridos, ácido fumárico y sebácico, también han resultado exitosas para mejorar la absorción oral de la insulina[40] [41]. Los autores atribuyen este resultado positivo a las propiedades mucoadhesivas de los polianhídridos y a la capacidad de las nanopartículas de ser internalizadas. No obstante también señalan la necesidad de incorporar PLGA a las nanoestructuras ya que las formulaciones preparadas sólo con polianhídridos o con PLGA no resultaron exitosas.

En cuanto a las formulaciones coloidales destinadas a mejorar la absorción intestinal de calcitonina, cabe destacar las nanopartículas de poliestireno recubiertas con derivados polivinílicos, las nanopartículas de PLGA conteniendo complejos de ácidos grasos y los liposomas de doble capa.

La asociación de calcitonina a nanopartículas constituidas a base de núcleos de poliestireno recubiertos por cadenas de derivados de poli-ácido metacrílico, poli-acrilamida, poli-vinilamida y poli-vinilacetamida permitió lograr un efecto hipocalcémico dependiente de la estructura química del material de recubrimiento[42, 43, 44]. Así pues, los mejores resultados se obtuvieron tras la administración de estas nanopartículas recubiertas por cadenas hidrofílicas catiónicas (cadenas de poli-N-isopropilacrilamida copolimerizadas con poli-vinilacetamida). El descenso del nivel de calcio observado con estas nanopartículas se atribuyó a sus propiedades

[40] Mathiowitz E., Jacob J.S., Jong Y.S., Carino G.P., Chickering D.E., Chaturvedi P., Santos C.A., Vijayaraghavan K., Montgomery S., Bassett M., Morrell C. Biologically erodable microspheres as potential oral drug delivery systems. Nature (1997) 386: 410-414.

[41] Carino G.P., Jacob J.S., Mathiowitz E. Nanosphere based oral insulin delivery. J. Control. Release (2000) 65: 261-269.

[42] Sakuma S., Suzuki N., Kikuchi H., Hiwatari K., Arikawa K., Kishida A., Akashi M. Oral peptide delivery using nanoparticles composed of novel graft copolymers having hydrophobic backbone and hydrophilic branches. Int. J. Pharm. (1997) 149: 93-106.

[43] Sakuma S., Suzuki N., Kikuchi H., Hiwatari K., Arikawa K., Kishida A., Akashi M. Absorption enhancement of orally administered salmon calcitonin by polystyrene nanoparticles having poly(N-isopropylacrylamide) branches on their surfaces. Int. J. Pharm. (1997) 158: 69-78.

[44] Sakuma S., Suzuki N., Sudo R., Hiwatari K., Kishida A., Akashi M. Optimized chemical structure of nanoparticles as carriers for oral delivery of salmon calcitonin. Int. J. Pharm. (2002) 239: 185-195.

mucoadhesivas[45] y a la capacidad de inhibir la degradación enzimática del péptido asociado[46].

En cuanto al éxito de las nanopartículas de PLGA para la administración oral de calcitonina (en forma de complejo con oleato sódico) cabe destacar un aumento importante de la concentración del péptido en sangre[47]. Este resultado fue atribuido al efecto protector de las nanopartículas frente a la actividad enzimática. Por otro lado, estudios realizados en el cultivo celular Caco-2 mostraron que las nanopartículas poseen capacidad para transportar la calcitonina a través del epitelio intestinal mediante transcitosis.

Por último, los estudios realizados tras la asociación de calcitonina a liposomas de doble capa con carga positiva, negativa y neutra mostraron que el máximo efecto hipocalcémico ocurría con los liposomas dobles cargados positivamente[48, 49]. Este efecto se atribuyó a la interacción entre el liposoma cargado positivamente y la mucosa intestinal. Hipotéticamente, dicha interacción daría lugar a un aumento del tiempo de residencia del sistema en el intestino, traduciéndose en una mejora en la absorción del péptido.

En definitiva, el conjunto de los resultados recogidos en la literatura relativos a la utilización de nanosistemas para mejorar la absorción de péptidos indican que el éxito de los mismos está directamente relacionado con su capacidad protectora del péptido encapsulado, así como también con su facilidad para

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interaccionar con la mucosa intestinal. Asimismo, en algunos casos, se señala el efecto de los polímeros constitutivos de los nanosistemas en las uniones estrechas intercelulares. No obstante, además de estos factores, un aspecto determinante, aunque no suficientemente discutido, de la eficacia de los nanosistemas reside en su estabilidad en los fluidos gastrointestinales y en su capacidad de control de la liberación. En efecto, estos nanosistemas no han de liberar el péptido asociado de forma prematura en los fluidos gastrointestinales; sin embargo han de procurar su liberación una vez en contacto con la mucosa intestinal. Por tanto, se podría concluir resaltando el interesante potencial de los nanosistemas a la vez que llamando la atención acerca de la necesidad de su optimización de forma individualizada, en función de las características del péptido a asociar.

Tabla 1: Estudios basados en la administración oral de péptidos encapsulados en nanoestructuras.

Sistema	**Composición**	**Fármaco**	**Parámetro analizado**	**Ref.**
Nanopartículas	PECL	Ciclosporina	Biodisponibilidad	[50]
Nanopartículas	Ácido esteárico	Ciclosporina	Biodisponibilidad	[51]
Nanopartículas	Lipídicas	Ciclosporina	Biodisponibilidad	[52]
Nanopartículas	Eudragit®	Ciclosporina	Biodisponibilidad	[53]
Nanopartículas	Eudragit® y ácidos grasos	Ciclosporina	Biodisponibilidad.	[54]
Nanopartículas	HPMCP	Ciclosporina	Biodisponibilidad	[55]
Nanocápsulas	PIBCA	Insulina	Respuesta farmacológica	[56, 57, 58]
Nanocápsulas	PIBCA	Insulina	Biodisponibilidad	[59]
Nanopartículas	PIBCA	Insulina	Respuesta farmacológica	[60, 61]
Nanopartículas	PECA	Insulina	Respuesta farmacológica	[62]
Hidrogeles	P(MAA-g-EG)	Insulina	Respuesta farmacológica Biodisponibilidad	[63, 64, 65]
Nanopartículas	P(FA:SA)	Insulina	Respuesta farmacológica	[66]
Nanopartículas	FAO:PLGA	Insulina	Respuesta farmacológica	[67]
Nanopartículas	PS-g-PNIPAA/ PS-g-PNIPAA-g-PVA	Calcitonina	Respuesta farmacológica	[68, 69, 70]
Nanopartículas	PLGA/ ácidos grasos	Calcitonina	Biodisponibilidad	[71]

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Abreviaturas

DMPC: Dimiristoil- fosfatidilcolina

DPPC: dipalmitoil-fosfatidilcolina

FAO:PLGA: Oligómeros de ácido fumárico: ácido poliláctico

HPMCP: Ftalato de hidroxipropil metilcelulosa

P(FA:SA): poli(ácido fumárico y sebácico)

P(MAA-g-EG): poli(ácido metacrílico-etilén glicol)

PECA: poli(etilcianoacrilato)

PECL: poli-ε-caprolactona

PIBCA: poli(isobutilcianoacrilato)

PIHCA: poli(isohexilcianoacrilato)

PLGA: poli(ácido láctico-glicólico)

PS-g-PNIPAA: poliestireno-poli-N-isopropilacrilamida

PS-g-PNIPAA-g-PVA: poliestireno-poli-N-isopropilacrilamida-g-polivinilamina

SA: estearilamina

Artículo de revisión: El potencial del quitosano para la administración oral de péptidos.

Artículo 1

The potential of chitosan for the oral administration of peptides

Cecilia Prego, Dolores Torres and María José Alonso*

Expert Opinion on Drug Delivery (aceptado)

Abstract

Over the last years, a major challenge in drug delivery has been the design of appropriate vehicles for the oral administration of macromolecular drugs (peptides and proteins). Indeed, despite the increasing market value of these complex molecules, their clinical use has been highly limited by their reduced oral bioavailability. Among the different delivery approaches explored so far, those based upon the use of the polysaccharide chitosan have opened promising alternatives towards this ambitious goal. This is due to the interesting physicochemical and biopharmaceutical properties of this polymer. This article describes the advances that have been made in the design of chitosan-based systems specially adapted for the oral administration of peptides. These systems include: solutions, microspheres, nanoparticles, nanocapsules and liposomes. More specifically, the article discusses the efficacy of the different delivery approaches for improving the absorption of peptides, and analyzes the various mechanisms that have been proposed for the understanding of their efficacy.

Keywords: chitosan, oral administration, delivery systems, peptides/proteins.

Introduction

Chitosan is a natural polymer synthesized by alkaline deacetylation of chitin, which is the second polysaccharide most abundant in nature after cellulose. This polymer can be found in the exoskeleton of crustacean, insects and some fungi. The main difference in the chemical structure between chitin and chitosan [α (1→4) 2-amino 2-deoxy β-D glucan] is related to the number of acetyl groups (Fig. 1). This small difference makes chitin insoluble in water or in the most common organic solvents, whereas chitosan base is soluble in acidic solutions.

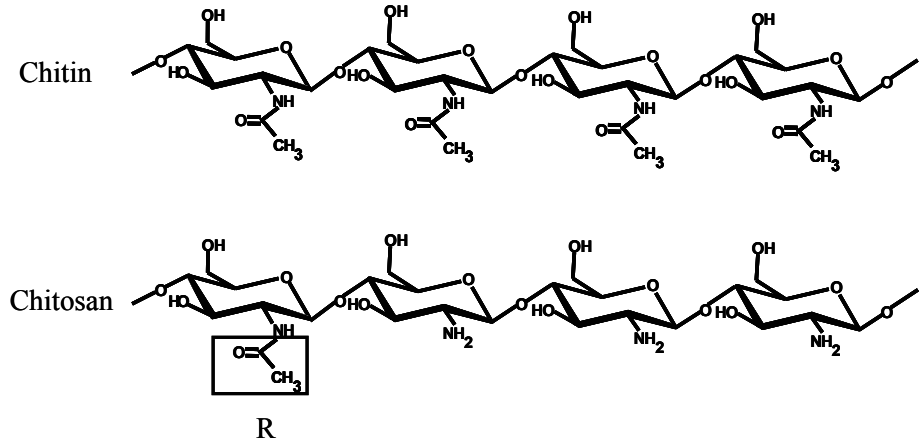

Figure 1: Chemical structure of chitin and chitosan. R: N-acetyl group (acetylation percentage usually < 50%).

Chitosan was discovered in 1859 by Rouget [1], however, most of the reports on its biological and pharmaceutical applications have been published in the last couple of decades. Biomedical and biological applications of chitosan include its use as a dietary supplement for weight loss and as a hypocholesterolemic [2], antimicrobial [3] and wound healing [4] agent. On the other hand, properties such as good biocompatibility, low toxicity [5, 6], biodegradation by lysozyme [5, 7] make of chitosan a new promising biomaterial.

From a pharmaceutical perspective, chitosan has also attracted significant attention for a variety of applications and modalities of administration. One area in which interest is growing is related to the use of chitosan as a material for transmucosal drug delivery. This is justified by some interesting characteristics of chitosan, such as bioadhesiveness and absorption promoting characteristics. These two properties have been exploited for achieving either, local or systemic drug delivery. For example, chitosan has become particularly well known because of its ability to increase the systemic absorption of drugs and vaccines administered intranasally [8-13]. A clear proof of its efficacy and acceptability is the fact that there are several formulations in different stages of clinical trials. For example, at this moment a chitosan-based liquid formulation is in clinical evaluation for nasal administration of several drugs and vaccines [14, 15]. Chitosan has also shown interesting potential as an ocular drug delivery agent [16]. For this specific modality of administration, the interest relies on the ability of chitosan-based systems to enhance the intensity and time of retention of topically applied drugs [17-19]. A wide range of applications have also been identified for the use of chitosan in oral drug delivery. A simple one would be its use as a hydrophilic excipient to increase the solubility of poorly soluble drugs [20]. Chitosan can also be presented in the form of microspheres (cross-linked or reacetylated) which have the ability to control the release of therapeutic agents along the intestinal tract [21, 22] or to deliver drugs, i.e. antibiotics, locally to the gastric wall [23]. Finally, a number of chitosan derivatives and chitosan-based carriers have been proposed to increase the systemic absorption of peptides by providing the polymer with a better solubility at the absorption intestinal pH or an enhanced mucoadhesive and permeation enhancing properties [24-26].

There are already several general reviews on the general potential of chitosan in the field of drug delivery [26-28] as well as specific reviews covering the application of chitosan for nasal [29] and ocular drug delivery [16]. Therefore, in this article we aimed to review a specific application of chitosan with a great market potential: the enhancement of the oral absorption of peptides. This is with no doubt one of the greatest challenges confronted by the pharmaceutical scientists in the last decade. A challenge that, on the other hand, is clearly justified by the increasing

number of macromolecular drugs approved and which have to be administered parenterally. Keeping this goal in mind, we will first describe the physicochemical and the biopharmaceutical properties of chitosan that are critical for the oral application of chitosan. Then, in the second part, we will present the features and efficacy of the different chitosan delivery systems evaluated so far for oral peptide delivery, making the distinction between solutions, microspheres and nanostructures. Finally, we will analyze the mechanisms suggested until now in order to explain the way these systems are able to enhance the bioavailability of orally administered peptides.

Chitosan properties

Bearing in mind that this review is focused on the use of chitosan as a material for oral peptide delivery, in the next paragraphs we will discuss about some physicochemical and biological properties which may affect this specific application of chitosan. In this sense, it is important to keep in mind that chitosan comprises a series of polymers, which vary in the percentage of N-acetyl groups, the degree of deacetylation, and in the molecular weight. Both characteristics are critical, not only from a physicochemical point of view, but also from a biological perspective.

Solubility properties

A property that affects greatly the solubility of chitosan is its deacetylation degree. Highly deacetylated chitosans (85%) are readily soluble up to a pH value of 6.5. However, their solubility decreases significantly with the deacetylation degree [30]. Consequently, most of the studies regarding the pharmaceutical use of chitosan have been performed with highly deacetylated chitosan.

Highly deacetylated and purified chitosans are commercially available, in a broad range of molecular weights, in the form of a base and also as a salt. The chitosan base form is soluble in acidic solutions such as hydrochloric, glutamic,

acetic and lactic acid solutions, in which the amino groups of chitosan become protonated leading to a positively charged polymer. Obviously, chitosan salts do not require the use of acids and are readily soluble in water. However, irrespective of the initial form, the solubility of chitosan decreases significantly when raising the pH to neutral or basic values. In addition to the pH, the ionic strength affects the solubility of chitosan. The higher the ionic strength is, the lower the solubility. In fact, a higher electrolyte concentration results in a salting-out effect which leads to the precipitation of the polymer [31].

This solubility behaviour is important from the perspective of the use of chitosan for oral administration. Indeed, chitosans administered orally in the form of an aqueous solution are expected to precipitate upon reaching the intestinal region due to the increase in the pH up to values in the range 6.5-7.5. On the other hand, chitosans administered as powders are supposed to dissolve in the acidic pH of the gastric cavity and, then, precipitate in the intestinal compartment. Consequently, the behaviour of classical solutions and powders justifies the search of optimized presentations of chitosan, either in the form of a chemical derivative that is soluble at the intestinal pH or, in the form of a device (nanoparticles/ microspheres) that is stable in the physiological conditions of the gastrointestinal tract.

Penetration enhancement properties

It is well known that chitosan can enhance the permeability of different compounds through the intestinal monolayer. This has been very clearly shown using the Caco-2 cell line for a variety of compounds such as buserelin, inulin, mannitol, and horseradish peroxidase [32-36]. The increase in the permeability has been generally related to a decrease in the transepithelial electric resistance (TEER) which was attributed to a partial disruption of the tight junctions. Moreover, the results of these studies have shown that the increase in the permeability of the monolayer and, hence, on the transport of drugs is dependent on a number of factors, such as the chitosan dose as well as the chitosan molecular weight and deacetylation degree [33-35]. More specifically, Schipper et al. [34] observed, using mannitol as a

model molecule, that chitosans with low acetylation degree (1-15%) showed a clear effect on the transport of the molecule, regardless the molecular weight. In contrast, for high acetylation degree of chitosan (>35%), a permeability enhancement of mannitol across the monolayers was only achieved with high molecular weight chitosans.

Despite the evidence of the penetration enhancement ability of chitosan, the mechanism of action remains unclear. Studies performed at the beginning nineties concluded that the exposure of the cell surface to chitosan solutions induces clear changes in the F-actin distribution [32]. Some years later, it was observed that chitosan induces not only a redistribution of F-actin but also that of the tight junction proteins zona occludens 1 (ZO-1) [33, 35]. In addition, it was found that the content of ZO-1 and occludin in the cytoskeleton increase, revealing the ability of chitosan to disrupt the epithelial cell tight junctions involving the translocation of the proteins ZO-1 and occludin from the membrane to the cytoskeleton [35]. Very recently, the same authors found that chitosan acts, at least in part, via an activation of protein kinase C (PKC). [37] Interestingly, the results of the previous studies [33-35] also indicated that the effect of chitosan on the Caco-2 cell monolayer is reversible and, hence, that the opening of the cellular barrier is transient. This specific behavior makes a great difference, in terms of toxicity, between chitosan and the classical penetration enhancers which are known to cause irreversible epithelial damage.

Within the context of this review article, we found it important to analyze this mechanism of action of chitosan with regard to its ability to enhance the absorption of peptides. However, as will be discussed later, the relevance of this mechanism on the efficacy of a specific chitosan-based oral drug delivery system will be obviously dependent on the characteristics of the device and, obviously, on the physical state of chitosan.

Mucoadhesive properties

One of the limitations of the oral route is the rapid transit that reduces the chances for the drug to interact with the absorptive epithelium. One of the approaches to deal with this limitation has been based upon the use of materials which favour the interaction of the drug with the mucus layer that covers the intestinal epithelium. Chitosan belongs to the category of these so-called mucoadhesive materials. Indeed, due to its positive charge, chitosan is able to interact with the negatively charged mucus components [38, 39]. This property has attracted significant attention to the use of chitosan for transmucosal drug delivery and, in particular, for nasal drug delivery. For example, in this case, the chitosan-mucus interaction leads to the formation of a viscous gel that reduces the mucociliary clearance and increases the residence time of the drug in the absorptive mucosa [29]. However, while the advantage of this property is clear for nasal application, its contribution to the potential of chitosan for oral drug delivery is uncertain. This is due to the fact that the mucus covering the intestinal wall undergoes a rapid turnover and, consequently, the mucoadhesion of chitosan does not necessarily imply a more intense and prolonged contact of the co-administered drug with the absorptive epithelium. Therefore, a critical point in the design of a chitosan-based mucoadhesive delivery device would be to achieve, first, a facilitated interaction with the mucus and, second, an adequate diffusion through the mucus layer towards the underlying epithelium. In addition, as mentioned above, classical chitosan solutions and powders are expected to precipitate upon reaching the intestinal tract and this uncontrolled precipitation may logically affect the inherent mucoadhesive properties of chitosan.

Irrespective of the importance of the role of mucoadhesion in the efficacy of chitosan-based delivery systems, there are some characteristics of chitosan that have been found to affect the intensity of the mucoadhesion phenomenon. Logically, as expected from the mechanism of mucoadhesion, the mucoadhesive character of chitosan is dependent on its acetylation degree. Indeed, a higher deacetylation degree of chitosan leads to a more important number of positive charges and, hence, to a more marked adhesiveness [39].

On the other hand, the molecular weight of chitosan has also been regarded as a parameter that affects the mucoadhesive properties of chitosan. For example, Kawashima et al. [40] observed, using a rat everted intestinal sac, that the mucoadhesion of chitosan increased with the molecular weight. The same conclusion was derived from a rheological study aimed at predicting the mucoadhesive properties of chitosan. The results of this study indicated that the mucoadhesion forces could be modulated by adjusting the chitosan molecular weight and concentration [41].

As in the case of the permeability enhancing property, it is worthwhile to mention that, despite the evidence of the chitosan inherent properties on its mucoadhesive character, the consequence that these properties may have on the behavior of chitosan-based delivery devices will obviously be highly dependent on the specific characteristics of the device.

Chitosan toxicity issues and regulatory status

The safety of chitosan has been investigated, showing its low toxicity and biocompatibility [5, 6, 42]. In fact, the oral LD50 of chitosan in rodents has been reported to be over 16 g/kg [42], showing that chitosan is safe following oral administration. On the other hand, the results in humans indicate that it is necessary to consume several grams of chitosan a day in order to observe signs of constipation or diarrhea [43, 44]. These amounts are far beyond those needed in pharmaceutical formulations and, consequently, it is accepted that the risk of side effects following oral administration of chitosan formulations is negligible.

With regard to the regulatory aspects, there is a monograph of chitosan hydrochloride in the European Pharmacopoeia (EP1774). In addition, The American Society for Testing and Materials (ASTM) has published a guideline (ASTM F 2103) for the characterization of chitosans for use in Tissue Engineered Medical Products (TEMPs). Finally, according to the information provided by the company

Novamatrix, a Drug Master File covering the chitosan salts and bases have been submitted to the US FDA in July 2004.

Chitosan-based systems for oral peptide delivery

Over the last years, a number of chitosan-based formulations (i.e. solutions, microspheres and nanostructures) have been developed for improving the oral administration of peptides and proteins. In this section we describe the performance of these vehicles in terms of their ability to enhance the intestinal absorption as well as the mechanistic details. The pharmacological efficacy obtained for the two model peptides, insulin and calcitonin, that have received the greatest attention as candidates for these chitosan-based carriers are summarized in Tables 1 and 2.

Table 1: Pharmacological efficacy obtained after oral administration to rats of insulin encapsulated in chitosan-based carriers.

Chitosan-based carrier	Drug	Dose (IU/kg)	R_{max}[a] (%)	t_{max}[b] (h)	Duration of effect	Reference
Chitosan nanoparticles[c]	Insulin	7, 14, and 21	60	10	From 8 until 24 h	Pan *et al.*, 2002 [68]
Chitosan nanoparticles[c]	Insulin	50 and 100	50	19	From 13 h until 24 hours	Ma *et al.*, 2001 [69]
Chitosan/glucomannan nanoparticles	Insulin	50	50	14	From 14h until 24 h	Alonso-Sande *et al.*, 2004 [73]
Chitosan-coated liposomes	Insulin	100	30	3	From 30 min until 12 h	Takeuchi *et al.*, 1996 [82]

[a]: Maximum pharmacological effect.
[b]: Time of the maximum pharmacological effect.
[c]: *In vivo* study performed in diabetic rats.

Table 2: Pharmacological efficacy obtained after oral administration to rats of calcitonin encapsulated in chitosan-based carriers.

Chitosan-based carrier	Drug	Dose (IU/kg)	R_{max}[a] (%)	t_{max}[b] (h)	Duration of effect	Reference
Enteric-coated chitosan-based microspheres	Calcitonin	500[c]	15	10	From 8 h until 12 h	Lamprecht *et al.*, 2004 [59]
Chitosan nanocapsules	Calcitonin	500	30	1	More than 24 h	Prego *et al.*, 2005 [76]
Chitosan-coated solid nanoparticles	Calcitonin	500	30	1	More than 24 h	Garcia-Fuentes *et al.*, 2005 [82]
Chitosan-coated PLGA nanoparticles	Calcitonin	125, 250 and 500	25	8	36 h	Kawashi-ma *et al.*, 2000 [40]
Chitosan-coated liposomes	Calcitonin	500	22	2	8 h	Takeuchi *et al.*, 2003 [84]

[a]: Maximum pharmacological effect.
[b]: Time of the maximum pharmacological effect.
[c]: Expressed by the authors in mg/kg and converted to IU taking the relation 1mg=5000IU.

Chitosan solutions

Most of the studies intended to evaluate the ability of chitosan solutions to improve the absorption of drugs across the intestinal epithelium were performed *in vitro,* either in cell culture [45] or in rat intestinal [46]. The *in vivo* evaluation of the effectiveness of chitosan solutions for oral peptide delivery is limited. This is understandable if we take into account that, as indicated above, chitosan precipitates at the pH of the intestinal tract (~6.5-7.5). In fact, from our literature search, we only found one study reporting the *in vivo* absorption enhancing effects of chitosan hydrochloride [47]. More concretely, the results showed an increase in the bioavailability of a nonapeptide following intraduodenal injection to rats. This positive result was attributed to the inherent capacity of chitosan to open the intercellular junctions.

The problem associated to the low solubility of chitosan at neutral and high pH values could be overcome by chemical modification of the chitosan molecule. For example, the derivative, N-trimethyl chitosan, was found to be readily soluble at neutral and basic pH values [48]. Unfortunately, the studies performed in Caco-2 cells showed that the mucoadhesive [49] and the promoting enhancing [46] properties of trimethylated chitosan were not as remarkable as those of the parent chitosan molecule. According to the authors [46, 49], this phenomenon could be related to a change in the conformation of the trimethylated chitosan that reduces the flexibility of the polymer molecules and, therefore, the interpenetration into the mucus layer. This reduced mucoadhesion could also be due to a decrease in the density of amino groups available for protonation subsequent to the chemical modification. Nevertheless, despite the reduced mucoadhesion of the chemically modified polymers in the *in vitro* cell line, the *in vivo* studies performed in rats or pigs revealed that trimethylated chitosan was significantly more efficient than chitosan hydrochloride at increasing the absorption of the peptide octreotide [50, 51]. This greater efficacy was attributed to the more important absorption enhancing effect of trimethylated chitosan at neutral pH values as compared to chitosan hydrochloride [52].

A quite successful approach has been the chemical modification of chitosan by the introduction of a thiol group [53]. Thiolated chitosans exhibited improved mucoadhesive properties *in vitro* [54] as well as an enhancement in the epithelial drug permeability [55]. Their in vitro behavior correlated well with their ability to increase the absorption of calcitonin or insulin following oral administration to rats [53, 56]. It should be, however, clarified that, in these studies, chitosan was presented in the form of a solid matrix (1.5 mm minitablets).

Chitosan-based microspheres

A different strategy towards increasing the systemic absorption of peptides administered orally has been designed specifically to deliver drugs in the colonic region. The strategy was proposed to take advantage of two critical facts: the limited

peptide enzymatic activity, as compared to that of the small intestine, and the markedly slower rate of colonic transit. Chitosan-based microspheres were chosen as candidate vehicles to achieve this goal due to the specific degradation of chitosan in the colonic microflora [57, 58], and to its mucoadhesive/absorption enhancing effects. Indeed, if conveniently designed, chitosan-based microspheres can travel intact along the gastrointestinal tract and reach the colonic region. Once in this region, the polymer matrix degrades and releases the peptide which, at this level, is free to cross the colonic mucosa.

The technological approach, adopted to prevent the alteration of chitosan-based microspheres during their gastrointestinal transit, was their entrapment or coating with pH-sensitive polymers (acrylic or cellulosic) [59-61]. Using fluorescent markers, i.e. carboxyfluorescein, or the peptide insulin, it has been shown that the pH-sensitive microspheres containing chitosan exhibit a pH-dependent release behavior. More specifically, when exposed to a pH-gradient they provide a negligible release until the colonic pH was reached (~6.5-7), and then, a continuous and controlled drug release [59-61].

Unfortunately, the limited number of *in vivo* studies intended to evidence the efficacy of these pH-sensitive chitosan systems do not permit us to extract clear conclusions regarding the efficacy of this approach. For example in the study performed by Lamprecht *et al.* [59], aimed at evaluating the performance of enteric-coated chitosan-based microspheres containing calcitonin, it was observed that the pharmacological response was not affected by the presence of chitosan. Consequently, the authors concluded that the success of the formulation was due to the enteric coating and that the role of chitosan was negligible in this type of formulation. These results differ from those observed by Tozaki et al. [62] who studied the efficacy of a large chitosan capsule, administered orally to rats. The chitosan capsule had an enteric polymer coating and contained insulin in association with an absorption enhancer and an enzyme inhibitor. A hypoglycemic response was noted at 6 hours post-administration, when the capsule reached the colon, giving an insulin bioavailability of 5.73%. The authors justified the success of this formulation to the specific disintegration of the capsules in the colonic region and to the

absorption enhancing properties of chitosan. In addition, they found that the positive effect of chitosan could be reinforced by the co-administration of other absorption enhancers.

Therefore, while this colonic delivery approach offers some potential, it is quite possible that the performance of a pH sensitive chitosan delivery system will be dependent on the type of peptide as well as on the specific composition of the final formulation.

Chitosan nanostructures

Over the last decade, a number of nanostructures based on chitosan and chitosan derivatives have been proposed for the oral administration of peptides. These nanostructures can be classified into three categories: chitosan-based self-assembling structures, chitosan-based nanoparticles and chitosan-coated nanosystems. Self-assembling nanostructures can be formed using specific chemical derivatives of chitosan. Chitosan-based nanoparticles can be composed of simply cross-linked chitosan or chitosan in combination with other hydrophilic polymers. Chitosan-coated nanosystems include chitosan-coated solid hydrophobic nanoparticles, chitosan-coated liposomes and chitosan-coated oily nanodroplets, also called nanocapsules.

Chitosan-based self assembling nanostructures

Recently, a number of authors have reported the chemical modification of chitosan with hydrophobic groups like palmitoyl, or linolenic or deoxycholic acid. The attachment of the hydrophobic moiety led the polymer to self-assemble forming a micelle-like nanostructure [63-65]. Moreover these systems have shown a capacity to entrap peptide molecules such as bovine serum albumin [65].

A different type of nanostructure was described by Ohya et al. [66]. According to these authors, the grafting of hydrophilic polymers such as PEG to the chitosan backbone provides the polymer with the ability to self-aggregate in aqueous media due to the formation of intermolecular hydrogen bridges. Moreover, these nanostructures could associate insulin and release it in a controlled fashion depending on the degree of PEGylation of chitosan.

Unfortunately, although these self-assembling nanostructures have been proposed as promising carriers for the delivery of labile molecules like peptides and proteins, so far there is no evidence of their performance following *in vivo* administration.

Chitosan-based nanoparticles

Chitosan has the ability to gel upon contact with specific polyanions. Taking advantage of this specific property, a few years ago, we developed nanoparticles made of solely chitosan or chitosan in combination with other hydrophilic polymers such as poly(ethyleneglycol) (PEG) [67]. These nanoparticles are formed immediately upon mixing two aqueous phases (one containing chitosan and the other one containing sodium tripolyphosphate) through inter and intramolecular linkages created between phosphates and chitosan amino groups. Besides the advantage of being produced under extremely mild conditions, these nanoparticles have shown a great capacity for the association of peptides and proteins like bovine serum albumin, tetanus toxoid and insulin [11, 12, 67]. The physical appearance of these nanoparticles as seen by transmission electronic microscopy is shown in figure 2A and compared to that of chitosan-coated systems, which will be described later.

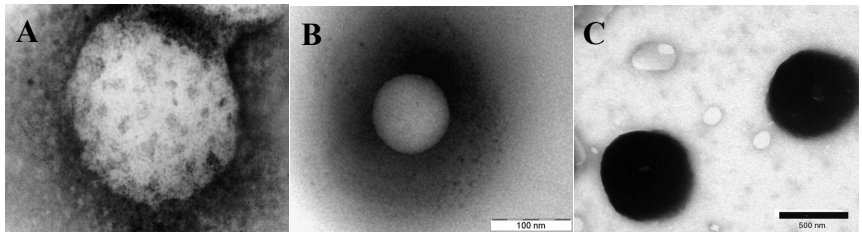

Figure 2: Transmission electron micrographs of: A) chitosan nanoparticles, B) chitosan nanocapsules and C) chitosan-coated tripalmitin nanoparticles*.* Figure from Garcia-Fuentes *et al.* [79].

More recently, these ionically cross-linked nanoparticles were tested for their efficacy as carriers for oral peptide administration [68]. The results obtained following oral administration of insulin-loaded chitosan nanoparticles to diabetic rats revealed their efficacy at improving the hypoglycemic response of insulin. More specifically, they observed a reduction of the blood glucose levels at 8 hours post-administration and this response was prolonged for up to 24 hours. In contrast, chitosan solution, used as a control, was totally inefficient at increasing the intestinal absorption of insulin. This report was coincident with that of Ma *et al.*, [69] who evaluated the efficacy of the same type of nanoparticles after oral administration to normal and diabetic rats. They observed that, while no significant hypoglycemic response was registered in normal rats, a marked hypoglycemic effect was observed in similarly dosed diabetic rats. The onset of action occurred at 10 hours post-administration and the effect was maintained for a few hours.

With respect to the mechanism by which chitosan nanoparticles are able to increase the hypoglycemic response of orally administered insulin, the authors [68, 69] have speculated about several possible effects: the protection of the peptide from degradation in the gastrointestinal tract, and the potential mucoadhesive and absorption enhancing properties of chitosan. Furthermore, the authors argued that the long-term response could be associated with the uptake of the nanoparticles by the M cells overlaying the Peyer's patches. Despite the advances made in this sense, the mechanism of action of these nanoparticles has not been fully elucidated. For example, using the Caco-2 model cell line we could observe that some chitosan

nanoparticles were able to enter into the cells and that this interaction with the monolayer was more important in the case of the mucus secreting cells (MTX-E12) [70]. Consequently, we concluded that the presence of mucus favoured the interaction of the particles with the underlying epithelium. Unfortunately, we can not directly translate this interpretation to the *in vivo* situation. However, we can reasonably accept that the nanoparticles may protect the peptide molecules from degradation and facilitate their interaction with the absorptive mucosa.

More recently, we have produced a different type of nanoparticles made of chitosan in association with the polysaccharide glucomannan, using the same principle of ionic cross-linking [71]. The rational for the design of this novel colloidal carrier was that glucomannan would improve the stability of nanoparticles in the gastrointestinal fluids and also facilitate the interaction of nanoparticles with mannose receptors present in the epithelial cells [72]. In order to validate this hypothesis we chose insulin as a model peptide and tested the efficacy of the carrier following oral administration to normal rats [73]. Interestingly, chitosan/glucomannan nanoparticles were able to elicit a delayed hypoglycemic response at 14 hours post-administration and this response was maintained for at least 10 hours. However, under the experimental conditions of this study, we could not observe a significant response for the insulin associated to chitosan nanoparticles, used as a control. The success of chitosan/glucomannan nanoparticles as compared to those made of chitosan could be related to the observed stabilizing effect of glucomannan [73]. In fact, very recently, we have observed a similar response for chitosan nanoparticles stabilized with poloxamer (non-published results). While the stabilization effect of glucomannan has been verified, its ability to improve the uptake of nanoparticles remains to be investigated.

Chitosan nanoparticles have also been explored for their efficacy to increase the systemic absorption of hydrophobic peptides such as cyclosporin-A [74]. In this study chitosan nanoparticles were administered orally to beagle dogs, and the currently available cyclosporin-A microemulsion (Neoral®) was used as a control. The results indicated that chitosan nanoparticles provided an improved absorption and, hence, a greater bioavailability of cyclosporine A, as compared to

the control microemulsion. The authors understood that this positive behaviour was due to a combination of the mucoadhesion and the ability to open the tight junctions of epithelial cells, inherent properties of chitosan. However, no mechanistic studies were undertaken in order to verify this proposed mechanism.

Chitosan-coated nanostructures

Chitosan has also been used as a coating material in order to change the surface properties of colloidal drug carriers. As indicated above, different types of nanosystems, oily nanodroplets, solid nanoparticles and liposomes have been selected as cores for the coating process. The purpose while designing these systems was to improve the interaction of the cores with mucosal surfaces. Obviously, the nature of the core is expected to affect the drug encapsulation and release properties of the system.

Chitosan nanocapsules

A few years ago, we developed chitosan-coated oily nanodroplets, also called nanocapsules [75]. The formation of these nanosystems was possible using the solvent displacement technique, but introducing an important modification: the incorporation of chitosan to the aqueous phase. The physical appearance of the coated systems is the one presented in figure 2B. Recently, we have investigated the efficacy of chitosan nanocapsules as carriers for oral peptide delivery using salmon calcitonin as a model peptide [76, 77]. We have monitored the reduction of serum calcium levels after oral administration of calcitonin-loaded chitosan nanocapsules and used an aqueous solution and a nanoemulsion containing calcitonin as controls. Interestingly, as shown in figure 3, a marked hypocalcemic response was noted when the peptide was associated to the nanocapsules, whereas no significant effect was observed after administration of the solution or the uncoated nanoemulsion. Therefore, a conclusion from these data was the chitosan coating was critical for the performance of the formulation. Moreover, the percentage of reduction of the serum

calcium levels achieved following administration of chitosan nanocapsules was maintained for, at least, 24 hours [76]. This pronounced and long-lasting hypocalcemic effect led us to speculate about the adhesion of the carrier and a sustained release of the associated peptide from the absorptive epithelium towards the blood stream.

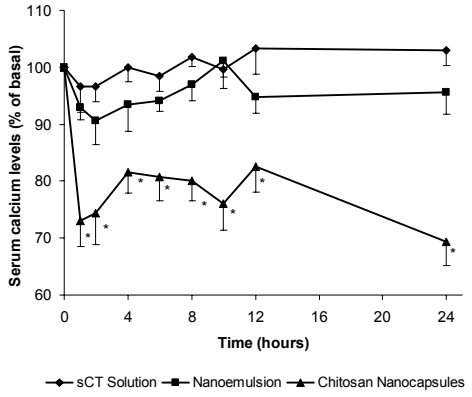

Figure 3: Hypocalcemic effect after oral administration in rats of chitosan nanocapsules as well as the control nanoemulsion and the aqueous solution of salmon calcitonin (mean ± s.e.; n=6).* Statistically significant differences from salmon calcitonin solution ($p<0.05$). Data from Prego *et al.* [76].

In order to obtain some evidence on the mechanism of action of chitosan nanocapsules we have performed some studies in the Caco-2 model cell line [76]. Two main conclusions were extracted from these experiments: (i) chitosan nanocapsules were able to reduce the TEER of the Caco-2 cell monolayer in a dose-dependent manner; however, the important concentration required to detect a significant change in permeability, led us to conclude that this phenomenon can not be taken as an explanation for the success of the formulation following *in vivo* administration; (ii) confocal experiments performed with rhodamine-labeled chitosan nanocapsules, suggested that both, the nanocapsules and the corresponding control emulsions, were able to enter Caco-2 cells monolayer; however, the level of internalization was very low and similar for coated and uncoated carriers and, consequently, these results do not justify the *in vivo* success of the chitosan-coated

systems. More recently, we tested chitosan nanocapsules using a co-culture of Caco-2 cells and mucus secreting cells (HT29-M6) and observed the fluorescence signals by confocal microscopy [78]. The images indicated that the level of interaction of chitosan nanocapsules was greatly enhanced by the presence of the mucus secreting cells. In addition, no transport of the particles across the monolayer was observed [78]. Therefore, overall, these results led us to suggest that the expected mucoadhesion of the chitosan-coated system could be responsible for the facilitated access of the drug to the underlying epithelium, and, hence for the pronounced and long-lasting hypocalcemic effect. An illustration of this mechanism is presented in figure 4.

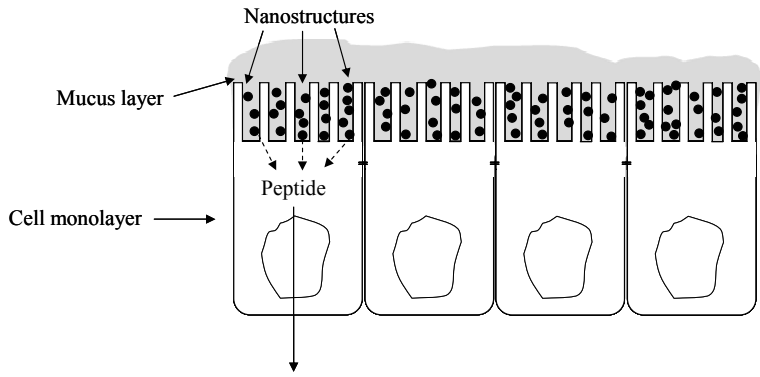

Figure 4: Schematic illustration of the interaction of chitosan-based nanostructures with the intestinal mucosa and the transport of the associated peptide

Chitosan-coated solid nanoparticles

In order to investigate the importance of the nature of the lipid core in the performance of chitosan-coated lipid systems, in our laboratory, we have also produced chitosan-coated tripalmitin nanoparticles [79]. First, calcitonin-loaded tripalmitin nanoparticles were prepared by the double emulsion-solvent emulsification method [80] and, then, coated with chitosan by simple incubation in a chitosan aqueous solution (Fig. 2C). After oral administration of calcitonin-loaded chitosan-coated tripalmitin nanoparticles to rats, the hypocalcemic effect was

evaluated. A great and long-lasting reduction of the serum calcium levels was obtained [81, 82]. This response was similar to the one observed for calcitonin-containing chitosan nanocapsules [76]. However, as in the case of non-coated nanoemulsion, tripalmitin cores were inefficient at reducing the serum calcium levels. An obvious conclusion from these observations was that the chitosan coating was critical for the success of the formulation. As in the case of the chitosan nanocapsules, our interpretation was that the presence of chitosan could facilitate the interaction with the overlying mucus layer leading to a prolonged site-specific delivery of calcitonin and, thus, an extended pharmacological response.

These results agree with those previously reported for chitosan-coated poly(lactic acid/glycolic acid) (PLGA) nanoparticles [40]. In this study, elcatonin-loaded PLGA nanoparticles were prepared by the emulsion solvent diffusion method. Then, the nanoparticles were isolated and incubated with a chitosan solution, forming chitosan-coated PLGA nanoparticles. The effectiveness of chitosan-coated PLGA nanoparticles was assayed in rats, showing a reduction of the serum calcium levels as compared to the peptide solution and the uncoated nanoparticles. As in the case of the chitosan-coated lipid nanoparticles, this positive *in vivo* behavior was attributed to the mucoadhesive character of the carrier and its intimate contact with the intestine. This explanation was justified by the observed mucoadhesion of the carrier using the everted intestinal sac model.

Chitosan-coated liposomes

Liposomes have been considered as candidate vehicles for oral peptide delivery due to their capacity to encapsulate peptides and to protect them from enzymatic degradation. One of approaches to improve their interaction with the intestinal mucosa and, hence, to increase the absorption of the associated peptide has been their coating with mucoadhesive polymers such as chitosan. For example, Takeuchi *et al.* prepared multillamelar liposomes coated with chitosan by hydration the lipid film with an aqueous solution of the polymer [83, 84]. As expected, these chitosan-coated liposomes exhibited a mucoadhesive character whose degree was

dependent on the amount of chitosan attached to their surface [83]. The efficacy of the coated liposomes as carriers for oral peptide delivery was tested *in vivo* for two model peptides, insulin and salmon calcitonin [83-85]. The results of these studies indicated that the chitosan-coated liposomes were more effective than the uncoated ones in terms of improving the pharmacological effect of the peptides administered orally to rats. This improved response was related to the mucoadhesive properties of the chitosan-coated liposomes. Moreover, the same authors tested the performance of the chitosan solutions for improving the absorption of calcitonin and observed that the simple presence of chitosan in solution did not help the absorption of this peptide. As a consequence, they concluded that the protection of the peptide in the liposomal core as well as their coating with the mucoadhesive polymer were critical for the success of the formulation.

Overall, the results obtained from chitosan-coated nanostructures underline the efficacy of this type of colloidal carrier. In general, the chitosan coating around the carriers showed a positive effect at improving the pharmacological response of the peptide, which was mainly attributed to the mucoadhesive properties of the polymer. However, the differences in the core material could also influence the level of protection of the associated peptide and also in its release from the carrier. As a consequence, these differences could have an impact on the intensity and duration of the pharmacological response.

Conclusion

Different chitosan-based drug delivery systems were revealed as promising peptide carriers. Among them, those based on chemically modified chitosan and also nanoparticulate carriers (nanoparticles and nanocapsules) have been particularly successful. Moreover, there is a clear evidence of the greater performance of nanoparticulate chitosan carriers, as compared to the solutions of non-modified chitosan, in terms of enhancing the absorption of peptides such as insulin and salmon calcitonin. The explanation to this phenomenon is that the particulate carriers are able not only to increase the peptide absorption due to the mucoadhesive

properties of chitosan, but also to offer protection from enzymatic degradation. Furthermore, while more work is needed to fully understanding the mechanism of action and the efficacy of these carriers, we can certainly conclude that chitosan-based systems have a promising future in oral drug delivery.

Expert opinion

At present, only the hydrophobic peptide cyclosporine A can be administered orally in the form of a microemulsion (Neoral®). However, the progress made over the last years, towards making feasible the oral administration of peptides, offers an optimistic perspective. Indeed, the Eligen® technology (based on using a low molecular weight delivery agent) has reached phase II-clinical trials for insulin and salmon calcitonin [86, 87]. Similarly, hexyl –insulin monoconjugate 2 (HIM2), a modified insulin conjugated to an amphiphilic polymer [88], as well as oral formulations of calcitonin and parathyroid hormone consisting of a combination of enzyme inhibitors, absorption enhancers, and enteric coating, have been evaluated in humans [89]. On the other hand, a number of particulate polymer and bioadhesive systems have given evidence of their effectiveness in large scale animals [90, 91]. Therefore, these delivery-based strategies are opening the way for future great developments, preferably based on nanosystems and polymers. This article presents the value of a more immature, but promising, strategy based upon the use of the bioadhesive polysaccharide chitosan. Chitosan as such (in solution or powder) can not be used because it is soluble in the gastric fluids but precipitates at the intestinal pH. However, a number of delivery approaches based on chitosan have shown a degree of success in small scale animals: (i) the use of chemically modified chitosan which is soluble at the intestinal pH; (ii) the use of nanosystems that protect the peptide and facilitate its interaction with the absorptive epithelium; and (iii) the design of devices that specifically deliver the peptide together with chitosan in the colonic region. Despite the difficulties to compare the results of these approaches, the specific characteristics of the nanosystems and the documented information about their efficacy lead us to the consideration of their special potential for oral

peptide administration. More detailed studies about their mechanism of action will help designing the way to proceed for their further optimization.

Five-year view

At present, there is the proof of concept that chitosan-based nanocarriers can enhance the absorption of model peptides such as insulin and salmon calcitonin in small scale animal models. It could be anticipated that these initial results will stimulate the optimization of oral peptide formulations based on chitosan delivery nanosystems. Further, this accumulated information is expected to lead to the evaluation of the efficacy of these nanosystems in large-scale animals in fed and fasting conditions. These results together with those of the efficacy of these nanosystems upon storage and also upon presentation in a solid dosage, will give very soon an indication of the potential of these nanosystems for clinical use.

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ANTECEDENTES, HIPÓTESIS Y OBJETIVOS

Antecedentes

1. La encapsulación de insulina en nanocápsulas constituidas por un núcleo oleoso y una cubierta polimérica permite mejorar la absorción sistémica de dicho péptido tras su administración por vía oral. Esta mejora se ha atribuido a la capacidad del núcleo oleoso para proteger la integridad del péptido en el tracto gastrointestinal[1, 2].

2. La utilización de sistemas coloidales que llevan en su composición el polisacárido quitosano ha permitido mejorar la absorción sistémica de fármacos peptídicos a través de la mucosa nasal e intestinal[3, 4].

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3. La modificación de la superficie de los sistemas coloidales con PEG ha permitido mejorar la estabilidad de los vehículos en los fluidos biológicos[5, 6] y la absorción sistémica de las proteínas asociadas a los mismos, tras su administración por vía nasal y oral [5, 7].

Hipótesis

1. Nanocápsulas constituidas por un núcleo oleoso y una cubierta de quitosano, pueden proteger el fármaco encapsulado de su degradación en fluidos biológicos así como, mejorar la absorción del fármaco a través de la mucosa nasal e intestinal.

2. La modificación de la cubierta polimérica mediante la peguilación del quitosano puede mejorar la estabilidad de los vehículos en los fluidos biológicos y, posiblemente, la absorción sistémica del péptido asociado.

Objetivos

Teniendo en cuenta lo previamente expuesto, el objetivo global del trabajo se ha dirigido a la evaluación del potencial que ofrecen las nanocápsulas de quitosano como sistemas para la administración de péptidos a través de la mucosa

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nasal e intestinal. A fin de facilitar el seguimiento de esta memoria, hemos desglosado este objetivo global en los siguientes objetivos parciales:

Desarrollo y evaluación del comportamiento *in vitro* de nanocápsulas de quitosano para la administración de péptidos por vía nasal

Este objetivo ha consistido en la optimización del proceso de preparación de las nanocápsulas de quitosano, evaluando la influencia de distintas variables tecnológicas sobre las características finales de los sistemas desarrollados. Asimismo, el objetivo se ha centrado en la incorporación de un péptido modelo, la calcitonina, a las nanocápsulas de quitosano para, posteriormente, explorar el potencial de dichos vehículos con el fin de mejorar la biodisponibilidad de la calcitonina por vía nasal.

Los resultados del trabajo correspondiente a este objetivo se recogen el artículo 2 titulado: "*Chitosan nanocapsules: a new carrier for nasal peptide delivery*" (sometido a evaluación).

Evaluación del potencial de las nanocápsulas de quitosano como sistemas para la administración oral de péptidos

Este objetivo ha consistido en evaluar si las nanocápsulas de quitosano pueden o no favorecer la absorción sistémica de calcitonina administrada por vía oral. Para ello se analizó la influencia de distintas variables como son el tipo de sal de quitosano y su peso molecular.

Los resultados correspondientes a este objetivo aparecen recogidos en los artículos titulados:

"*Transmucosal macromolecular drug delivery*"; Journal of Controlled Release 101 (2005) 151–162.

"*Chitosan nanocapsules as carriers for oral peptide delivery: Effect of chitosan molecular weight and type of salt on their in vitro behaviour and in vivo effectiveness*" (sometido a evaluación).

"Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery" (sometido a evaluación).

Evaluación del potencial de las nanocápsulas de quitosano-PEG como sistemas de administración de péptidos por vía oral

El objetivo de esta etapa de trabajo ha sido el de evaluar si la peguilación del quitosano podría mejorar la estabilidad de las nanocápsulas en los fluidos gastrointestinales y, como consecuencia, su eficacia desde el punto de vista de mejorar la absorción intestinal de la calcitonina. Asimismo, se ha considerado como objetivo adicional la evaluación de la toxicidad y del mecanismo de interacción de las nanocápsulas de quitosano-PEG con la línea celular Caco-2.

Los resultados del trabajo correspondiente a este objetivo se recogen el artículo 5 titulado: "*Chitosan-PEG nanocapsules as new carriers for oral peptide delivery. Effect of chitosan pegylation degree"* (sometido a evaluación).

Estudio del mecanismo de interacción de las nanocápsulas de quitosano con células Caco-2 y con células goblet (HT29-M6)

El objetivo de este estudio ha sido el de investigar el papel del recubrimiento con quitosano en la interacción con la línea celular Caco-2 y con el cocultivo Caco-2:HT29-M6. Para ello se analizó el efecto de las nanocápsulas en la viabilidad celular, en la modificación de las uniones intercelulares, así como, en la interacción de las nanocápsulas con las células. Finalmente, con la finalidad de conocer la influencia de la presencia de mucus en la interacción de los sistemas con las células, se evaluó el comportamiento de las nanocápsulas de quitosano en el

cocultivo Caco-2:HT29-M6, constituido por enterocitos (Caco-2) y células secretoras de mucus (HT29-M6).

Los resultados correspondientes a este objetivo aparecen recogidos en los artículos ya mencionados:

"*Chitosan nanocapsules as carriers for oral peptide delivery: Effect of chitosan molecular weight and type of salt on their in vitro behaviour and in vivo effectiveness"* (sometido a evaluación).

"Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery" (sometido a evaluación).

TRABAJO EXPERIMENTAL

Parte 1

Evaluación de las nanocápsulas de quitosano como nuevos sistemas para la administración nasal de péptidos.

Artículo 2

Chitosan nanocapsules: a new carrier for nasal peptide delivery

Cecilia Prego, Dolores Torres and María José Alonso

Sometido a evaluación

Abstract

This work describes the preparation and characterization of chitosan (CS)-coated oil nanodroplets (CS nanocapsules) as well as the evaluation of their potential for enhancing the nasal absorption of peptide drugs. For this purpose, salmon calcitonin (sCT) was chosen as the model peptide. CS nanocapsules were obtained by the solvent displacement technique which involves mixing an organic polar phase with and aqueous phase. The coating of the oily nanodroplets was feasible due to the interaction between negatively charged phospholipid, lecithin, and the positively charged CS, which allowed the stabilization of the system. For the formation of this coating, CS was added to the aqueous phase either before or after the mixing with the organic phase. In both cases, the influence of lecithin and CS concentration on the physicochemical properties of the nanocapsules was evaluated. CS nanocapsules displayed a mean particle size in the nanometer range, between 200 and 570 nm, depending on the formulation variables. The presence of the CS coating was noted by transmission electron microscopy and confirmed by the increase in size and the inversion in the zeta potential of the nanocapsules, as compared with the respective values observed for the uncoated nanoemulsions. The association efficiency of sCT to the nanocapsules was high and affected by the presence of the CS coating. The effectiveness of CS nanocapsules for nasal delivery of sCT was evaluated by measuring the plasma calcium levels in rats. The hypocalcemic effect observed following nasal administration of sCT-loaded CS nanocapsules was significantly enhanced and prolonged in comparison to that corresponding to the uncoated sCT-loaded nanoemulsion or to the sCT aqueous solutions containing CS. Furthermore, the area above the hypocalcemic effect-time curve ($AAC_{0\text{-}12h}$) obtained for CS nanocapsules and the control nanoemulsion was 2 and 1.3 times, respectively, higher than the one corresponding to the aqueous solution of sCT. Consequently, the results highlight the critical role of the CS coating in enhancing the transport of the associated peptide and, hence, the potential utility of CS nanocapsules for nasal peptide delivery.

Keywords: Nasal administration, peptide delivery, nanocapsules, chitosan

Introduction

Despite the advances made in the last decade, the use of a non-parenteral route is still, in most cases, a non viable or deficient option for peptide administration. The most important biopharmaceutical problems inherent to these macromolecules are their instability in the biological environment and their low permeability across biological barriers. Within this frame, most of the studies have been directed towards making the delivery of these macromolecules across mucosal surfaces feasible (1-4). Among these, 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. In fact, currently there are a few peptide formulations being administered by this route, such as buserelin, desmopresin, oxitocin, calcitonin and nafarelin. However, despite these promising results, we must admit that while the permeability of the nasal mucosa is acceptable for small molecules, unfortunately, it falls off sharply with the increase of the molecular weight of the drug (5). Indeed, the bioavailability of peptides such as salmon calcitonin (sCT) (3200 Da), has been reported to be very limited (only 1.6% with respect to the intramuscular administration) (6).

Several strategies have been explored so far to enhance the absorption efficacy of peptides by the nasal route. A frequently chosen option has been the use of penetration enhancers (7, 8). However, a major limitation of the majority of the enhancers is related to their ability to induce morphological damage on the nasal mucosa and/or inhibition of the ciliary movement (9, 10). Among the penetration enhancers, a special case is represented by the polysaccharide chitosan (CS). Even though this polysaccharide may cause a reduction of the ciliary movement, when applied as a solution, it has been reported that the penetration enhancing effect of CS is reversible and, hence, does not compromise the integrity and functionality of epithelia (11, 12). In addition to this property, CS is known as a mucoadhesive material. The efficacy of this material in terms of increasing the nasal absorption of peptides has already been illustrated for insulin, salmon calcitonin, leuprolide, parathyroid hormone (12-14).

Another strategy for improving nasal peptide absorption has been based upon the use of colloidal systems such as liposomes, micelles, nanoemulsions and nanoparticles (15-18). The absorption enhancement ability of these nanosystems has been attributed to different mechanisms, which include (i) the facilitated interaction of the peptide-containing nanosystem with the mucosa (ii) the transport of the peptide-containing nanosystem across the nasal mucosa (iii) the enhancement of the mucosal permeability caused by some of components of the nanocarrier, i.e. lipids, CS. For example, we have shown that the presentation of CS in the form of nanoparticles leads to a significant enhancement in the nasal absorption of insulin (18). Moreover, we have recently reported that low molecular weight CS nanoparticles enhance the nasal transport of large proteins, such as tetanus toxoid, leading to enhanced and long-lasting immune responses (19).

Here we present a new alternative nanocarrier for nasal peptide delivery, called CS nanocapsules, which could offer some potential advantages as compared to those previously reported. CS nanocapsules are composed of an oily core surrounded by a CS coating. The selection of the coating was justified by the positive behavior reported for CS solution which, in our understanding, could be reinforced by the presentation of the polymer in the form of a nanostructure. On the other hand, it was our hypothesis that the presence of an oily core could offer some beneficial effects in terms of stabilizing the associated peptide and enhancing its absorption (20, 21). Thus, the main objective of the present study was to develop and evaluate the potential of CS nanocapsules as a new colloidal system for nasal peptide delivery. sCT was selected as a model peptide in order to test the *in vivo* efficacy of the proposed system.

Materials and Methods

Materials

CS, in the form of hydrochloride salt (Protasan® Cl 110, Mw= 140 kDa, deacetylation degree: 86%) was purchased from Pronova Biopolymer, A.S., (Norway).

Miglyol 812® (caprylic/capric triglycerides) was supplied by Lemmel (Spain). The surfactant soybean L-α-lecithin and poloxamer 188 (Pluronic F-68®) were provided by Sigma-Aldrich (Spain). Salmon calcitonin (sCT) was kindly donated by Almirall Prodesfarma, S.A. (Spain).

Preparation of CS and sCT-loaded CS nanocapsules

CS nanocapsules were prepared by the solvent displacement technique (22) as follows. A variable amount of lecithin (0.4, 0.8 and 1.2% w/v) and 0.125 ml of Miglyol 812® were dissolved in 10 ml of acetone. This organic phase was, then, poured under moderate agitation into 20 ml of an aqueous phase containing poloxamer 188 (0.25% w/v) and different amounts of CS (0, 0.1, 0.2 and 0.3%). The mixture turned milky immediately as a result of the formation of CS nanocapsules. Finally, the solvent was evaporated and the suspension concentrated to 10 ml under vacuum. Alternatively, CS was added to the external aqueous medium upon mixing the organic phase with the aqueous phase containing poloxamer 188 (0.25% w/v) followed by the solvent evaporation.

The variables investigated in the development of CS nanocapsules were: lecithin concentration in the organic phase (0.4, 0.8 and 1.2% w/v), CS concentration in the aqueous phase (0, 0.1, 0.2 and 0.3% w/v) and the way of CS incorporation, either during the nanocapsules preparation step or by addition to the previously formed nanoemulsion followed by incubation for 1 hour at room temperature. The nanocapsules were isolated by ultracentrifugation at 120,000 xg for 1 hour.

The formulations variables investigated for the association of sCT to CS nanocapsules were: the concentration of lecithin (0.4 and 1.2% w/v) and the concentration of CS (0 and 0.1% w/v).

Characterization of CS nanocapsules

The particle size and polydispersion index of the nanocapsules were determined by photon correlation spectroscopy (PCS). Samples were diluted to the appropriate concentration with filtered ultrapure water. Each analysis was performed at 25ºC with an angle detection of 90º. The zeta potential was calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry (LDA). Samples were diluted with KCl 1mM and placed in the electrophoretic cell where a potential of ±150 mV was established. The PCS and LDA analysis were performed using a Zetasizer® 3000 HS (Malvern Instruments, Malvern, UK). Each batch was analyzed in triplicate.

The morphological examination of CS nanocapsules and nanoemulsions was performed using a transmission electron microscopy (TEM), (Philips CM12, Eindhoven, Netherlands), following staining with a 2% w/v phosphotungstic acid solution.

sCT encapsulation efficiency

Due to the important affinity of sCT for the lipids, the attempts directed to extract or separate the encapsulated sCT from the nanocapsules and nanoemulsions were unsuccessful. Consequently, the encapsulation efficiency of sCT in the nanocapsules was calculated by the difference between the total amount of sCT used in the encapsulation process and the amount of free sCT that remained in the aqueous suspending medium. The amount of free sCT was determined in the supernatant following separation of nanocapsules from the aqueous medium by a combined ultrafiltration-centrifugation technique (Centricon YM-100, Millipore,

USA) at 1000 x g for 1h. The supernatant was diluted with pH=4 acetate buffer and assayed for sCT content by HPLC at 220 nm (Agilent Technologies, Germany) as described in the British Pharmacopoeia, 1998 (column: Vidac 218TP). A calibration curve was made with solutions of sCT in pH=4 acetate buffer at concentrations ranging from 5 to 100 μg/ml. Each sample was assayed in triplicate.

In vitro release of sCT from CS nanocapsules

In vitro release studies of sCT from CS nanocapsules and control nanoemulsions were performed by incubating the formulations in acetate buffer (pH= 4.0) in a shaking water bath at 37°C. At appropriate time intervals, individual samples were ultrafiltered at 1,000x g for 1 h. The amount of sCT released at each time point was determined by HPLC.

Nasal administration of sCT-loaded CS nanocapsules

Male Sprague-Dawley rats (225-275 g), from the Central Animals House of the University of Santiago de Compostela (Spain), were fasted for 12 h before experiments, but allowed water *ad libitum*. Animals were kept conscious during the experiments. These experiments were approved by the Ethical Committee of the University of Santiago de Compostela. The following formulations were instilled intranasally to rats by means of a micropipette inserted into the nostrils: (1) isolated sCT-loaded CS nanocapsules and (2) non-isolated sCT-loaded CS nanocapsules (3) sCT-loaded nanoemulsion, (4) sCT aqueous solution and (5) sCT aqueous solution containing CS. The dose of sCT administered was, in all cases, 15 UI/kg in a volume of 20 μl (10μl in each nostril). Blood samples were collected from the tail vein 30 min prior to the nasal administration, in order to establish the baseline calcium levels, and at different times after dosing. The serum was separated by centrifugation at 3,000x g for 5 min. Hypocalcemic effects were determined in serum samples by a colorimetric method at 570 nm (Kit OR-cresolphtalein v/v, Spinreact, Spain). The area above the hypocalcemic effect-time curve ($AAC_{0\text{-}12h}$)

was calculated by means of the trapezoidal method. Results are shown as the mean values of serum calcium levels (± s.e.) of 6 animals.

Statistical analysis

Particle size and zeta potential were statistically analyzed by the analysis of variance (ANOVA) combined with a lineal regression using the program SPSS 11.5. Differences were considered to be significant at a level of $p < 0.01$.

The mean serum calcium levels determined in samples collected before sCT administration were taken as the baseline levels. Using these values, the statistical comparison of the percentages of calcium reduction at each time was performed by the ANOVA test followed by the Student-Newman-Keuls test for multiple comparisons between treatments. Differences were considered to be significant at a level of $p < 0.05$.

Results and discussion

The main goal of the present work was to investigate some formulation factors involved in the preparation of CS-coated oily nanodroplets and to evaluate their potential for the association and delivery of peptides following nasal administration. With this purpose in mind, sCT was selected as a model peptide. The rational behind this work was that the combination of an oily core with a CS coating, in the form of a colloidal system, would help protect the associated peptide from degradation while favoring its interaction with the nasal mucosa.

Development and characterization of CS nanocapsules

The supporting hypothesis for the preparation of CS nanocapsules was that the ultradispersion of a lipid solution in a CS aqueous solution would facilitate the

ionic interaction between the negatively charged lipids and the positively charged CS molecules. This hypothesis was verified a number of years ago (22). In the present work, we aimed to elucidate the factors that were determinant in the formation of these CS nanocapsules. Hence, the variables evaluated were: the concentration of lecithin in the organic phase, the concentration of CS in the aqueous phase and the way CS was incorporated into the aqueous phase, either before or after mixing the organic and the aqueous phase. The analysis of the particle size by PCS indicated that all formulations displayed a size in the nanometer range, between 200 and 570 nm, depending on the values of the variables selected (Figure 1 and 2). In addition, the results indicated that, irrespective of the way CS was incorporated, both lecithin and CS concentration had a statistically significant influence on the particle size, the presence of CS being the main factor affecting the particle size. More specifically, figure 1 depicts the surface response showing the simultaneous influence of the variables selected on the size of CS nanocapsules formed by incorporating CS prior to the oil-in-water mixing process. It can be noted that the addition of a small amount of CS to the external aqueous phase (0.1-0.2%) did not significantly change the size of the oily droplets, however, a further increase in the CS concentration (0.3%), led to a significant enlargement in the particle size (from 200 to 570 nm). On the other hand, in figure 1 it can also be noted that the influence of the lecithin concentration was determined by the CS concentration. Concretely, for the lowest CS concentration, an increase in the lecithin concentration did not lead to a significant change in the particle size. However, for the upper limit of the CS concentration, the particle size decreased significantly when the lecithin concentration increased. This size decrease was attributed to the presence of a population of CS-lecithin nanocomplexes that accompanied the formation of the nanocapsules. In order to corroborate this hypothesis, these high lecithin-content formulations were ultracentrifuged at 120,000 x g for 1 hour. The result of this process was a three-phase system consisting of a foam caused by the association of nanocapsules, a precipitate attributed to the formation of complexes between lecithin and CS and the aqueous suspending medium. In contrast, for the low lecithin content formulations only a-two phase system consisting of a foam and aqueous suspending medium was observed. Consequently, these results indicate that there is a critical

value for the parameters investigated, lecithin and CS concentration, in order to obtain a unique population of CS nanocapsules.

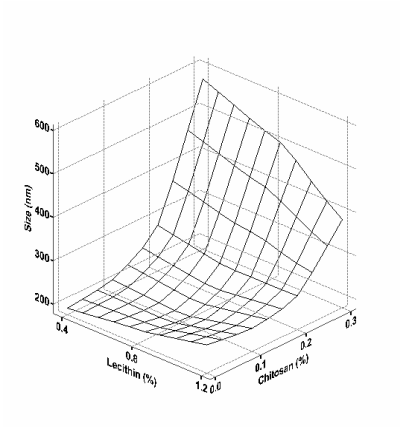

Figure 1: Response surface showing the effect of lecithin and CS concentrations on the particle size of CS nanocapsules obtained by adding CS to the external aqueous medium prior to the mixing with the organic solution of the lipids.

Mean particle size = 211.231 + 181.462 X_1 – 1426.453 X_2 + 9581.111 X_2^2 – 1269.295 X_1X_2; r = 0.966.

X_1 = Lecithin concentration in the organic phase.

X_2 = CS concentration in the aqueous phase.

Figure 2 shows the influence of the concentration of both CS and lecithin, on the size of CS nanocapsules formed by incorporating CS after the dispersion of the lipids in the aqueous medium. The size of the nanosystems was affected by the variables investigated, CS and lecithin concentration, although to a different extent. As observed in the previous method, the increase in the CS concentration led to a significant enlargement in the particle size. In both cases, this effect, which was slightly affected by the concentration of lecithin, was attributed to the deposition of increasing amounts of CS around the oily nanodroplets. In fact, as shown in figure 3, the size enlargement occurred simultaneously with a progressive inversion in the zeta potential. A similar effect was previously observed when increasing either the amount of CS or its molecular weight (22, 23). In contrast, the lecithin concentration had an opposite effect on the size as compared to that observed in the previous

method: for the highest CS concentration, the size became larger as the lecithin concentration increased. This could be simply attributed to a more important deposition of CS onto the lipid cores. However, as occurred with the previous method, the presence of lecithin-CS complexes was detected upon ultracentrifugation of the formulations prepared with the highest lecithin concentration. Consequently, the increase in the size could be attributed not only to the more important deposition of CS but also probably to some lecithin-CS complexes attached to the nanoemulsion during the incubation step.

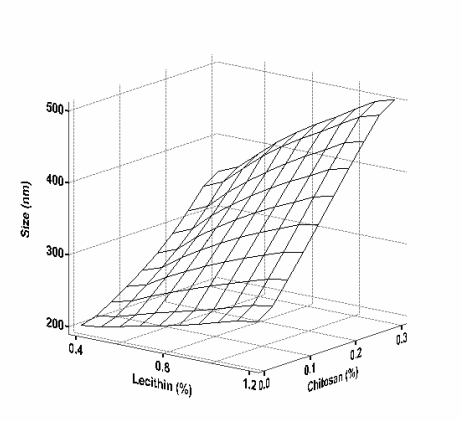

Figure 2: Response surface showing the effect of lecithin and CS concentration on the particle size of CS nanocapsules prepared by adding CS to the external aqueous medium after the formation of the submicron emulsion.
Mean particle size = 122.624 + 173.027 X1 + 217.725 X2 – 60.299 X12 + 420.139 X22 + 5.163 X1 X2; r = 0.994.
X1 = Lecithin concentration in the organic phase.
X2 = CS concentration in the aqueous phase.

Finally, irrespective of the moment of addition of CS, the particle size distributions were close to a monomodal distribution for the lowest CS concentration (polydispersion index < 0.2), however for higher CS concentration values, a less homogeneous population was obtained, as confirmed by the polydispersion index values which evolved to a value of 0.4. The increase in the polydispersion index could also be related to the presence of different populations corresponding to the nanocapsules and the nanocomplexes.

The appearance of CS nanocapsules and the corresponding control nanoemulsion is presented in figure 3. In the case of the nanoemulsion, we could only appreciate a spherical morphology. However, for CS nanocapsules we visualised the presence of an oily core surrounded by a CS coating, regardless the concentration of lecithin.

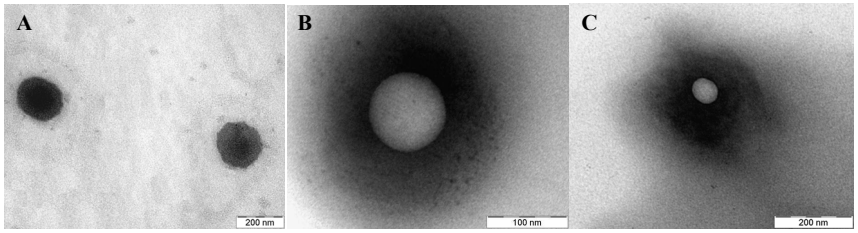

Figure 3: Transmission electron micrographs of: (**A)** uncoated nanoemulsion, (**B)** CS nanocapsules prepared with 0.4% w/v of lecithin and (**C)** CS nanocapsules prepared with 1.2% w/v of lecithin.

In addition to the results of the particle size analysis, the evidence of the CS coating around the oily nanodroplets was corroborated by the measurement of the zeta potential of the nanocapsules. As shown in figure 4, the uncoated nanoemulsions (0% CS) exhibited a high negative charge that was inverted upon coating with CS. Moreover, it can be noted that the concentration of CS was the most relevant factor on the evolution of the surface charge of the nanocapsules. This inversion from highly negatives values (about -60 mV) to highly positive values (about +50 mV) was certainly due to the presence of the cationic polymer on the surface of the nanodroplets. On the other hand, the lecithin concentration did not have a significant effect on the surface charge. As observed in a previous study (22), a minimum amount of lecithin is enough to facilitate the formation of the CS coating, and thus to confer the nanocapsules with a positive charge. After the inversion of the charge, neither the lecithin concentration, nor the CS concentration produced significant changes on the surface charge.

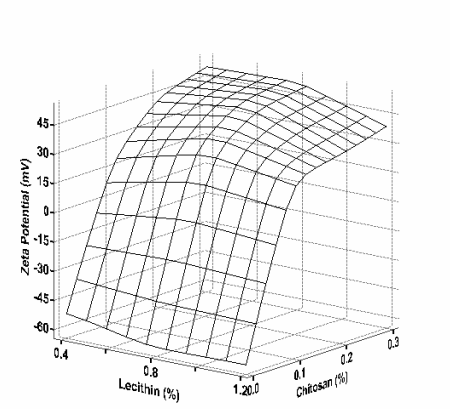

Figure 4: Response surface showing the effect of lecithin and CS concentration on the zeta potential of CS nanocapsules prepared by adding CS to the external aqueous medium after the formation of the submicron emulsion.
ζ Potential = -54.340 + 923.767 X_2 – 1981.667 X_2^2; r = 0.977.
X_2 = Chitosan concentration in the aqueous phase.

Characterization of sCT-loaded CS nanocapsules

An important goal of this work was to associate the peptide sCT to CS nanocapsules. To achieve this goal the peptide was dissolved in 50 µl of water and then added to the organic phase containing the lipids. Using this approach, it was thought that the interaction between the peptide and the lipids would be facilitated due to the positive charge of sCT at the selected pH (pKa= 10.4). As describe in the Materials and Methods section, these peptide-loaded formulations were prepared by the method involving the addition of CS upon mixture of the oily and water phases.

The results presented in table 1 indicate that more than 90% of sCT was associated to the control nanoemulsion, regardless of the lecithin concentration. The reason for this high association could be related to the strong electrostatic interaction between the negatively charged lecithin and the positively charged sCT, as previously described for other lipid systems containing sCT (24, 25). Moreover, sCT has some hydrophobic regions in their structure enabling them to interact with lipidic structures by means of some specific interactions (26). On the other hand, as can be noted in *table 1* that the association of sCT to the nanocapsules was reduced

by the presence of CS and that this reduction was affected by the lecithin concentration (values of encapsulation efficiency between 44 and 52%). This effect was ascribed to the positive character of both sCT and CS, which may be a result of a competition between the polymer and the peptide for the anionic binding sites in the oily core. Theoretically, this displacement effect should affect the sCT molecules which are associated to the surface of the oily droplets, since the attachment of CS occurred upon encapsulation of sCT in the lipid droplets. In order to verify the displacement of sCT molecules due to the presence of CS, some control experiments were performed. The experiment involved the incubation sCT-loaded nanoemulsions in the presence of a cation (0.1% w/v calcium chloride) and the evaluation of the sCT displacement. The results indicated that, as in the case of CS, calcium competed with sCT leading to its displacement from the surface of the oily nanodroplets. An additional control experiment consisted of incubating an uncoated nanoemulsion with sCT and subsequently with CS (0.1% w/v). The results showed that sCT was greatly adsorbed onto the nanoemulsion and further displaced following incubation with CS. Therefore, overall these results indicate that the surface association of the cationic peptide is reduced upon coating of the system with CS. These results agree well with those previously reported by Kawashima *et al*. (27) who observed that the coating of PLGA nanospheres with CS reduced the elcatonin encapsulation. Nevertheless, despite the above mentioned displacement effect, it must be noted that the association efficiency of sCT to the nanocapsules remains sufficiently important.

Table 1: Physicochemical properties and encapsulation efficiency of chitosan nanocapsules and uncoated nanoemulsions containing sCT (mean ± s.d.; n=3).

% Lecithin (w/v)	% Chitosan (w/v)	Particle Size (nm)	ζ Potential (mV)	Encapsulation efficiency (%)
0.4	0	193.3±1.2	-52.0±1.1	> 90
	0.1	224.7±0.6	+20.2±0.4	44.1±3.2
1.2	0	243.7±2.1	-59.9±2.0	> 90
	0.1	333.0±6.1	+28.3±2.1	51.9±1.9

With regard to the physicochemical properties (size and zeta potential) of the sCT-loaded systems, the results in table 1 show that, as expected, the coating with CS increased the particle size of the oily nanodroplets and led to an inversion of the surface charge. These changes were more pronounced for the highest lecithin concentration.

In vitro release of sCT from CS nanocapsules

Figure 5 displays the release profiles of sCT from CS nanocapsules prepared with different lecithin concentration (0.4 and 1.2% w/v). These profiles indicate that a certain amount of sCT was rapidly released in the first 30 minutes and then, no further release was observed in the 6 hours of the study. The initial release was attributed to the surface-associated peptide whereas the second slow phase would correspond to the release of the well entrapped peptide molecules showing a high affinity for the oily core. Interestingly, the initial burst effect observed for CS nanocapsules was higher for those prepared with 1.2% lecithin compared to those prepared with 0.4% lecithin (15% vs 50%). This difference may be related to the presence of complexes lecithin-CS in those prepared with the highest concentration of lecithin. Indeed, some sCT molecules could be associated to these complexes and be rapidly released upon incubation in the release medium. In contrast, the release of sCT from lecithin nanoemulsions was negligible irrespective of the lecithin concentration, probably due to the peptide binding affinity to the lipid. Similarly, the leakage of calcitonin from positively liposomes was greater than for negative or neutral ones, suggesting that the charge repulsion played an important role in the leakage of calcitonin from positive liposomes (24).

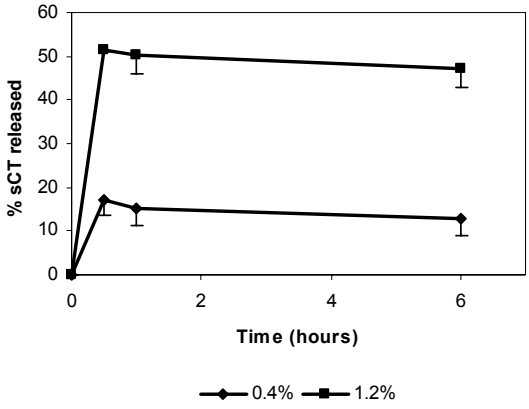

Figure 5: *In vitro* release profiles of sCT from CS nanocapsules prepared with different lecithin concentrations, 0.4% w/v (♦) and 1.2% w/v (■) (mean ± s.d.; n=3).

Nasal administration of sCT-loaded CS nanocapsules

The reduction in the serum calcium levels obtained after intranasal administration to rats of sCT included in CS nanocapsules (0.4% w/v lecithin), as well as in the uncoated nanoemulsion, and in an aqueous solution (with or without CS) are shown in figure 6. The results show that the hypocalcemic effect observed after administration of the colloidal systems, (uncoated nanoemulsion and CS nanocapsules) was significantly greater than that obtained with the sCT aqueous solutions containing CS or not. In fact, the presence of CS in the sCT solution with a similar amount to that incorporated in the nanocapsules did not have any effect on the response of sCT. The significant decrease in the calcium levels observed for the uncoated nanoemulsion might be related to the lipids stabilizing and/or absorption-enhancing effects. Indeed, Mitra *et al*. 2000 (17) reported that the insulin absorption could be enhanced by its incorporation into an o/w emulsion. They found that the presence of a small fraction of oil droplets along with the peptide in the aqueous phase appeared to play an important role in the nasal absorption enhancement mechanism. Similarly, this potential absorption promoting effect of the lipid vehicles has been extensively studied by Muranishi (21). On the other hand, it has

also been shown that lipids are able to protect peptide drugs from protease degradation, especially upon contact with gastrointestinal fluids (20, 28).

Nevertheless, besides the possible absorption-enhancing and/or protective effects of the nanoemulsion, the results of the present study clearly show that the coating of the oily nanodroplets with CS markedly improved the hypocalcemic effect of sCT. Consequently, these results confirm the importance of the CS coating at promoting the interaction of the colloidal system with the nasal mucosa, thus leading to a further enhancement of the sCT transport. This interpretation agrees well with the results reported for other CS-based nanocarriers (18, 19, 24). Indeed, CS nanoparticles have been shown to increase the nasal absorption of insulin and also the immune response of tetanus toxoid administered intranasally (18, 19). Similarly, CS-coated liposomes have displayed a capacity of enhancing the absorption of salmon calcitonin (24).

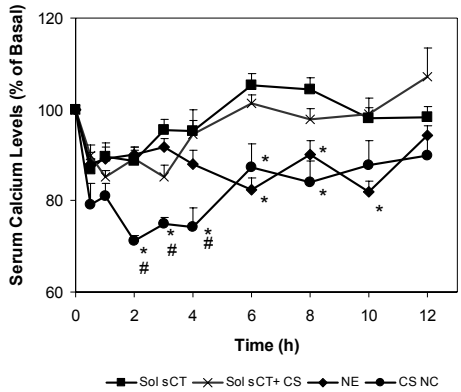

Figure 6: Serum calcium levels in rats after nasal administration of sCT (dose: 15 UI/kg) in aqueous solution (with or without CS) or encapsulated in the control nanoemulsion (NE) or in non- isolated CS nanocapsules (CS NC); (mean ± s.e.; n=6).
* Significantly different from sCT solutions ($p< 0.05$).
# Significantly different from nanoemulsion ($p< 0.05$).

An additional observation from *figure 6* is the lack of effect observed for CS solutions. Even though this observation could be considered in disagreement with the previously observed positive effect of CS in the nasal absorption of insulin and salmon calcitonin (12, 13)**,** it does correlate well with the negligible effect that we have previously observed for the CS solutions containing insulin (18). These differences in the efficacy of CS solutions could be related to the different experimental conditions of the different reports (different animal model and different CS dose) but they could also be related to the different molecular weight of the polymer used in the various experiments.

Interestingly, a similar decrease in the serum calcium levels was observed following administration of both types of nanocapsules, isolated or not (Figure 7). The differences between both types of administrations are related to the presence of free sCT, free CS, and Poloxamer 188. Consequently, the similarity of the behaviour could be attributed to the crucial role of the CS nanocapsules at facilitating the absorption of the free peptide.

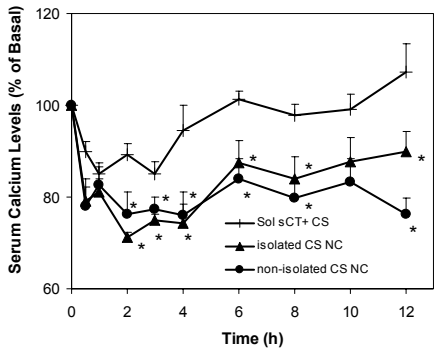

Figure 7: Serum calcium levels in rats after nasal administration of sCT (dose: 15 UI/kg) encapsulated in isolated or in non-isolated CS nanocapsules (CS NC) and sCT in CS aqueous solution; (mean ± s.e.; n=6)
***** Significantly different from sCT solution ($p < 0.05$).

Finally, the comparison of areas above the hypocalcemic effect curve (AAC $_{0\text{-}12h}$) obtained following administration of the different formulations (Figure 8) confirms the significantly greater pharmacological effect elicited by CS nanocapsules in comparison to those corresponding to the sCT solution containing CS or not and the uncoated nanoemulsion. In fact, the AAC $_{0\text{-}12h}$ of the serum calcium levels-time curve after nasal administration of CS nanocapsules (isolated or not) was significantly higher than that of sCT control solution with or without CS. Additionally, this hypocalcemic effect was maintained within 12 hours post-administration. In the case of CS nanocapsules, the AAC $_{0\text{-}12h}$ was increased by about 1.5, 1.7 and 2 fold when compared with the uncoated nanoemulsion, the CS solution containing sCT and the sCT aqueous solution, respectively. Overall, these results highlight the crucial role of the combination of CS and lipids in the form of a nanocarrier in terms of improving the nasal absorption of sCT.

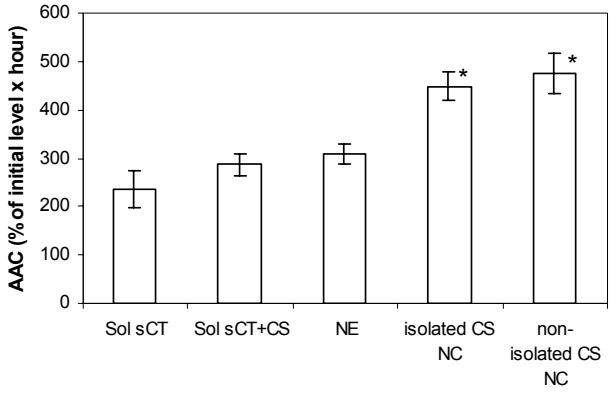

Figure 8: Area above hypocalcemic effect curve (AAC $_{0\text{-}12\ hr}$) after nasal administration to rats of sCT (dose: 15 UI/kg) incorporated in different formulations: aqueous solution (sol sCT), CS aqueous solution (sol sCT+ CS), uncoated nanoemulsion (NE) and isolated and non- isolated CS nanocapsules (CS NC).
* Significantly different from sCT solutions and uncoated nanoemulsion ($p < 0.05$).

Conclusions

CS nanocapsules consisting of a hydrophobic core (oily nanodroplets) and a hydrophilic coating have shown an excellent capacity for enhancing the absorption of sCT across the nasal mucosa. This was confirmed by the significant and prolonged reduction in the serum calcium levels which was obtained with these nanocarriers compared to the sCT control solutions or uncoated nanoemulsion. Therefore, this innovative system could be considered as a promising carrier for improving the nasal absorption of peptide drugs, such as sCT.

Acknowledgements

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Parte 2

Evaluación del potencial de las nanocápsulas de quitosano como sistemas para la administración oral de péptidos.

Artículo 3

Transmucosal macromolecular drug delivery

Cecilia Prego, Marcos García, Dolores Torres and María José Alonso

Adaptado de: Journal of Controlled Release (2005) 101: 151–162

Abstract

Mucosal surfaces are the most common and convenient routes for delivering drugs to the body. However, macromolecular drugs such as peptides and proteins are unable to overcome the mucosal barriers and/or are degraded before reaching the blood stream. Among the approaches explored so far in order to optimize the transport of these macromolecules across mucosal barriers, the use of nanoparticulate carriers represents a challenging but promising strategy. The present paper aims to compare the characteristics and potential of nanostructures based on the mucoadhesive polysaccharide chitosan (CS). These are CS nanoparticles, CS-coated oil nanodroplets (nanocapsules) and CS-coated lipid nanoparticles. The characteristics and behaviour of CS nanoparticles and CS-coated lipid nanoparticles already reported [A. Vila, A. Sanchez, M. Tobio, P. Calvo, M.J. Alonso, Design of biodegradable particles for protein delivery, J. Control. Release 78 (2002) 15–24; R. Fernandez-Urrusuno, P. Calvo, C. Remuñan-Lopez, J.L. Vila-Jato, M.J. Alonso, Enhancement of nasal absorption of insulin using chitosan nanoparticles, Pharm. Res. 16 (1999) 1576–1581; M. Garcia-Fuentes, D. Torres, M.J. Alonso, New surface-modified lipid nanoparticles as delivery vehicles for salmon calcitonin (submitted for publication).] are compared with those of CS nanocapsules originally reported here. The three types of systems have a size in the nanometer range and a positive zeta potential that was attributed to the presence of CS on their surface. They showed an important capacity for the association of peptides such as insulin, salmon calcitonin and proteins, such as tetanus toxoid. Their mechanism of interaction with epithelia was investigated using the Caco-2 model cell line. The results showed that CS-coated systems caused a concentration-dependent reduction in the transepithelial resistance of the cell monolayer. Moreover, within the range of concentrations investigated, these systems were internalized in the monolayer in a concentration-dependent manner. This uptake was slightly enhanced by the presence of the CS coating but, as compared with previously published results [M. Garcia-Fuentes, C. Prego, D. Torres, M.J. Alonso, Triglyceride-chitosan nanostructures for oral calcitonin delivery: evaluation in the Caco-2 cell model and in vivo (submitted for publication)], highly dependent on the nature of the lipid core. Nevertheless,

these differences in the uptake of the CS-coated systems (solid lipid core or oily core) by the Caco-2 cells did not have a consequence in the in vivo behaviour. Indeed, both CS-coated systems (nanocapsules and CS-coated nanoparticles) showed an important capacity to enhance the intestinal absorption of the model peptide, salmon calcitonin, as shown by the important and long-lasting decrease in the calcemia levels observed in rats.

Keywords: chitosan; nanoparticles; nanocapsules; transmucosal transport; peptide/protein carriers.

Introduction

Over the last decades significant efforts have been dedicated to explore new routes, alternative to injection, for the administration of macromolecules such as peptides and proteins. Among them, the transmucosal routes such as the nasal, pulmonary and oral routes, are those which have received the most important deal of attention [1–5]. As a consequence of this activity, the pulmonary administration of the peptide insulin has recently become a reality in clinical practice [5]. In addition, the number of peptides that are being administered intranasally increases year by year. However, the possibility to administer large macromolecules by the oral route needs still further investigations. The harsh conditions of the gastrointestinal tract and the limited permeability of the intestinal barrier make the situation very complex, and the solution to these problems a great challenge [6]. Nevertheless, despite the past pessimistic view, the knowledge accumulated during recent years regarding the mechanisms of interaction between nanostructured biomaterials and biological surfaces, as well as the discovery of new nanotechnologies and characterization approaches, has led to a new promising perspective on the use of nanocarriers for transmucosal macromolecular drug delivery [7–9]. The hypothesis behind this strategy has been that nanosystems, due to their colloidal size, are able to cross and, hence, transport the associated drug through the mucosal barrier, thus acting as transmucosal macromolecular nanocarriers. Indeed, at present, there is no doubt on the fact that the size is a critical parameter for the nanosystems to cross biological barriers [10,11]. However, the question that remains to be answered is how the ability of these systems to overcome these barriers can be optimized.

By accepting the premise that not only the size but also the surface properties and composition of the nanocarriers may affect their stability in biological fluids and their interaction with mucosal surfaces, we have focused our activity to the design of new types of nanocarriers especially adapted for transmucosal drug delivery [12–16]. The first relevant conclusion from this work was that a hydrophilic polyethylene glycol (PEG) coating around the nanosystem has a very positive effect on its in vivo success as a macromolecular drug carrier. More specifically, using tetanus toxoid as a model of a large protein, we showed that its transport across the

nasal and intestinal barriers was greatly improved following its nanoencapsulation into PEG-coated poly(lactic acid) (PLA) nanoparticles [12,13]. More importantly, we observed that the positive behaviour of the nanoparticles was mainly due to the presence of a PEG coating around them. The efficacy of these PEG-coated nanoparticles as nasal protein and DNA carriers was further corroborated by studying the immune response generated by the nanoencapsulated protein and plasmid DNA [14–16]. The positive effect of the hydrophilic coating around the nanosystems was not exclusive of PEG. In fact, another early discovery from our work was that a coating consisting of the hydrophilic polysaccharide CS significantly improved the ability of polyester nanocapsules to transport drugs across the corneal epithelium [17]. More recently, we have compared the effect of a CS vs. a PEG coating on the transport of these nanocapsules across the corneal epithelium [18]. While the mechanistic details need to be further investigated, the results indicated that both types of coatings affected significantly the interaction of these nanosystems with the ocular mucosa. Therefore, despite the different nature of the two hydrophilic coatings, PEG and CS, these initial studies led us to the overall conclusion that an adequate selection of the biomaterials and a specialized design of the nanosystems are required in order to optimize the capacity of nanocarriers to transport drugs across mucosal surfaces.

The purpose of the present report was to compare the characteristics and potential of different nanostructures having as a common material the Mucoadhesive polysaccharide CS. These nanosystems are CS nanoparticles, CS-coated oil nanodroplets (nanocapsules) and CS-coated lipid nanoparticles. By comparing these systems, the idea was to evaluate the role of the CS alone or in combination with lipids, and also the importance of the structural organization of these materials, in their behaviour as transmucosal drug carriers.

The selection of CS was based on its excellent properties including biocompatibility, mucoadhesion [19] and ability to reversibly alter the tight junctions [20]. On the other hand, for the selection of the lipid cores we have taken into account the promising results obtained for solid lipid nanoparticles [21] as well

as for oily polyisobutylcyanoacrylates nanocapsules with regard to their use as transmucosal drug carriers [22].

Materials and methods

Chemicals and animals

The materials and methods used for the preparation and evaluation of CS nanoparticles have been described in Refs. [14,23]. Similarly, the materials and methods used for the preparation and evaluation of CS-coated lipid nanoparticles have been described in Refs. [24–26]. The chemicals and animals used for the preparation and evaluation of the CS-coated oil nanodroplets are reported below.

CS with 85% degree of deacetylation and a viscosity of 16 mPa was purchased from FMC Biopolymer/ Novamatrix, (Norway). Miglyol 812®, a triglyceride formed from medium chain fatty acids was supplied by Lemmel (Spain). The surfactant soybean l-α-lecithin and Poloxamer 188 (Pluronic F- 64®) were supplied from Sigma-Aldrich (Spain). Salmon calcitonin (sCT) was kindly donated by Almirall Prodesfarma (Spain). Rhodamine was purchased from Sigma (Spain).

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, UK). Minimum Eagle Medium (MEM), fetal bovine serum (FBS), non-essential amino acids, l-glutamine, 100 UI/ml Penicillin/100 μg/ml Streptomycin solution, 0.05% Trypsin/0.02% EDTA solution, Dulbecco's phosphate buffered saline (DPBS) and Hank's basal salt solution (HBSS) were purchased from Sigma (Spain).

Male Sprague–Dawley rats (225–275 g), from the Central Animals House of the University of Santiago de Compostela (Spain) were used. They were kept in a 12-h light–dark cycle and at a temperature of 20±2ºC. The animals were allowed access to food and water *ad libitum*.

Preparation of CS nanocapsules

CS-coated oily nanodroplets (here called CS nanocapsules) were prepared according to the procedure previously described by our group, in two steps. More specifically, firstly, we prepared a nanoemulsion using a solvent displacement technique [27]. For this purpose, 125 μl of Miglyol 812 were added to an organic phase composed of 40 mg of lecithin dissolved in 0.5 ml of ethanol and 9.5 ml of acetone. This organic phase was added to an aqueous phase containing Poloxamer 188 (0.25% w/v). The mixture immediately turned milky due to the formation of the nanoemulsion. Then, the solvents were evaporated under vacuum. Secondly, this nanoemulsion was coated with CS by simple incubation in the polymer solution [28,29]. More specifically, 5 ml of the control nanoemulsion were incubated with 1 ml of CS aqueous solution (0.5% w/v) for 1 h, leading to the formation of CS nanocapsules.

Physicochemical characterization of CS-coated systems

The different colloidal carriers were characterized with regard to morphology, particle size, zeta potential and encapsulation efficiency. The association efficiency of sCT to the nanosystems was calculated from the concentration of the non-encapsulated peptide. The separation of the nanocapsules from the non-encapsulated peptide was performed by ultrafiltration (Centricom® YM-100, Millipore, Spain) for CS nanocapsules. The concentration of sCT was determined by HPLC as described (BP 1998).

Cell culture experiments

Caco-2 cells were cultivated in 80 cm^2 flasks (Nunc, Denmark) using MEM supplemented with 10% FBS, 1% l-glutamine, 1% nonessential amino acids and Penicillin/Streptomycin solution. Cells were maintained in a controlled atmosphere at 37ºC with 95% of relative humidity and 5% CO_2. The culture medium was

changed every two days for approximately 5–6 days until cells reached approximately 80–90% confluency. After the passage operation, cells were seeded approximately at $2.5x10^5$ cells per flask. For the experiments, cells with passage numbers between 30 and 35 were used.

Cytotoxicity studies

The effect of the different nanosystems on their in vitro biocompatibility was investigated at different concentrations using Caco-2 cell cultures. Cells were seeded on 96 well plates with a cell density of 10^4 cells/cm^2 for 7 days until cell monolayer was obtained. After 7 days, the medium was replaced by the nanocapsule suspensions at concentrations between 0.01 and 2 mg/ml in an appropriate volume to obtain a density of nanocapsules between 6 and 1212 μg/cm^2. As a positive control we used HBSS and as a negative control 2% sodium dodecyl sulphate (SDS). Nanocapsules and the controls were in contact with the monolayers for 90 min, at 37ºC and 5% CO_2. After this time, the nanocapsule suspensions were removed. The cell viability was determined using a colorimetric method (MTS, Promega), which determines the intracellular dehydrogenase activity. The absorbance was measured at 490 nm.

Measurement of the transepithelial electrical resistance

The measurement of the transepithelial electrical resistance (TEER) was performed on Transwell® plates with a cell density of $5.5x10^4$ cells 21 days post-seeding. The integrity of the monolayers was checked on the 21st day in MEM. The TEER measurements were performed with a Millicell®ERS connected to a pair of chopstick electrodes (Millipore, Spain). After these measurements, the medium was changed by HBSS (HBSS pH 6 in the apical side in the case of CS nanocapsules) in order to perform the experiments in buffer. After 1 hour of equilibrium, the apical solutions were replaced by the nanocapsule suspensions and the respective controls (HBSS or HBSS pH 6). The concentration of nanocapsules was 250 μg/ml in two

different cell growing areas, 1.1 and 4.5 cm^2 leading to a nanocapsule dose of 204.5 and 80 $\mu g/cm^2$, respectively. Nanocapsule suspensions were incubated for 100 min and TEER measurements were performed every 25 min. After this time, the solutions were removed and replaced by MEM in order to check the recuperation of the TEER values at 24 h.

Quantitative uptake studies

Caco-2 cells were seeded with a density of $5.5x10^4$ cells/cm^2 on 12-well plates with an area of 3.5 cm^2 and left to grow until full differentiation (19–21 days). The culture medium was replaced by rhodamine-loaded nanostructures in HBSS (or HBSS pH 6) at concentrations of 0, 250, 500 and 1000 μg/ ml (the corresponding dose per surface area values were: 0, 71.43, 142.86 and 286.71 $\mu g/cm^2$, respectively). The suspensions were incubated for 1 h. After incubation, the nanocapsule suspensions were removed and the monolayers washed five times with ice-cold DPBS. Monolayers were finally incubated for 15 min with 5 mM EDTA in DPBS adjusted to pH=5 ("acid wash"), a medium intended to detach the nanocapsules associated to the monolayer surface. Caco-2 monolayers were dried overnight and dissolved in a 2% SDS/50 mM EDTA/ DPBS at pH=6 ("lysis medium"). The nanocapsules-associated fluorescence internalized by the monolayers was quantified by fluorimetry. The calculation of the amount of fluorescent carriers taken up by the cells was performed using the corresponding calibration curves (different concentrations of fluorescence nanocapsules incubated in the lysis medium). The fluorescence of monolayers without nanocapsules (0 mg/ml samples) in lysis medium was substracted from the experimental values.

In vivo studies

Male Sprague–Dawley rats (225–275 g) were fasted for 12 h before experiments, but allowed water ad libitum. Animals were kept conscious during the experiments.

The following formulations were administered intragastrically to rats: (1) sCT aqueous solution, (2) nanoemulsion, and (3) CS nanocapsules. The dose at sCT administered was 500 IU/kg in a volume of 0.5 ml. Blood samples were collected from the tail vein 30 min prior to the oral administration to establish the baseline calcium levels and at different times after dosing. The serum was separated by centrifugation at 3000xg for 5 min. Hypocalcemic effects were determined in serum samples by a colorimetric method at 570 nm (Kit OR-cresolphtalein v/v, Spinreact, Spain).

Statistical analysis

The statistical significance of the differences between parameters of the oily-based nanosystems was tested by the analysis of variance (ANOVA) together with the multiple comparison Student–Newman– Keuls method.

The mean serum calcium levels determined in samples collected before sCT administration was taken as the baseline levels. Using these values, the statistical comparison of the percentage of calcium reduction at each time after dosing was performed by the one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons between treatments. Differences were considered to be significant at a level of $p<0.05$.

Results and discussion

The main goal of the present report was to provide an overview of the potential of different nanosystems consisting of solely CS or CS in combination with solid and liquid lipids as carriers for the transmucosal (nasal and oral) transport of macromolecules. The common feature of these nanosystems is the presence of CS in their composition. As illustrated in Fig. 1, the key differences between these systems are the following: (i) CS is in the form of solid nanoparticles (nanogel network) or as a soluble coating around lipid cores, (ii) the lipid core of the CS-coated systems is

either an oil (Miglyol® 812) or a solid lipid (tripalmitin). Here, we describe the physicochemical properties of these systems (size and zeta potential) as well as their ability to associate and release macromolecules. In addition, we provide information about the in vitro viability of Caco-2 cells exposed to CS nanocapsules and about their mechanism of interaction with this cell model and compared with that reported for CS-coated lipid nanoparticles and CS nanoparticles. Finally, we present the efficacy of the CS-based systems as transmucosal macromolecular drug carriers.

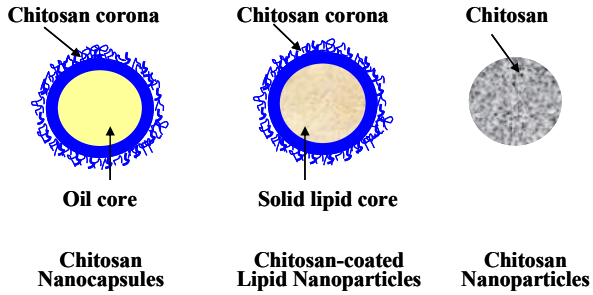

Figure 1: Illustration of the different chitosan-based nanosystems described in the present report.

Preparation and physicochemical characterization of the CS-based nanosystems

We have previously reported [14,27–30] the optimal conditions for the formation of CS nanoparticles and CS nanocapsules. The formation of the nanoparticles was achieved according to an ionic gelation process of CS upon contact with a counter anion, i.e. sodium tripolyphosphate. On the other hand, the formation of CS-coated oily droplets (nanocapsules) was achieved by means of an ionic complexation process between the negatively charged lecithin and the positively charged CS. This polymer layer was found to be responsible for the stabilization of the oily core of the nanoemulsions [27,28]. The same approach was recently applied to the formation of CS coated-lipid nanoparticles [25,26]. In this latter case, the coating was formed by simple incubation of the lipid nanoparticles with an aqueous solution of CS. The attachment of CS was due to its interaction

with lecithin and probably with other negative lipids that are present in the core lipid nanoparticles.

Table 1 shows the physicochemical properties (size and zeta potential) of the different systems we have designed and those of the corresponding control nanoemulsion. In addition, the table presents some macromolecules that have been associated to the systems and the modality of administration for which we have tested them as carriers for transmucosal macromolecular drugs. These results indicate that, despite their different composition, all CS-based systems have a colloidal size and a positive zeta potential. Moreover, it can be noted that the zeta potential was inverted from highly negative values for the uncoated nanoemulsion (about -52 mV) to highly positive values for CS nanocapsules (about +34 mV). The positive zeta potential can be logically explained by the presence of the cationic polysaccharide CS on the surface of these nanosystems. Simultaneously to the inversion of the zeta potential, we observed an increase in the size that was attributed to the presence of a CS coating.

Table 1: Physicochemical properties and encapsulation efficiency of different macromolecules into a nanoemulsion, chitosan-coated lipid nanoparticles chitosan nanocapsules and chitosan nanoparticles as well as the route of administration of the nanosystems (mean ± s.d.; n=3).

Carrier	Size (nm)	ζ Potential (mV)	Encapsulated peptide	Administration route	Encapsulation efficiency (%)
Nanoemulsion	195.8±1.1	-52.0±1.1	Salmon calcitonin	Oral Nasal	>98
Chitosan nanocapsules	266.6±7.6	+34.8±0.6	Salmon calcitonin	Oral Nasal	44.1±3.2
Chitosan-coated lipid nanoparticles*	537±16	+29.2±6.2	Salmon calcitonin	Oral	30.7±2.3
Chitosan nanoparticles**	337±14	+36.9±0.3	Insulin	Nasal	94.7±2.1

* Data from Garcia-Fuentes et al, (ref. 26, 33).
** Insulin/chitosan ratio (w/w): 0.4. Data from Urrusuno et al, 1999 (ref. 23).

The presence of this coating could also be visualized by transmission electron microscopy (Fig. 2). It can be seen that both, the control nanoemulsion and

CS nanocapsules, have a spherical shape. However, the nanocapsules show a corona that was attributed to the CS molecules attached to the surface of the oily droplets. Interestingly, besides the potential mucoadhesive characteristics that CS confers to the nanosystems, it was recently observed that this CS corona enhanced the stability of the oily nanodroplets in the gastric fluids [31].

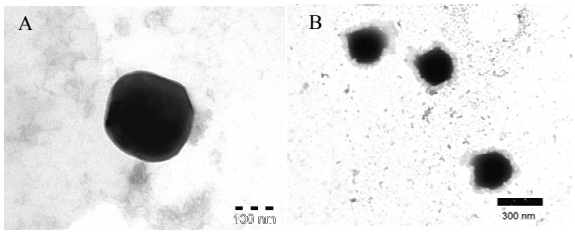

Figure 2: Transmission electron micrographs of (A) the control nanoemulsion and (B) chitosan nanocapsules.

The three CS-based systems showed a great capacity to associate macromolecules. For example, CS nanoparticles have a high loading capacity for acidic proteins such as insulin (I.P.=5.4) and tetanus toxoid (I.P.=4.4–5.9), reaching association efficiencies as high as 97% and 55%, respectively [14,23]. The reason for this high association was related to the affinity of these negatively charged molecules with the positively charged CS molecules. The association of other types of macromolecules such as the lipophilic peptide cyclosporin A has also been possible through the adjustment of the formulation conditions. On the other hand, the CS-coated lipid systems have also shown an ability to associate insulin as well as the cationic peptide sCT (I.P.=10.4) [25,26,29]. The association of insulin was attributed to its affinity for lipid materials and also for CS, whereas the association of sCT was facilitated by the lipids but slightly inhibited by CS. This inhibition of sCT association was ascribed to the positive character of both sCT and CS, that may compete for the anionic binding sites of the lipid cores. Overall, these results indicated that a variety of macromolecules, either hydrophilic or lipophilic, can be efficiently associated to these CS-based nanosystems.

With respect to the in vitro release behaviour, our previous work on CS nanoparticles led us to the conclusion that the chemical structure of the protein, and its interaction with CS in the release medium conditions, were major factors governing the release process. For example, in the case of insulin, we observed a fast release that was associated to its easy detachment from CS in buffered conditions [23]. In contrast, in the case of tetanus toxoid-loaded CS nanoparticles, we noted an initial burst of release followed by a slow release that was dependent on the CS molecular weight [32]. This slow release was justified by the affinity of the toxoid for CS and the characteristics of the release medium. Similarly, the release of the peptide sCT from the CS-coated lipid systems followed a biphasic pattern consisting of an initial burst followed by a continuous and slow release [26,29]. The burst of release was attributed to the surface-associated peptide whereas the second slow phase corresponds to the release of the well-entrapped peptide molecules. These results suggest that more studies are needed in order to fully understand the factors that govern the release from the different nanostructures. However, we must keep in mind that the physiological conditions under which these nanosystems release their content in vivo are expected to be quite different from those in vitro and, consequently, in vitro–in vivo correlations should not be expected.

Cytotoxicity of CS-based nanosystems in Caco-2 cells

In order to investigate the cytotoxicity of CS associated to a nanosystem, we determined the intracellular dehydrogenase activity in Caco-2 cells exposed to different concentrations of CS nanocapsules and to the control nanoemulsion. Fig. 3 shows that, as it was the case for the lipid nanoparticles [33], the control nanoemulsion did not affect cell viability in the range of concentrations assayed. On the other hand, following exposure of Caco-2 cells to CS nanocapsules, we observed a dose-dependent cell viability [34]. More specifically, the cellular viability decreased when the concentration varied between 250 μg/ml (152 μg/cm^2) and 1000 μg/ml (606 μg/cm^2), being the media lethal concentration (LC50) around 1000 μg/ml of nanocapsules suspension (606 μg/cm^2), a value that corresponds to approximately 80 μg of chitosan/ml (48.49 μg/cm^2). A similar dependence of cell viability with CS dose was observed for CS-coated lipid nanoparticles [33] and CS

[35]. A conclusion that can be drawn from this study is that the toxicity values are not clearly dependent on the nanocarrier structure (polymer solution, or CS-coated nanosystems). This conclusion is also corroborated by a recent study that showed similar cellular toxicity for CS nanoparticles and CS solutions [36]. On the other hand, from the perspective of the potential in vivo toxicity, it is worthwhile to mention that the toxicity of these new formulations is low if we take into account the surface area of the intestinal barrier (1 m2 for rats, 200 m2 for humans) [37].

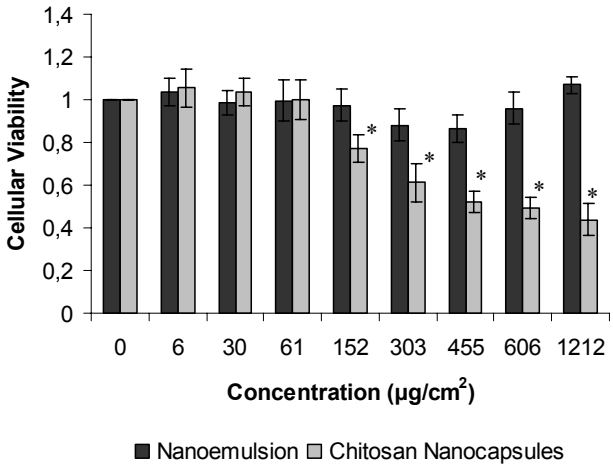

Figure 3: Cytotoxicity profiles of chitosan nanocapsules and the control nanoemulsion in Caco-2 cell monolayer (Mean±SD, n=4–8). *Statistically significant differences from the nanoemulsion ($p<0.01$).

Interaction of CS-based nanosystems with the Caco-2 cells monolayer: measurement of the transepithelial electrical resistance (TEER)

It is well known that CS solutions cause a significant and dose-dependent decrease of the TEER of the Caco-2 cell monolayers. The first evidence of this phenomenon was reported in the early 1990s [20]. These initial studies intended to explore the mechanism of interaction between CS and the intestinal epithelium concluded that the loss of tight junction integrity was caused by a reorganization of the actin rings [20]. However, a more recent hypothesis points to an interaction between chitosan and the tight junction protein ZO-1, leading to its translocation to

the cytoskeleton [38]. In addition to these mechanistic details, some studies have shown that the ability of CS to modify the TEER of Caco-2 monolayers is dose-dependent [38,39]. Unfortunately, quantitative comparisons between studies are difficult due to the different experimental conditions used and also to the different types of CS tested (different deacetylation degrees and molecular weights). For example, very marked TEER reductions (up to 80%) were observed for high CS concentrations (5 mg/ml) [38]. However, significant reductions in the TEER values after prolonged exposure to low CS concentrations 0.5 mg/ml were also reported [39].

Based on this information, we found it important to evaluate if the association of CS to a colloidal carrier would modify its interaction with the intestinal epithelial cells. With this purpose in mind, we have measured the TEER of the monolayer exposed to different doses of CS nanocapsules and to the corresponding controls. In Fig. 4 we can notice that neither the controls (HBSS and HBSS pH 6) nor the control nanoemulsion influenced the TEER values after incubation for 100 minutes. On the other hand, following exposure of the cells to CS nanocapsules, we detected a reduction in the TEER values of the monolayer, however, the extent of this decrease was remarkable only for high concentrations of CS nanocapsules. More precisely, when the dose of CS nanocapsules was 80 μg/cm2 the TEER value was slightly reduced (10%), and a decrease of around 35% was only observed for a high dose of CS nanocapsules (204.5 $\mu g/cm^2$). Higher doses were not tested since the dose of 204.5 $\mu g/cm^2$ led to a decrease in cell viability of 20–30%. An additional observation was that in all the cases, the TEER values returned gradually to the normal values (total recuperation after 24 h in MEM). A comparable decrease in TEER was observed with similar CS doses in the form of CS-coated lipid nanoparticles [33]. Despite the difficulties to compare experiments only on the basis of the polymer concentrations, globally CS nanosystems and solutions seem to have similar dose- TEER reduction profiles [39]. This indicates that CS-coated lipid systems mostly maintain the intrinsic permeabilizing properties of the CS polymer solutions. In contrast, preliminary data using CS nanoparticles suggested that these nanosystems do not display this permeabilizing property at least at concentrations below 250 μg/ml. At this point it is tempting to speculate that the

different presentation of CS, in the form of a soluble coating or as solid CS nanoparticles, may be responsible from this different behaviour.

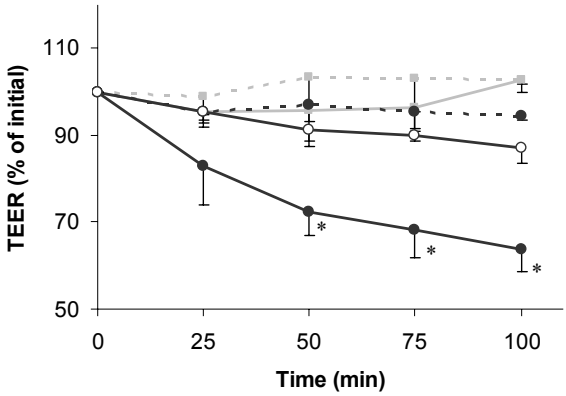

Figure 4: Transepithelial electric resistance (TEER) of Caco-2 monolayer exposed to chitosan nanocapsules with two doses of formulations per surface area, 80 μg/cm^2 (solid black line with open circles, o) and 204.5 μg/cm^2 (solid black line with close circles, ●), the nanoemulsion (solid grey line, —), or their respective controls of Hank´s balanced salt solution (HBSS) (HBSS pH 7.4; dotted grey line, - - -, and HBSS pH 6; dotted black line, - - -). (Mean±SD, n=3). *Statistically significant differences from chitosan nanocapsules with the lowest dose per surface area, the nanoemulsion and both HBSS ($p<0.01$).

Interaction of chitosan-based nanosystems with the Caco-2 cells monolayer: quantitative uptake studies

We have previously investigated the mechanism of interaction of CS nanoparticles with the Caco-2 cells [40]. The results of this study made clear that CS nanoparticles were internalized by the cells and also that this internalization process was saturable (30 min approx.) as well as energy and temperature-dependent. Moreover, these previous results showed that the presence of mucus in the monolayers (MTX-E12cells) strongly increased the association of the nanoparticles.

In the study reported here, we aimed to investigate the behaviour of CS nanocapsules and, hence, to elucidate if the composition of the carrier would affect the interaction of CS-based systems with the Caco-2 cells monolayer (Fig. 5). For

this purpose, we used fluorescent rhodamine-loaded CS nanocapsules and quantified the percentage of internalized fluorescence as a function of the dose per area of nanocapsules added to the incubation medium. The results showed a linear dose-dependent uptake for the range of concentrations between 250 and 1000 μg/ml (71–286 μg/cm^2). It was also noted that the uptake was low and similar for the nanocapsules (maximum value of uptake of 7 μg/cm^2) than for the corresponding nanoemulsion (5 μg/cm^2). Interestingly, a much higher association was observed for both CS-coated lipid nanoparticles and the corresponding control lipid nanoparticles as compared to that observed for the nanocapsules [33]. Consequently, these results suggest that, not only the CS coating but mainly the nature of the core (either oil or tripalmitin) influences the systems uptake. On the other hand, we observed that the low association values of CS nanocapsules to the Caco-2 cells monolayer are also comparable to those we have observed for CS nanoparticles [40]. The differences observed for the various nanosystems in the Caco-2 cells association profiles, as well as the suggested dependence on the structure of the system, underline the necessity of additional studies in order to gain a better understanding about the role of CS in the interaction of nanosystems with the intestinal epithelium.

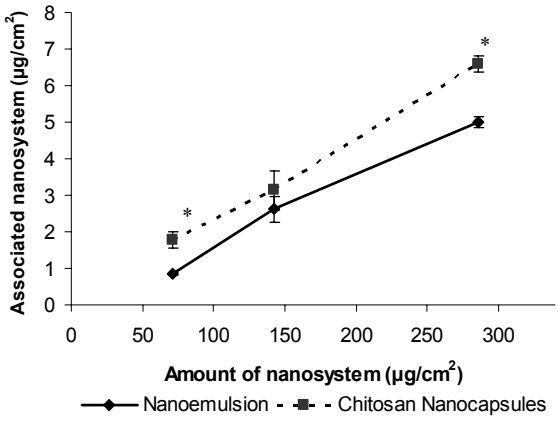

Figure 5: Percentages of fluorescent nanoemulsions and chitosan nanocapsules associated to the Caco-2 monolayer following their incubation for 1 hour at different concentrations (Mean±SD, n=3). The dose of the incubated formulations per surface area values were: 0, 71.43, 142.86 and 286.71 μg/cm^2. *Statistically significant differences from the nanoemulsion ($p<0.01$).

In vivo efficacy of CS-based nanosystems as transmucosal macromolecular drug carriers

Bearing in mind that the main goal of our work has been to design new transmucosal macromolecular drug carriers, we have tested different CS-based nanosystems for their ability to increase the transport of peptides and proteins across the nasal and intestinal mucosa. We showed the first evidence of this capacity for CS nanoparticles containing insulin. Indeed, these nanoparticles led to a significant decrease of the glycemia levels, as compared to a control solution of insulin and CS, following intranasal administration to rabbits [23]. Moreover, we observed that this increased absorption was maintained after freeze-drying and further resuspension of the nanoparticles [41]. Recently, we also showed that chitosan nanoparticles were able to increase the nasal transport of antigens, i.e. tetanus toxoid [32]. More importantly, the results of the increasing and long-lasting IgG levels generated following intranasal administration of tetanus toxoid-loaded CS nanoparticles led us to hypothesize that the nanoparticles facilitated the delivery of the antigen to the immunocompetent cells.

As an alternative vehicle for nasal administration, we have also evaluated the efficacy of CS nanocapsules for enhancing the systemic absorption of sCT [29]. With this purpose in mind, we determined the hypocalcemic effect of sCT in aqueous solution or sCT associated to a control nanoemulsion and to CS nanocapsules after nasal administration to rats. The results showed a negligible response for the controls (either the aqueous sCT solution or the nanoemulsion) but a significant reduction in the calcemia levels for the nanocapsules.

The potential of CS nanoparticles for oral peptide administration has also been reported [42]. The results of this previous study, which was performed in rats, showed that CS nanoparticles were efficient in improving the response of insulin administered orally. The authors speculated that the hypoglycaemic response obtained with CS nanoparticles could be attributed to the protection of the peptide by the formulation and to the potential mucoadhesive and absorption enhancing properties of CS.

Very recently, we have evaluated the potential of CS-coated lipid systems (CS nanocapsules and CS-coated lipid nanoparticles) for oral administration of sCT, using a rat animal model [25,28,33]. In order to elucidate the role of the CS coating we have compared the behaviour of the CS-coated systems with that of the control formulations (nanoemulsion and lipid nanoparticles). In Fig. 6 it is shown that the reduction of the serum calcium levels following oral administration of sCT in the form of an aqueous solution or a nanoemulsion was insignificant. However, the hypocalcemic response was greatly enhanced for CS nanocapsules. More importantly, the percentage of reduction of the serum calcium levels (27% for CS nanocapsules reached at 1 h post-administration) was maintained for 24 h. This long-lasting hypocalcemic response was similar to the one observed for sCT-containing CS-coated lipid nanoparticles [25,33]. Given the lack of efficacy of the controls (non-coated nanoemulsion and lipid nanoparticles), the important hypocalcemic effect elicited by the new formulations was, logically, attributed to the presence of the CS corona around the nanosystems. These results agree with those previously reported for CS-coated poly(lactic acid/glycolic acid) nanoparticles and CS-coated liposomes containing sCT [43,44] and underline the positive effect of a CS coating around the colloidal peptide carriers.

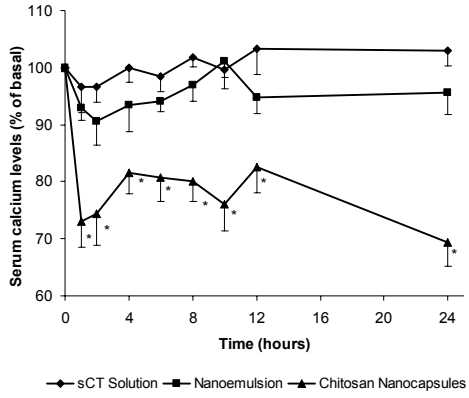

Figure 6: Hypocalcemic effect observed following oral administration of chitosan nanocapsules, a control nanoemulsion and an aqueous solution of sCT to rats (mean±s.e.; n=6). The dose of sCT administered was 500 IU/kg. *Statistically significant differences from sCT solution ($p<0.05$).

The overall conclusion from these studies is that CS-based nanosystems are efficient vehicles for improving the transmucosal transport of peptides. The explanation to this positive effect of CS could be found at different levels: (i) first, as suggested by Kawashima et al. [43] this effect could be related to the mucoadhesive properties of CS and, hence, to its positive interaction with the negatively charged mucosal surfaces; (ii) there is also the possibility that some of these nanosystems interact with the underlying epithelium entering the cells and also opening the tight junctions; (iii) the peptide, either in a free form or associated to the nanosystem, reaches the blood or lymphatic vessels. If we take into account that the quantity of particles that enter the Caco-2 cells (with no mucus layer) was low for both, uncoated and CS-coated systems, while only those coated with CS were effective in vivo, we could speculate that, indeed, the mucoadhesion properties of CS might be determinant in the efficacy of these nanosystems. Nevertheless, more detailed studies will have to be performed in order to fully understand the in vivo behaviour of these novel nanocarriers.

Conclusions

The present study reports the efficacy of three different CS-based nanosystems, i.e., CS nanoparticles, CS nanocapsules and CS-coated lipid nanoparticles, as carriers for nasal/oral administration of macromolecules. Moreover, it gives some mechanistic details that help to understand the mechanism of action of these new systems. The presence of CS on the systems provides a modification of the transepithelial resistance and enhances slightly the uptake of the nanosystems by the Caco-2 model cell line. However, the great enhancement of the in vivo response to a model peptide, sCT, associated to the nanosystems suggests that additional mechanisms, which remain to be elucidated, are responsible for this positive behaviour.

Acknowledgments

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Artículo 4

Chitosan nanocapsules as carriers for oral peptide delivery: Effect of chitosan molecular weight and type of salt on their *in vitro* behaviour and *in vivo* effectiveness

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Sometido a evaluación

Abstract

Very recently, we reported preliminary data that showed the efficacy of chitosan nanocapsules as carriers for oral peptide delivery. In the present work, our aim was to investigate the influence of some chitosan properties, such as molecular weight and type of salt, on the *in vitro* behaviour of these nanocapsules in terms of their interaction with the Caco-2 cells and also on their *in vivo* effectiveness.

Chitosan nanocapsules were prepared by the solvent displacement technique using high (450 kDa) and medium (160 kDa) molecular weight chitosan glutamate as well as high molecular weight chitosan hydrochloride (270 kDa). The results of their *in vitro* characterization indicated that the size of the nanocapsules was dependent on the chitosan molecular weight, varying between 250 and 344 nm, whereas the zeta potential (around +30 mV) and the association efficiency of salmon calcitonin (40% approx.) were not affected by the chitosan properties. Upon incubation with the Caco-2 cells, chitosan nanocapsules exhibited a dose-dependent cellular viability, which was hardly affected by, either the chitosan molecular weight or, the type of salt. In addition, it was observed that the transepithelial resistance of the Caco-2 monolayer was not significantly modified upon their exposure to a dose of chitosan nanocapsules which does not compromise the cellular viability.

The results of the *in vivo* response achieved following oral administration to rats indicated that chitosan nanocapsules were able to reduce significantly the serum calcium levels, in comparison to the uncoated nanoemulsion, and to prolong this reduction for at least 24 hours. Moreover, this response was not affected by the type of chitosan salt nor by its molecular weight.

In conclusion, this study corroborates the efficacy of chitosan nanocapsules for improving the oral absorption of salmon calcitonin. Moreover, it indicates that changes in chitosan properties such as chitosan type of salt and molecular weight did not affect either the interaction or the permeability of the Caco-2 monolayer or their *in vivo* efficacy.

Keywords: chitosan nanocapsules, chitosan properties, Caco-2 cell culture, oral efficacy.

Introduction

There is no doubt that the oral route is the more convenient way to deliver drugs to the body. Unfortunately, up until now the oral administration of peptides and proteins has not been feasible due to their instability in the gastrointestinal tract and also to their low permeability across the epithelium. However, the important efforts that many researchers have dedicated over the last few years to the design of approaches intended to overcome these problems have crystallized in very positive results. In fact, nowadays there are a number of peptide formulations in clinical trials which hold great promise and open optimistic prospects towards the oral peptide administration (1-3). These technologies include the chemical modification of peptides, but also the use of carriers that facilitate the transepithelial transport of macromolecules (Eligen technology) as well as the use of a combination of enzyme inhibitors, absorption enhancers and enteric coating.

Apart from the technologies undergoing clinical trials, one of the approaches that has led to great expectations is one based upon the use of nanosystems (4-7). Indeed, in the late 80s it was shown for the first time that poly(alkylcyanoacrylate) nanocapsules were able to protect insulin from degradation and to facilitate its transport across the intestinal barrier (8). Following this original study, a number of reports have made it clear that the size is critical for the interaction of the delivery carrier with the absorptive site (9, 10), and also that the biological behaviour of the constituent of the carrier is a determining factor for its efficacy. More specifically, it has been found that polymers which exhibit mucoadhesive properties are very good candidates for the design of a trasmucosal drug nanocarrier (11-13). The efficacy of this approach is well illustrated with the results reported for the nanosystems based on the polysaccharide chitosan (14, 15). Indeed, this hydrophilic and cationic polymer has been presented in the form of nanomatrices (16, 17) and also as a coating of different types of nanosystems (12, 18, 19). In all cases, the nanostructural presentation of chitosan has resulted in a very positive behaviour in terms of their application for transmucosal drug delivery and, in particular for ocular (20), nasal (21) or intestinal (19, 22) drug delivery. While the mechanistic details need to be further investigated, the hypothesis is that the known

mucoadhesive and penetration enhancing properties inherent to chitosan may play a significant role in their performance (23, 24).

Chitosan is commercially available in the form of different types of salt and with different molecular weights. Some authors have investigated the influence of these properties, using chitosan as an aqueous solution, on its *in vitro* cell toxicity and penetration enhancing properties (25-29). Unfortunately, the analysis of these results makes it difficult to extract clear conclusions. For example, Schipper et al, 1996 (25) observed that soluble chitosans are able to promote the drug transport across the Caco-2 monolayer, regardless the molecular weight of the polymer (ranging between 4.7 and 19 kDa). However, Chae et al, have shown an effect of the molecular weight of chitosan on the ability to modify the tight junctions, chitosan olygomers being (molecular weigh <10 kDa) those that produced the lowest decrease in the transepithelial resistance values (26). With respect to the effect of the type of salt on the penetration enhancing properties, the *in vitro* transport study performed by Kotze et al. (27) indicated the superiority of chitosan hydrochloride with respect to glutamate.

On the other hand, from the analysis of the influence of chitosan molecular weight on its cytotoxicity in Caco-2 cells, it can be deduced that there is a tendency for the cytotoxicity to increase with the molecular weight (25, 26, 28). However, despite the difficulties of comparing these data, the differences in the toxicity values do not appear to be important. Similarly, there is preliminary evidence that the type of salt in which chitosan is presented may affect its cytotoxicity, chitosan hydrochloride being more toxic than glutamate, glycol or lactate (29).

Finally, with respect to the influence of chitosan properties on its *in vivo* performance, the results reported up until now, either for chitosan solutions or chitosan nanoparticles have not made such influence evident (21, 30, 31). For example, we have previously shown that the ability of chitosan nanoparticles in enhancing the nasal absorption of insulin (21) or eliciting an immune response against tetanus toxoid administered intranasally was not or hardly affected by the molecular weight of chitosan (31).

Besides these previous reports, the influence of the above indicated parameters on the toxicity and efficacy of chitosan nanocapsules has never been reported. Therefore, taking into account this information, the aim of the present work was to investigate the effect of chitosan molecular weight and chitosan salt on the behaviour of chitosan nanocapsules upon contact with the Caco-2 cell monolayer. In addition, we studied the influence of these properties on the potential of chitosan nanocapsules as carriers for oral peptide administration using salmon calcitonin as a model peptide.

Materials and Methods

Materials

Salmon calcitonin (sCT) was kindly donated by Almirall Prodesfarma, S.A. (Spain). Miglyol 812®, a triglyceride formed from medium chain fatty acids was supplied by Lemmel (Spain). The surfactant soybean L-α-lecithin and Poloxamer 188 (Pluronic F-68®) were supplied from Sigma-Aldrich (Spain). Chitosans with an acetylation degree of 15% and different salts (glutamate and chloride) and viscosities (<20 mPa and 20-200 mPa) were purchased from FMC Biopolymer/ Novamatrix (Norway). The molecular weight of these different polymers is presented in table 1. For the purpose of this work, we consider low a molecular weight <100 kDa, medium, a molecular weight between 100-200 kDa and high, a molecular weight >200 kDa.

Table 1: Physicochemical characterization of different types of chitosan.

Chitosan brand name	Salt form	Viscosity (mPa)	Molecular weight (kDa)
Protasan® UP Cl 213	Chloride	71	270
Protasan® UP G113	Glutamate	16	160
Protasan® UP G213	Glutamate	133	450

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, UK). Minimum Eagle Medium (MEM), fetal bovine serum (FBS), non-essential amino acids, L-glutamine, 100 UI/ml penicillin/ 100 µg/ml streptomycin solution, 0.05% trypsin/0.02% EDTA solution and Hank's balanced salt solution (HBSS) were purchased from Sigma (Spain).

Preparation of chitosan nanocapsules

Chitosan nanocapsules were prepared according to the procedure previously described by our group (22, 32), in two steps. First, we prepared a nanoemulsion (33) as a reference formulation by the solvent displacement technique. Secondly, this colloidal carrier was incubated with chitosan solutions leading to the formation of chitosan nanocapsules. Briefly, 125 µl de Miglyol were added to an organic phase composed of 40 mg of lecithin dissolved in 0.5ml of ethanol and 9.5 ml of acetone. This organic solution was poured into 20 ml of an aqueous phase containing Poloxamer 188 (0.25% w/v). The mixture immediately turned milky due to the diffusion of the acetone towards the aqueous phase and the consequent formation of colloidal particles. Then, the solvent was evaporated under vacuum and the nanoemulsion was concentrated to a final volume of 10 ml. Finally, this nanoemulsion was coated by different types of chitosan (See Table 1) by simple incubation in polymer solutions. More specifically, 4 ml of the control nanoemulsion were incubated with 1 ml of chitosan aqueous solution (0.5% w/v) for 1 h, leading to the formation of chitosan nanocapsules.

Characterization of chitosan nanocapsules

The mean particle size of the colloidal systems was analyzed by photon correlation spectroscopy (PCS). For the determination, samples were diluted to the appropriate concentration with filtered ultrapure water. Each analysis was performed at 25ºC with an angle detection of 90º.

The zeta potential was calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry (LDA). Nanocapsule suspensions were diluted with KCl 1mM and placed in the electrophoretic cell where a potential of ±150 mV was established. The PCS and LDA analysis were performed using a Zetasizer® 3000 HS (Malvern Instruments, Malvern, UK). Each batch was analyzed in triplicate.

Salmon calcitonin encapsulation efficiency

The encapsulation of sCT in chitosan nanocapsules made with different types of chitosan (see Table 1) as well as the control nanoemulsion was performed by incorporating 50 µl of an aqueous solution of sCT to the organic phase. The amount of sCT associated to the nanocarriers was indirectly calculated from the amount of free sCT in the supernatant of the nanocarriers. The non-encapsulated sCT was determined following separation of nanocapsules from the aqueous medium by a combined ultrafiltration-centrifugation technique (Centricon YM-100, Millipore, USA) at 1000 x g for 1h. The supernatant was diluted with pH=4 acetate buffer and assayed for sCT content by HPLC at 220 nm (Agilent Technologies, Germany), as described in the British Pharmacopoeia, 1998 (column: Vidac 218TP). A calibration curve was made with solutions of sCT in pH=4 acetate buffer at concentrations ranging from 5 to 100 µg/ml. Each sample was assayed in triplicate.

In vitro release studies

In vitro release studies of sCT from nanocapsules made of chitosan chloride and chitosan glutamate of different molecular weight were performed by incubating 3 ml of the loaded nanocapsules in 3 ml of acetate buffer (pH= 4.0) at 37°C, under horizontal shaking (100-110 cycles min^{-1}). At appropriate time intervals, the supernatant of chitosan nanocapsules was collected by ultrafiltration at 1000 xg for 1 h. The amount of sCT released in each time-interval was determined by HPLC.

Cell culture experiments

Caco-2 cells were cultivated in 80 cm^2 flasks (Nunc, Denmark) using MEM supplemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids and penicillin/ streptomycin solution. Cells were maintained in a controlled atmosphere at 37ºC with 95% of relative humidity and 5% CO_2. The culture medium was changed every second day for approximately 5-6 days until cells reached approximately 80-90% of confluence. After the passage operation, cells were seeded approximately at 2.5 x 10^5 cells per flask. For the experiments, cells with passage numbers between 30 and 40 were used.

Cytotoxicity studies

Caco-2 cells were seeded at a cell density of 10^4 cells/cm^2 into 96 multiwell plates and were allowed to grow for 7 days, until cell monolayer was obtained. Then, the medium was replaced by the formulations of chitosan nanocapsules (CS of different viscosities and salt form). The concentrations of nanocapsules assayed varied between 0.01 and 2 mg/ml, in a final volume of 0.2 ml. As a positive control we have used HBSS pH 6 and as a negative control 2% sodium dodecyl sulfate (SDS). Chitosan nanocapsules and the controls were put in contact with the monolayers for 90 min at 37ºC and 5% CO_2. After this, the nanocapsule suspensions were removed and cell viability was determined using a colorimetric method (MTS, Promega), which determines the intracellular dehydrogenase activity. The absorbance was measured at 490 nm.

Transepithelial electrical resistance studies

The measurement of the transepithelial electrical resistance (TEER) was performed on Transwell™ plates with a cell density of 5.5 x 10^4 cells 21 days post-seeding. The integrity of the monolayers was checked on the 21st day in MEM. The TEER measurements were performed with a Millicell®-ERS connected to a pair of

chopstick electrodes (Millipore, Spain). After these measurements, the medium was changed by HBSS in order to perform the experiments in buffer. After 1 hour of equilibrium, the apical solutions were replaced by the chitosan nanocapsule suspension (CS of different viscosities and salt form) and its respective control, HBSS pH 6. The concentration of chitosan nanocapsules was 250 and 450 µg/ml in a cell growing areas of 4.5 cm^2 leading to a nanocapsule dose of 80 and 150 $\mu g/cm^2$. Nanocapsule suspensions were incubated for 100 minutes and TEER measurements were performed every 25 minutes. After this, the solutions were removed and replaced by MEM in order to check the recuperation of the TEER values at 24 hours.

In vivo efficacy of chitosan nanocapsules

Male Sprague-Dawley rats (225- 275 g), from the Central Animals House of the University of Santiago de Compostela (Spain), were fasted for 12 h before experiments, but allowed water *ad libitum*. Animals were kept conscious during the experiments.

We have administered intragastrically chitosan nanocapsules with two different viscosities of chitosan (16 and 133 mPa). As controls we used the previously assayed sCT aqueous solution and the nanoemulsion (22). The dose administered in all the cases was 500 UI/kg in a volume of 0.5 ml.

Blood samples were collected from the tail vein 30 min prior to the oral administration to establish the baseline calcium levels and at different times after dosing. The serum was separated by centrifugation at 3000 xg for 5 min. Hypocalcemic effect was determined in serum samples by a colorimetric method at 570 nm (Kit OR-cresolphtalein v/v, Spinreact, Spain).

Statistical analysis

Data from the *in vitro* experiments were analyzed using the statistical software package SPSS 11.5. Statistically differences were considered to be significant at a level of $p < 0.01$.

The mean serum calcium levels determined in samples collected before sCT administration were taken as the baseline levels. At each time interval the reduction of calcium percent was calculated. Using these values, the area above the time curve of the hypocalcemic effect was calculated and the statistical comparison was performed by ANOVA followed by the Student-Newman-Keuls test for multiple comparisons between treatments using the software SPSS 11.5. Differences were considered to be significant at a level of $p < 0.05$.

Results and discussion

Chitosan nanocapsules were developed for oral peptide delivery with the idea of protecting the associated macromolecule from enzymatic attack and facilitating the interaction of the peptide with the intestinal mucosa. In a previous paper, we have presented preliminary data showing the role of a chitosan coating around a lipid core, either oily or solid, in the enhancement of the oral absorption of sCT (22). Indeed, in this previous work, we showed that the presence of the chitosan coating was critical for the effectiveness of chitosan nanocapsules and chitosan-coated tripalmitin nanoparticles, since the uncoated formulations did not lead to a significant absorption of the associated peptide. In the present paper, we aimed at investigating how the physicochemical properties of chitosan (different salts and molecular weight) could affect the *in vitro* and *in vivo* behaviour of chitosan nanocapsules.

Characterization of chitosan nanocapsules

Chitosan nanocapsules were prepared by the solvent displacement technique (see properties of chitosan on Table 1) (22, 32). A control nanoemulsion

was also formed under the same conditions but avoiding the process of coating with chitosan. The process of coating with chitosan is based upon the ionic interaction between the negatively charged lecithin and the positively charged chitosan. The resulting complex lecithin-chitosan is responsible for the stabilization of the oily core (34).

The influence of different types of salt (chloride and glutamate) and molecular weight (between 160 and 450 kDa) of chitosan (Table 1) on the physico-chemical properties of chitosan nanocapsules is shown in Table 2. Additionally, this table shows the characteristics of the nanoemulsion used as a control. All the colloidal carriers presented a particle size in nanometre range. The coating of the nanoemulsion with chitosan caused an increase in the size of the system that was attributed to the thickness of the coat. The hydrodynamic diameter of chitosan nanocapsules was influenced by the chitosan molecular weight; however, the type of salt had no significant effect. More specifically, we observed that chitosan nanocapsules prepared with the larger chitosan molecular weight were larger than those prepared with the smaller molecular weight (330nm *vs*. 250 nm, respectively). These results are in agreement with those reported by Calvo et al (33) who found that the increase on the size of chitosan-coated poly-ε-caprolactone nanocapsules was dependent on the size of the chitosan molecules attached to the surface of the carrier.

Table 2: Physicochemical properties and encapsulation efficiency of chitosan nanocapsules and nanoemulsions containing sCT (mean ± s.d.; n=3).

Carrier	Size(nm)	ζ Potential (mV)	Encapsulation efficiency (%)
NE	195.8±1.1	-52.0±1.1	>98
CS Cl213 NC	344.3±15.0	+32.0±0.8	37.93±2.65
CS G113 NC	250.5±7.9	+34.4±0.7	39.17±3.83
CS G213 NC	330.7±5.8	+31.3±1.6	38.30±1.56

Despite the effect of chitosan molecular weight on the size of the resulting nanocapsules, their surface charge did not suffer a significant change. Indeed, the zeta potential values were in all cases close to +30 mV, independent of the chitosan properties. However, a remarkable difference was noted when comparing these values with those corresponding to the control nanoemulsion, which displayed a high negative charge. This inversion in zeta potential confirmed the presence of chitosan adsorbed onto the surface of the colloidal carriers and suggested that this cationic polymer forms a coating around the oily cores, hence forming a reservoir structure.

Salmon calcitonin encapsulation efficiency

A comparison of the encapsulation efficiency of sCT in the different chitosan nanocapsules and the control nanoemulsion formulation is shown in Table 2. The ability of these nanocarriers to entrap sCT into their structure was dependent on their composition. In fact, the control nanoemulsion showed a total encapsulation of sCT due to the strong binding affinity of the peptide, which is positively charged, for the anionic sites present in the nanoemulsion. This electrostatic interaction was diminished after incubation of sCT-loaded nanoemulsion with the different chitosan solutions, forming sCT-loaded chitosan nanocapsules. In this case, it was found that a certain amount of sCT was expelled from the system due to a competition between chitosan and sCT, both positively charged. The resulting encapsulation efficiency of sCT in chitosan nanocapsules was around 40%, independent of the physicochemical properties of chitosan (type of salt and molecular weight). The lack of influence of the chitosan properties on the encapsulation efficiency could be related to the fact that the surface charge of chitosan nanocapsules was the same regardless the structural properties of chitosan.

In vitro release studies

The *in vitro* release behaviour of sCT from chitosan nanocapsules was investigated using acetate buffer pH=4. This buffer was selected because it is the optimum pH for the preservation of the stability of sCT during the incubation process (35).

The release profiles of sCT from chitosan nanocapsules prepared with chitosan with different physicochemical properties is shown in figure 1. All the formulations displayed a release profile characterized by an initial burst and no further peptide release for up to 6 hours. It can also be noted that this initial release-phase was not affected by the chitosan type of salt nor by the molecular weight. The initial release phase could correspond to the release of the amount of sCT that was entrapped on the chitosan coating, rather than in the core of the nanocapsules. The low affinity of this cationic peptide for chitosan could be an explanation for this fast initial release. On the other hand, the great affinity of the peptide for the lipids would justify the lack of further release under the experimental conditions of the study.

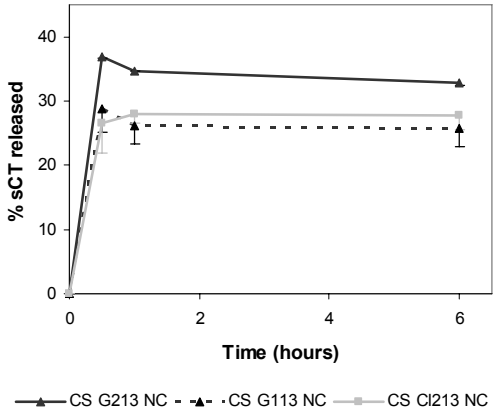

Figure 1: In vitro release profiles of salmon calcitonin from chitosan nanocapsules prepared with different salts and molecular weights of chitosan (mean ± s.d.; n=3).

Cytotoxicity studies

An important aspect of the investigation of the potential of chitosan nanocapsules for oral delivery is the analysis of their toxicity upon interaction with the intestinal epithelium. It has been previously shown that the viability of Caco-2 cells decreases in a dose-dependent manner after incubation with chitosan (25, 26, 28). Additionally, it has been reported that specific properties of chitosan, such as salt form and molecular weight may influence this cytotoxic effect (25, 26, 28, 29). However, as indicated in the introduction, the influence of these properties has not been clearly elucidated. This is due, on the one hand, to the difficulties in comparing published data; and, on the other hand, to the fact that these differences are not remarkable. Moreover, it is important to keep in mind that the relevance of these properties may be dependent on the physical presentation of chitosan (as a solution, as a coating material or as a nanomatrice).

Thus, in the present work we have evaluated the cellular viability of Caco-2 monolayer after exposure to chitosan nanocapsules for up to two hours (Fig. 2). More specifically, we studied the effect of chitosan coating properties, salt form and molecular weight, on the intracellular dehydrogenase activity in Caco-2 cells. As shown in Figure 2, the first observation was that chitosan nanocapsules presented concentration-dependent cytotoxicity. Upon exposure of Caco-2 cells to concentrations of nanocapsules higher than 0.25 mg/ml (152 $\mu g/cm^2$), the cellular viability started to decrease, reaching the 50% lethal concentration (LC50) at a concentration between 1 and 2 mg/ml. In addition, it can be observed that either the salt form of chitosan or its molecular weight have only a minor effect on the cell viability. These results agree with those previously reported for chitosan solutions and chitosan nanoparticles prepared with chitosan of molecular weights ranging between 4.7 and 213 kDa (25, 28). However, these results should not lead to the conclusion that chitosan molecular weight does not influence the cellular viability, but only that this effect was not seen within the range of chitosan molecular weight investigated (between 160-450 kDa). In fact, it has been recently reported that chitosan olygomers (molecular weight <10 kDa) have less cytotoxicity effects than high molecular weight chitosans (molecular weight 230 kDa) (26).

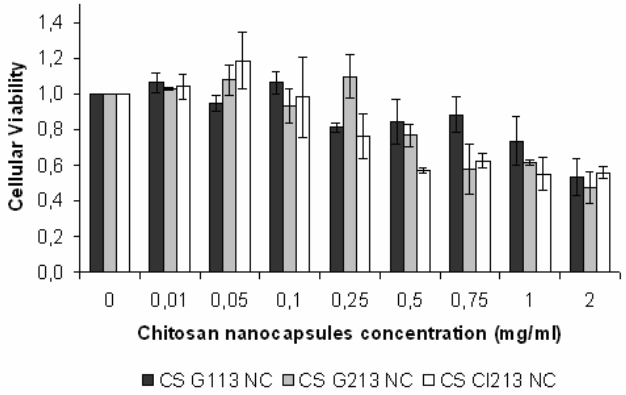

Figure 2: Effect of the different formulations of chitosan nanocapsules on the cellular viability of Caco-2 cells (mean ±d.e., n≥4).

Transepithelial electrical resistance studies

Chitosan is known for its ability to enhance the penetration of drugs across the epithelium by a mechanism involving the interaction of the positively charged amino groups of chitosan with the negatively charged sites on the cell surfaces and tight junctions (24, 25, 36, 37). A way to study the intensity of this effect is the measurement of the transepithelial electrical resistance of the monolayer. For example, in a previous work, we have shown that the exposure of the Caco-2 monolayer to chitosan nanocapsules may lead to a reduction of the TEER (22). However, the results of this work also indicated that it was necessary to use a high concentration of nanocapsules (which are at the toxic level) in order to observe a significant reduction of the TEER value. Unfortunately, the comparison of these results with those obtained for chitosan solutions is unviable due to the different experimental conditions among experiments and to the absence of meticulous information concerning these conditions.

Thus, in this work, our objective was to evaluate whether or not chitosan properties (molecular weight and salt form) affect the interaction (TEER value) of the nanocapsules with the monolayer. Additionally, we have analyzed the effect of the dose of chitosan nanocapsules on the TEER values, up to the limit to the

maximum dose which does not compromise cell viability. In figure 3, we can appreciate the evolution of TEER values after exposing the cells to chitosan Cl213 nanocapsules and the previously assayed chitosan Cl110 nanocapsules (22). We can observe that the TEER values of the Caco-2 monolayer decreased slowly, over the time, upon exposure to high molecular weight chitosan hydrochloride (Protasan® Cl 213; Mw: 270 kDa) nanocapsules, reaching a 10% reduction after 75 min. This slight reduction was similar to the one observed for medium molecular weight chitosan (Protasan® Cl 113; Mw: 140 kDa) nanocapsules (22).

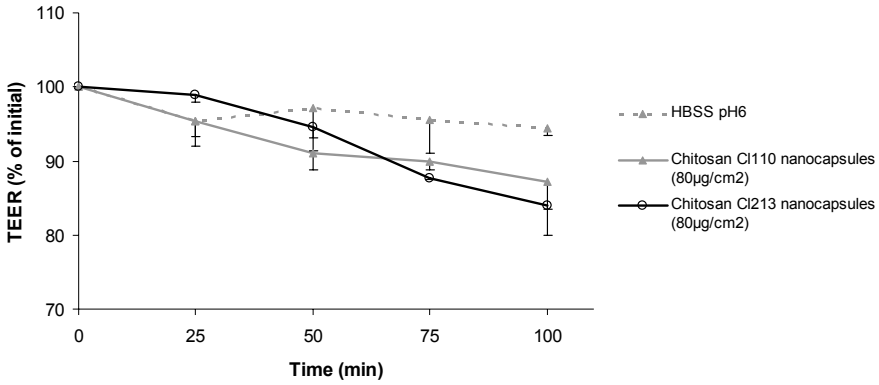

Figure 3: Transepithelial electrical resistance (TEER) of Caco-2 cells after incubation with 250µg/ml of chitosan Cl 213 nanocapsules, chitosan Cl 110 nanocapsules* and the control HBSS pH 6 in a surface area of 4.5 cm^2 (mean ± s.d.; n= 3). *Data from Prego *et al* 2005 (22).

The effect of chitosan glutamate nanocapsules, with two different molecular weights of chitosan (160 and 450 kDa), on their ability to modify TEER values is shown in figure 4. Additionally, this graph shows the influence of the dose of chitosan G113 nanocapsules (80 and 150 µg/cm^2) on the TEER values. As it occurred in the case of chitosan hydrochloride nanocapsules, the different chitosan glutamate molecular weights (160 and 450 kDa) used to prepare chitosan nanocapsules led to a slight and similar modification of the TEER values at the dose

per surface area of 80 μg/cm^2. An additional observation from this figure is that an increase in the dose of chitosan G113 nanocapsules from 80 to 150 μg/cm^2 led to a significant and progressive reduction of the TEER values, reaching the 20% of reduction after 50 min of exposure to the cells.

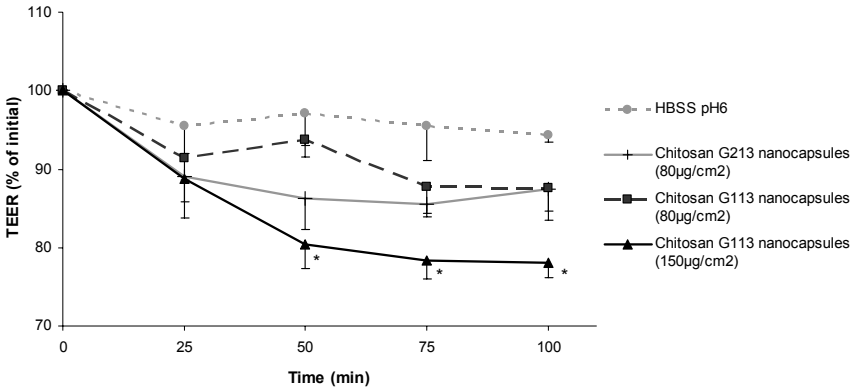

Figure 4: Transepithelial electrical resistance (TEER) of Caco-2 cells after incubation with of chitosan G 113 nanocapsules, chitosan G 213 nanocapsules, at two does per surface area, 80 and 150 μg/cm^2, as well as the control HBSS pH 6 (mean ± s.d.; n= 3). * Significantly different from HBSS pH6 ($p < 0.01$).

Consequently, these results led us to conclude that, within the chitosan molecular weight range of 140- 450 kDa and the salt form of chitosan chloride or glutamate, chitosan nanocapsules have a minor effect on the TEER values of the Caco-2 monolayer. Only when the dose of nanocapsules was increased up to the limit which does not compromise cell viability, we could observe a significant reduction (20%) in the TEER values. Despite the difficulties for a strict comparison of these results with those previously reported for chitosan solutions, there is an agreement in the irrelevance of chitosan properties on the transepithelial resistance values (38). It is also worthwhile to mention that, in all cases, there is a gradual recuperation of the original TEER values.

In vivo efficacy of chitosan nanocapsules

As indicated above, we have already performed some preliminary studies, which showed that chitosan Cl110 nanocapsules are able to improve the pharmacological effect of sCT administered orally (22). In this study, we have attempted to evaluate this effect further by studying the behaviour of nanocapsules prepared with different types of chitosan (different salts and molecular weight). Fig. 5 depicts the hypocalcemic profiles following oral administration of sCT associated to different formulations of chitosan nanocapsules. As reference formulations, we have also tested a sCT solution and a nanoemulsion (22). The results showed that the oral administration of sCT alone or associated to the nanoemulsion did not lead to a change in the serum calcium levels. On the contrary, chitosan nanocapsules showed an important hypocalcemic effect which was prolonged for, at least, 24 h. Moreover, it can be noted that the chitosan molecular weight did not affect this capacity of the nanocapsules to enhance the absorption of the associated peptide.

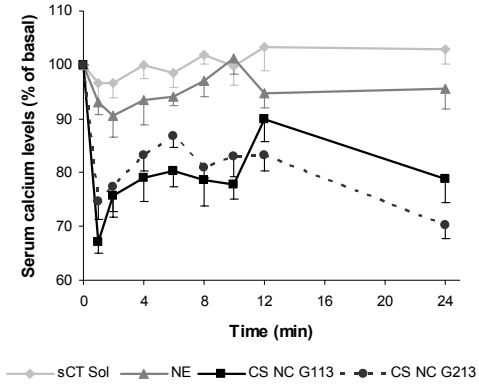

Figure 5: Hypocalcemic effect observed for salmon calcitonin-loaded chitosan nanocapsules prepared with chitosan glutamate of different molecular weight (CS G113 NC and CS G213 NC) as well as for a control nanoemulsion (NE) and for an aqueous solution of salmon calcitonin (sCT Sol), following oral administration to rats (mean ± s.e.; n=6). The dose of salmon calcitonin administered was 500UI.

Finally, the comparison of areas above the hypocalcemic effect curve ($AAC_{0\text{-}24h}$) obtained following administration of the different formulations (Fig. 6) confirms the significantly greater pharmacological effect of chitosan nanocapsules

with different chitosan molecular weights in comparison to the sCT aqueous solution and the nanoemulsion.

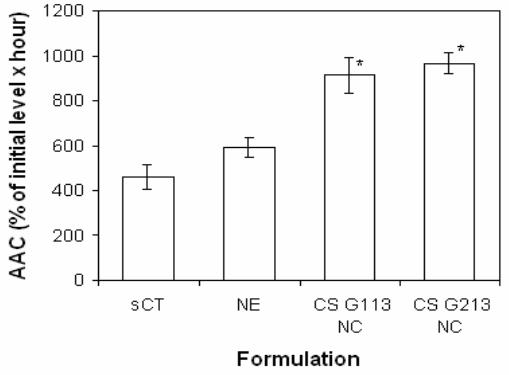

Figure 6: Area above hypocalcemic effect curve ($AAC_{0\text{-}24\ hr}$) after oral administration to rats of salmon calcitonin in solution or incorporated in the nanoemulsions and chitosan nanocapsules made of chitosan glutamate of different molecular weight.* Significantly different from salmon calcitonin solution and the control nanoemulsion ($p < 0.05$).

Therefore, a general conclusion from these *in vivo* studies is that the presence of chitosan was essential to obtain a pharmacological response. These results are in accordance with those previously found for other chitosan-coated based nanosystems such as chitosan-coated PLGA nanoparticles (12), chitosan-coated liposomes (18) and chitosan-coated lipid nanoparticles (19) in the sense that all these colloidal systems led to an increase in the systemic absorption of sCT. However, an interesting observation from this comparative analysis is that the high response attained for nanostructures composed of a lipid core and a chitosan coating is more sustained and prolonged than that corresponding to chitosan-coated PLGA nanoparticles (12), chitosan-coated liposomes (18).

Regarding the mechanism of action of these nanocapsules, we could speculate about their penetration enhancing effect. However, this hypothetic mechanism does not appear to be relevant given the minor effect in the TEER observed *in vitro*. A more probable mechanism would be the one related to the mucoadhesive properties of chitosan and, hence, to the efficient interaction of the

nanocapsules with the intestinal mucosa. In this sense, it is worthwhile to mention the fact that chitosan forming a coating around a colloidal carrier is critical. Indeed, previous studies performed by other groups (18) as well as by our own group have shown that chitosan in the form of a solution or in the form of spray-dried microparticles were not efficient at increasing the systemic absorption of sCT. It should also be taken into account that the fact that the peptide is entrapped in an oily core may help protect the peptide from hydrolytic and enzymatic degradation in the gastro-intestinal tract.

Conclusions

The results of this work confirm the potential of chitosan nanocapsules as carriers for improving the oral absorption of sCT. Additionally, they indicate that this positive behaviour is not affected either by the different type of salt used (hydrochloride or glutamate chitosan) or by the use of different chitosan molecular weights ranging between 140-450 kDa.

Acknowledgments

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Parte 3

Evaluación del potencial de las nanocápsulas de quitosano-PEG como sistemas de administración de péptidos por vía oral.

Artículo 5

Chitosan-PEG nanocapsules as new carriers for oral peptide delivery. Effect of chitosan pegylation degree

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Sometido a evaluación

Abstract

We have previously reported the formation and characterization of chitosan-coated oily nanodroplets, called chitosan nanocapsules, and showed their ability to enhance and prolong the oral absorption of peptides, i..e. salmon calcitonin. In the present work, our goal was to prepare a new type of nanocapsules, using chitosan chemically modified with poly(ethylene glycol) (PEG) (0.5 and 1% pegylation degree) and to investigate the consequences of this modification on the *in vitro* and *in vivo* behaviour of the nanocapsules. In addition, the properties of these chitosan-PEG nanocapsules were compared with those of PEG nanocapsules, used as a control. The hypothesis was that the presence of PEG in the coating of the nanocapsules would be advantageous in terms of improving their stability in contact with the gastrointestinal fluids as well as their cellular biocompatibility.

Chitosan-PEG and PEG nanocapsules could be obtained using the solvent displacement technique. The size of the resulting nanocapsules was in the range of 160-250 nm. Their zeta potential was greatly affected by the nature of the coating, being positive for chitosan-PEG nanocapsules and negative in the case of PEG nanocapsules. The presence of PEG, whether alone or grafted to chitosan, improved the stability of the nanocapsules in the gastrointestinal fluids. In addition, using the Caco-2 model it was observed that the pegylation of chitosan reduced the cytotoxicity of chitosan nanocapsules and also that these nanocapsules did not cause a significant change in the transepithelial resistance of the monolayer. Finally, following oral administration of chitosan-PEG nanocapsules containing salmon calcitonin, it was found that chitosan-PEG nanocapsules have the capacity to enhance the intestinal absorption of salmon calcitonin. Additionally, the results showed that an increase in the pegylation degree (from 0.5% to 1%) led to a reduction in the pharmacological response of the associated peptide. Therefore, modulating the pegylation degree of chitosan, it should be possible to obtain nanocapsules with a good stability, a low cytotoxicity and high absorption enhancing properties.

Keywords: PEG, Chitosan-PEG, nanocapsules, colloidal stability, cell culture, oral peptide absorption.

Introduction

Despite the efforts dedicated over the last decades towards making the oral administration of peptides and proteins feasible, the actual fact is that this objective still remains a challenge. This is quite understandable if we take into account the great barriers that need to be overcome, such as the metabolic activity and the low permeability of the intestinal epithelium (1). However, in spite of these difficulties, the success of the formulations undergoing clinical trials (2-4) offers an optimistic prospect towards reaching this objective.

The use of colloidal carriers such as nanoparticles and nanocapsules (5-7) has been reported to be a promising way to improve the oral bioavailability of peptides and proteins. The proposed mechanisms explaining the efficacy of these nanocarriers are: the great surface interaction of the nanocarrier with the absorptive epithelium, which can be further enforced by the use of bioadhesive materials, and the protective effect of the carrier for the associated peptide. Indeed, the effectiveness of nanocarriers at improving the absorption of labile macromolecules depends strongly on their polymer composition. For example, the simple surface modification of a nanocarrier using polymers with specific properties may be an easy approach to modulate its interaction with the epithelium. In this sense, an interesting polymer with a promising future in the biopharmaceutical and biomedical fields is the positively charged polysaccharide chitosan. In fact, chitosan has already been used in the form of nanoparticles and nanocapsules aimed at improving the transmucosal delivery of drugs across different mucosal surfaces (8-12). Among these different transmucosal drug delivery applications, we have found the use of chitosan nanocapsules for oral peptide delivery quite promising (13, 14). More specifically, following oral administration of chitosan nanocapsules containing salmon calcitonin, we have observed a great improvement on the pharmacological effect.

Taking this previous information into account, the main purpose of this work was to explore the possibilities to further improve the surface properties of chitosan nanocapsules in terms of their interaction with the gastro-intestinal

environment. For this purpose, we chose as an additional polymer, poly(ethylene glycol) (PEG). The choice of this polymer was justified because of two main reasons. First, the chemical modification of chitosan with PEG was seen as a way of improving the biocompatibility of chitosan (15); second, we have previously observed that a PEG coating around the nanocarrier helps to improve their stability in the biological fluids and, as a consequence, facilitates the transport of bioactive macromolecules across the intestinal and nasal epithelium (10, 16, 17).

Therefore, the objectives of the present work were: first, to obtain and characterize nanocapsules made of chitosan chemically modified with PEG, with two different substitution degrees (0.5 and 1%) and, second, to evaluate the effect of pegylation of the *in vitro* and *in vivo* behaviour of the resulting chitosan-PEG nanocapsules. More specifically, we evaluated the cytotoxicity of chitosan-PEG nanocapsules and their possible effect on the TEER of the Caco-2 cells monolayer and, finally, we investigated their efficacy at improving the intestinal absorption of the model peptide salmon calcitonin.

Materials and Methods

Materials

Salmon calcitonin was kindly donated by Almirall Prodesfarma, S.A. (Spain). Miglyol 812®, a triglyceride formed from medium chain fatty acids was supplied by Lemmel (Spain). The surfactant soybean L-α-lecithin and poloxamer 188 (Pluronic F-68®) were supplied from Sigma-Aldrich (Spain). Chitosan-PEG was synthesized from chitosan hydrochloride salt (Protasan® CL110) which was purchased from FMC Biopolymer/ Novamatrix, (Norway). Polyethylene glycol 5000 monomethyl ether (PEG) was supplied by Fluka (Spain). NHS (*N*-hydroxysuccinimide) and EDC [*N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride] were purchased from Fluka (Spain).

Synthesis of Chitosan-g-PEG

We have synthesized chitosan-PEG with two different degrees of substitution, 0.46% and 1.19%, called in this paper chitosan-PEG 0.5 and 1%, respectively. First of all, we characterized the polymers used to synthesize chitosan-PEG: chitosan and PEG. The degree of acetylation of chitosan determined by ^{1}H-NMR according to our modification of the Hirai procedure (18, 19) was 7%. The average molecular weight of chitosan (Mw) was determined by size exclusion chromatography-multiangle laser-light scattering (SEC-MALLS). An Iso Pump G1310A (Hewlett Packard) was connected to a PSS Novema GPC column 10 μ (8 x 50 mm) and a PSS Novema 3000 column (8 x 300 mm). A PSS SLD7000 MALLS detector (Brookhaven Instruments Corporation) operating at 660 nm and a G1362A refractive index detector (Agilent) were connected on line. A 0.15M NH_4OAc/0.2M AcOH buffer (pH = 4.5) was used as eluent. Polymer solutions (0.1% w/v) were filtered on 0.45 μm pore size membranes (Millipore) before injection. Refractive index increment d*n*/d*C* was set at 0.1922 according to Schatz *et al*. (20). The resulting Mw was 87000 Da. MeO-PEG-CH_2CO_2H was synthesized from MeO-PEG-OH (Fluka, Mn 5055.5, Mw 5087.8 determined by MALDI-TOF) according to Royer *et al*. (21).

To perform the synthesis, chitosan·hydrochloride (80 mg, 0.40 mmol) was dissolved in 11.5 ml of water. MeO-PEG-CH_2CO_2H (Mn 5114.5, 14.2 mg, 2.79 μmol) and NHS (1.6 mg, 0.014 mmol) were added to the solution. Then, EDC (21.7 mg, 0.113 mmol) was added in small portions. The resulting solution was stirred at room temperature for 22 hours and afterwards ultrafiltered (Amicon, YM30) and lyophilized [degree of pegylation 0.46%, determined by ^{1}H-NMR (2% DCl in D_2O)].

Chitosan-PEG with a-1.19% substitution degree (determined by ^{1}H-NMR (2% DCl in D_2O)) was synthesized by the procedure indicated above from chitosan·hydrochloride (80 mg, 0.40 mmol), MeO-PEG-CH_2CO_2H (117 mg, 0.023 mmol), NHS (13.5 mg, 0.117 mmol) and EDC (22.4 mg, 0.117 mmol).

Preparation of chitosan-PEG nanocapsules

Chitosan-PEG nanocapsules were prepared in two steps. First, we prepared a nanoemulsion, which was taken as a reference formulation, by solvent displacement technique, as previously reported (22, 23). Secondly, this colloidal carrier was coated with chitosan-PEG with two pegylation degrees of chitosan, 0.5 and 1%, by simple incubation in polymer solutions. More specifically, we prepared the nanoemulsions by adding an organic phase, composed of 125 µl de Miglyol, 40 mg of lecithin dissolved in 10 ml of acetone was added to an aqueous phase containing poloxamer 188 (0.25% w/v). The mixture turned milky immediately due to the formation of the nanoemulsion. Then, the solvents were evaporated under vacuum. Finally, 4 ml of the nanoemulsion were incubated for one hour with 1 ml of chitosan-PEG aqueous solution (0.5% w/v) with different pegylation degrees of chitosan (0.5 and 1%), leading to the formation of chitosan-PEG 0.5% and chitosan-PEG 1% nanocapsules, respectively.

For the preparation of PEG nanocapsules we adopted the conditions described for the formation of the control nanoemulsion but introducing a little modification, which consisted in adding 40 mg of PEG-stearate to the organic phase prior to the mixing with the aqueous phase.

Characterization of chitosan-PEG nanocapsules

The size and polydispersion index of the colloidal systems were analyzed by photon correlation spectroscopy (PCS). For this analysis, samples were diluted to the appropriate concentration with filtered ultrapure water. Each analysis was performed at 25ºC with an angle detection of 90º.

The zeta potential was determined by laser Doppler anemometry (LDA). For the determination of the electrophoretic mobility, samples were diluted with KCl 1mM and placed in the electrophoretic cell where a potential of ±150 mV was established.

The PCS and LDA analysis were performed using a Zetasizer® 3000 HS (Malvern Instruments, Malvern, UK). Each batch was analyzed in triplicate.

The morphology of the nanocarriers was visualized by transmission electron microscopy (TEM) (Philips CM12, Eindhoven, Netherlands). Images were taken following staining of the isolated chitosan-PEG nanocapsules with 2% w/v phosphotungstic acid for one minute and left drying overnight.

Salmon calcitonin encapsulation efficiency

Salmon calcitonin-loaded chitosan-PEG nanocapsules were prepared as described previously by incorporating 50 µl of an aqueous solution of salmon calcitonin to the organic phase containing the oil and phospholipids. Then, this organic phase was added to the external aqueous phase containing poloxamer 188 (0.25%).

The encapsulation efficiency of salmon calcitonin in the nanocapsules and control nanoemulsion was calculated by the difference between the total amount of salmon calcitonin used to prepare the loaded systems and the remaining amount of free salmon calcitonin in the aqueous medium. The amount of free salmon calcitonin was determined in the supernatant following the separation of nanocapsules from aqueous medium by a combined ultrafiltration- centrifugation technique (Centricon YM-100, Millipore, USA) at 1000 x g for 1h. The supernatant was diluted with pH=4 acetate buffer and assayed for salmon calcitonin content by HPLC at 220 nm (Agilent Technologies, Germany) as described in the British Pharmacopoeia, 1998 (column: Vidac 218TP). A calibration curve was made with solutions of salmon calcitonin in pH=4 acetate buffer at concentrations ranging from 5 to 100 µg/ml. Each sample was assayed in triplicate.

In vitro release studies

In vitro release studies of salmon calcitonin from chitosan-PEG nanocapsules was performed by incubating 3 ml of loaded nanocapsules in 3 ml of pH=4 acetate buffer under horizontal stirring at 37°C. At appropriate time intervals, individual samples were ultrafiltered at 1000 x g for 1 h. The amount of salmon calcitonin released at each time point was determined by HPLC.

Stability of the nanocapsules in simulated gastrointestinal fluids

Taking into account that these nanostructures were developed as carriers for oral administration of peptides, we consider it important to evaluate their stability in the gastrointestinal fluids. Consequently, chitosan-PEG nanocapsules, PEG nanocapsules, the unpegylated chitosan nanocapsules and the control nanoemulsion were incubated at 37ºC under moderate stirring, either in simulated gastric medium (USP XXVII, pH=1.2, pepsin 0.32% w/v) or in simulated intestinal medium (USP XXVII, pH=6.8, pancreatin 1% w/v). Samples were collected after 1h-incubation period and centrifuged for 2 min at 3000xg in order to precipitate the aggregates and enzymes. Finally, the mean particle size of the remaining non-aggregated nanocapsules was determined by PCS.

Caco-2 cell culture experiments

Caco-2 cells were cultivated in 80 cm^2 flasks (Nunc, Denmark) using minimum essential medium (MEM) supplemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids and penicillin/ streptomycin solution. Cells were maintained in a controlled atmosphere at 37ºC with 95% of relative humidity and 5% CO_2. The culture medium was changed every second day for approximately 5-6 days until cells reached approximately 80-90% of confluence. Then, these cells with passage numbers between 30 and 40 were seeded approximately at 2.5 x 10^5 cells per flask.

Cytotoxicity studies

To investigate the biocompatibility of both types of chitosan-PEG nanocapsules, an *in vitro* experiment was performed using Caco-2 cell culture. Cells were seeded on 96 well plates with a cell density of 10^4 cells/cm^2 for 7 days until cell monolayer was obtained. After 7 days, the medium was replaced by the nanocapsule suspensions at concentrations between 0.01 and 20 mg/ml in a final volume of 0.2 ml. As a positive control we have used Hank´s balanced salt solution (HBSS) pH 6 and as a negative control 2% sodium dodecyl sulfate (SDS). Chitosan-PEG nanocapsules and the controls were in contact with the monolayers for 90 min at 37ºC and 5% CO_2. Afterwards, the nanocapsule suspensions were removed. The cell viability was measured using a colorimetric method (MTS, Promega), which determines the intracellular dehydrogenase activity. The absorbance was measured at 490 nm.

Transepithelial electrical resistance (TEER) studies

Transepithelial electrical resistance was performed on Transwell® plates with a cell density of 5.5 x 10^4 cells 21 days post-seeding. The integrity of the monolayers was checked on the 21st day in MEM. The TEER measurements were performed with a Millicell®-ERS connected to a pair of chopstick electrodes (Millipore, Spain). After these measurements, the medium was changed by HBSS and let to equilibrate for 1 hour. Then, the apical solutions were replaced by the chitosan-PEG 0.5% nanocapsule suspension and its respective control, HBSS pH 6. The concentration of chitosan-PEG 0.5% nanocapsules was 250 µg/ml in a cell growing areas of 4.5 cm^2 leading to a nanocapsule dose of 80 µg/cm^2. Nanocapsule suspensions were incubated for 100 minutes and TEER measurements were performed every 25 minutes. After this, the solutions were removed and replaced by MEM in order to check the recuperation of the TEER values at 24 hours.

In vivo efficacy of chitosan-PEG nanocapsules

Male Sprague-Dawley rats (225- 275 g) from the Central Animals House of the University of Santiago de Compostela (Spain) were fasted for 12 h before experiments, but allowed water *ad libitum*. Animals were kept conscious during the experiments.

Chitosan-PEG nanocapsules with two different degrees of pegylation degree of chitosan, 0.5 and 1% were administered intragastrically to rats. As a control, we have included the previously assayed nanoemulsions and salmon calcitonin solution (13). The dose administered in all the cases was 500 UI/kg in a final volume of 0.5 ml.

Blood samples were collected from the tail vein 30 min prior to the oral administration to establish the baseline calcium levels and at different times after dosing. The serum was separated by centrifugation at 3000 x g for 5 min. The calcium levels in serum samples were determined by a colorimetric method at 570 nm (Kit OR-cresolphtalein v/v, Spinreact, Spain). The area above the time curve of the hypocalcemic effect ($AAC_{0\text{-}24h}$) was calculated by means of the trapezoidal method. Results are shown as the mean values of serum calcium levels (± s.e.) of 6 animals.

Statistical analysis

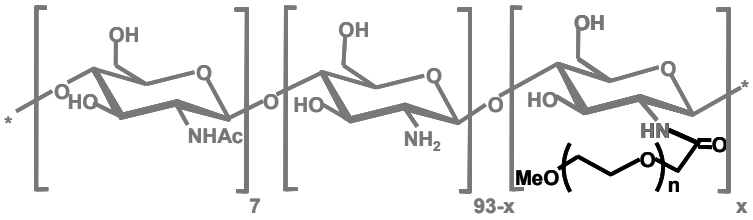
Data from the *in vitro* cell culture experiments were analyzed by an ANOVA test followed by the Student-Newman-Keuls with the statistical software package SPSS 11.5. Differences were considered to be significant at a level of $p < 0.01$.

The mean serum calcium levels determined in samples collected before salmon calcitonin administration were taken as the baseline levels. At each time interval the percent of calcium reduction was calculated. Using these values, the area above the time curve of the hypocalcemic effect was calculated and the statistical

comparison was performed by ANOVA followed by the Student-Newman-Keuls test for multiple comparisons between treatments using the software SPSS 11.5. Differences were considered to be significant at a level of $p < 0.05$.

Results and discussion

We have recently shown that chitosan nanocapsules are able to enhance the nasal and the intestinal absorption of peptides (13, 14, 23). On the other hand, previous work by our group has evidenced the positive role of a PEG coating around polyester nanocarriers with regard to their performance as transmucosal drug carriers (10, 16, 17). More specifically, this PEG coating was found to enhance the stability of the nanocarriers upon contact with mucosal fluids and, their subsequent transport across epithelia (10, 16). In addition, we have reported the comparative behaviour of chitosan-coated polyester nanocapsules vs. PEG-coated nanocapsules in terms of their capacity of overcoming the ocular mucosa (24). The results indicated that both types of coatings have a positive effect on the interaction of the nanocarriers with the mucosal surface (24). In the present work, our idea was that the combination of these two polymers, chitosan and PEG -pegylation of chitosan-, could be advantageous in terms of improving the cellular toxicity of chitosan as well as the stability of the oily cores in the gastrointestinal fluids. Therefore, with this idea in mind, we used three different polymers: PEG and chitosan-PEG with two degrees of substitution of chitosan, 0.5 and 1%, which means that 0.5 or 1% of the chitosan monomers have been covalently linked to a PEG chain of 5000 Da. The structure of chitosan-PEG comb-type polymer and that of the expected nanocapsule is shown in Figure 1.



x= degree of pegylation of chitosan, 0.5% or 1%.

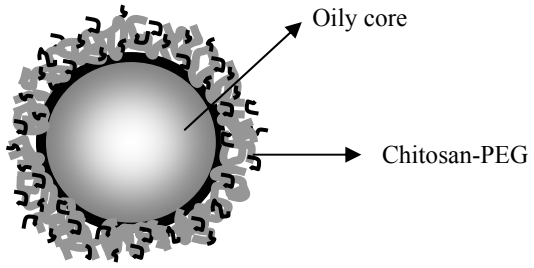


Figure 1: Chemical structure of chitosan-PEG comb polymer and illustration of the theoretically resulting chitosan-PEG nanocapsule.

Characterization of chitosan-PEG

Chitosan-PEG with two degrees of substitution, 0.46 and 1.19%, was obtained under aqueous conditions by a classical amide formation with EDC between the amino groups of chitosan and a carboxylic acid derived PEG. The resulting copolymers were characterized by ^{1}H-NMR (in 2% DCl solution), which clearly indicated the incorporation of PEG by the appearance of an intense signal at 3.70 ppm corresponding to the PEG methylene units. The degree of pegylation was determined from the ^{1}H-NMR spectrum by integration of the multiplet at 2.90-4.40 ppm (H2-H6 protons of chitosan, and PEG protons) against the acetyl signals (1.95-2.20 ppm; NAc, and AcOH from hydrolysis). As a representative example, the ^{1}H-NMR spectrum of chitosan-PEG 1.19% is included: ^{1}H-NMR (2% DCl in D_2O, 500 MHz): δ 2.06-2.08 (m, 20.8 H, $COCH_3$), 3.20 (br s, 95 H, H2 from $GluNH_2$), 3.37 (s, MeO from PEG), 3.55-3.95 (m, 1040H, H2 from GluNHAc; H3, H4, H5, H6 from $GluNH_2$ and GluNHAc, CH_2O from PEG), 4.62 (br s, H1 from GluNHAc), 4.91 (d, *J*=6.7 Hz, H1 from $GluNH_2$).

Characterization of chitosan-PEG nanocapsules

The results of the physico-chemical characterization of the different systems developed are shown in Table 1. As a means of comparison, the oily cores, named nanoemulsions were also included. It can be seen that the size of the chitosan-PEG nanocapsules is significantly larger than that of the control nanoemulsion. In agreement with the observations made for chitosan nanocapsules (13, 23), this increase in particle size was a first indication of the attachment of the polymers to the surface of the oily cores, as illustrated in figure 1. This can be easily understood by the fact that chitosan-PEG nanocapsules were prepared by simple incubation of the nanoemulsion with the polymers solutions. The chitosan-PEG coating of the nanocapsules was formed due to the complexation between the negatively charged lecithin and the positively charged chitosan-PEG, this complex being responsible for the stabilization of the oily core. An additional observation from table 1 is that the size of PEG-coated nanocapsules was slightly smaller (161 nm) than that corresponding to the control nanoemulsion (196 nm). This reduction of the size, despite the presence of a PEG coating was attributed to the tensoactive properties of PEG-stearate used for the formation of these nanocapsules.

The second evidence of the chitosan-PEG coating comes from the results of the zeta potential. The nanoemulsion has a marked negative charge (-52 mV) which, after incubation with both chitosan-PEG polymers, was inverted to highly positive values (+30 mV), regardless of the pegylation degree of chitosan (Table 1). This inversion of the superficial charge of the system confirms the presence of chitosan-PEG coating around the dispersed phase of the nanoemulsions, leading to a reservoir structure. In addition, the lack of influence of the chitosan pegylation degree could be an indication of the excess of positively charged chitosan around the nanostructure. On the other hand, it can be noted that the zeta potential of PEG nanocapsules was less negative (-45 mV) than that of the nanoemulsions (-52.0 mV). This slight reduction in the negative charge of the nanoemulsions was attributed to a greater shift of the shear plane of the diffused nanocapsules layer due to the PEG coating (25).

Table 1: Physico-chemical properties of PEG nanocapsules, chitosan-PEG 0.5 and 1% nanocapsules and the control nanoemulsion (mean ± s.d.; n=3).

Type of nanosystem	Size (nm)	ζ Potential (mV)	Encapsulation efficiency (%)
NE[1]	195.8±1.1	-52.0±1.1	>98
PEG NC	161.2±3.3	-45.6±1.8	---
CS-PEG 0.5% NC	247±7	+32.8±0.5	44.13±3.09
CS-PEG 1% NC	251±17	+29.7±3.1	52.10±3.23

[1]Data from Prego *et al*, (23).

The morphological appearance of chitosan-PEG 0.5% nanocapsules and the control nanoemulsion was visualized using TEM (Figure 2). It could be observed that both types of nanostructures presented a spherical morphology.

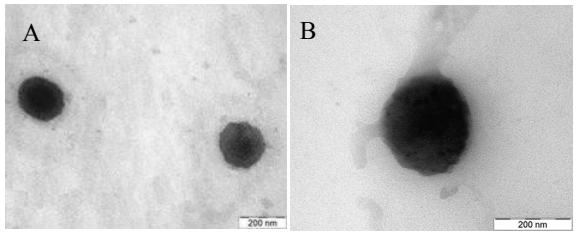


Figure 2: Transmission electron micrographs of A) nanoemulsion and B) chitosan-PEG 0.5% nanocapsules.

Salmon calcitonin encapsulation efficiency

The encapsulation efficiency of salmon calcitonin in chitosan-PEG nanocapsules with both pegylation degrees is shown in Table 1. As comparison, we have also included the encapsulation efficiency of salmon calcitonin in the oily core. The results show that the association of salmon calcitonin was dependent on the composition of the carrier. Indeed, the peptide exhibited a high affinity for the

nanoemulsions, its encapsulation efficiency being close to 100%. However, the encapsulation efficiency in chitosan-PEG nanocapsules was reduced and affected by the chitosan pegylation degree (44% and 52% for 0.5 and 1% pegylation degree respectively). These results agree with those previously found for chitosan nanocapsules (13, 23) and suggest that, under the experimental conditions of the study, the attachment of chitosan-PEG leads to a displacement of the peptide molecules associated to the surface of the oily cores. This could be justified by the positive charge of both, salmon calcitonin and chitosan-PEG. This mechanism would also explain the fact that the chitosan with less positive charges (more pegylated) has a lower interference in the encapsulation of salmon calcitonin. Overall, these results indicate that the attachment of polymers onto the surface of nanoemulsions may interfere with the surface localization of some peptide molecules.

In vitro release studies

In agreement with the observed effect of the pegylation degree of chitosan on the encapsulation efficiency of salmon calcitonin, we found that the extra PEG coating also affects the release behaviour of the resulting nanocapsules. Indeed, as shown in Figure 3, both types of chitosan-PEG nanocapsules presented an initial fast release of salmon calcitonin followed by a non-release phase for up to 6 hours. It should be noted that the percentage of peptide released in the initial phase from chitosan-PEG 0.5% nanocapsules was moderate (20%) and similar to that observed for chitosan nanocapsules (23). However, an increase in the pegylation degree of chitosan led to a significant decrease in the percentage of salmon calcitonin released in the initial phase (10%). In other words, the pegylation of the chitosan coating facilitated the retention of the peptide in the nanocapsules. This could be related to both the greater amount of peptide associated to chitosan-PEG nanocapsules and to the different composition and organization of the coating.

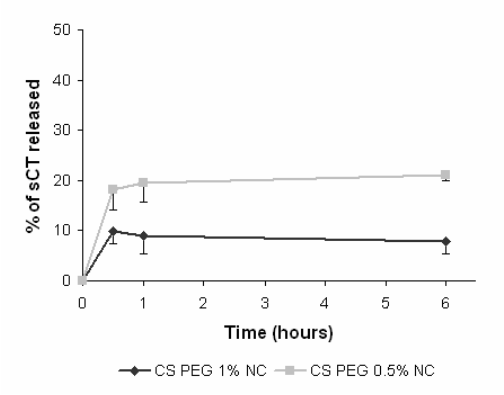


Figure 3: In vitro release profiles of salmon calcitonin from chitosan-PEG 0.5% nanocapsules (grey line) and chitosan-PEG 1% nanocapsules (black line). (Mean ± s.d.; n=3).

Stability of the nanocapsules in simulated gastrointestinal fluids

It is known that nanocarriers are susceptible to aggregation in media with high ionic strength, extreme pH or high enzyme/protein content, and that the surface composition of the nanocarrier plays an important role in its stability. In this study, we have investigated the effect of PEG at improving the stability of the nanoemulsions and chitosan nanocapsules in the gastrointestinal fluids. Accordingly, we have monitored the size of PEG nanocapsules and chitosan-PEG nanocapsules with 0.5 and 1% of pegylation degree, after incubation in gastrointestinal fluids (Figure 4 and 5). As a means of comparison we also investigated the unpegylated chitosan nanocapsules and the nanoemulsion. As shown in Figure 4b, in the gastric medium without enzymes, all nanocarriers maintained their original size. A different situation was observed in the gastric medium containing enzymes (Figure 4a) where the presence of PEG, chitosan or chitosan-PEG attached to the oily core was found to improve the stability of the nanoemulsion, which otherwise aggregated massively upon dilution in the incubation medium. The increase on size observed for the nanoemulsion was attributed to the presence of pepsin, since the nanoemulsion maintained its particle size in absence of enzymes. In fact, taking into account that the isoelectric point of the pepsin is 3.5 (26) and the pH of the simulated gastric fluid

is 1.2, the enzyme was probably adsorbed onto the negatively surface of the nanoemulsion. Consequently, and in agreement with the PEG stabilizing effect previously reported by Tobio *et al*. (16), the explanation of the positive effect of either PEG or chitosan-PEG coatings was the reduction in the interaction of the nanocarrier with the digestive enzymes. With regard to the ability of chitosan in preventing the desestabilization of the systems, previous studies showed the positive effect of this polymer at stabilizing poly-ε-caprolactone nanocapsules in the presence of lysozyme (27).

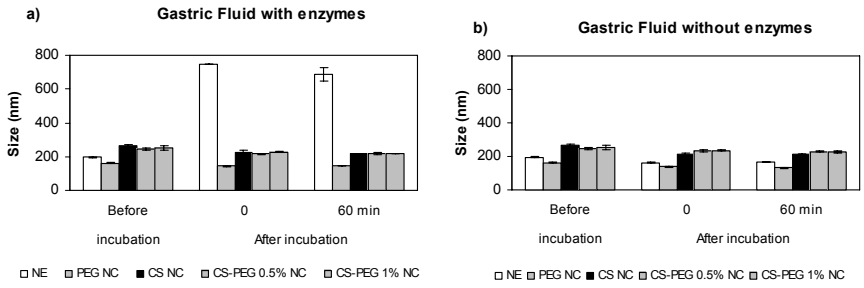


Figure 4: Stability of PEG nanocapsules and chitosan-PEG nanocapsules with 0.5% and 1% pegylation degrees, in simulated gastric fluid with enzymes (a) and without enzymes (b) (Mean ± s.d.; n=3).

The behaviour of the nanocarriers upon incubation in simulated intestinal fluids was quite different (Figure 5). In this medium, PEG nanocapsules and the nanoemulsion were stable, however, the stability of chitosan nanocapsules was influenced by the degree of pegylation of chitosan (Figure 5a). Chitosan nanocapsules were massively aggregated and precipitated after one hour of incubation in intestinal fluids. A pegylation degree of chitosan of 0.5% did not lead to a particle precipitation although a certain aggregation was noticed, whereas those with a 1% PEG were totally stable. An explanation of these results could be that PEG grafted to chitosan would reduce the interaction of the nanocarrier with the intestinal enzymes probably by a steric stabilization effect. In order to verify this hypothesis, we incubated the nanocapsules in intestinal fluid without enzymes (Figure 5b). The results showed that as expected, the size of PEG nanocapsules and

chitosan-PEG 1% nanocapsules remained unchanged, however a significant size increase, although much less significant than in the presence of enzymes, was noted for chitosan-PEG 0.5% nanocapsules. In the case of the unpegylated chitosan nanocapsules, they were not massively aggregated although a size increase was clearly distinguished.

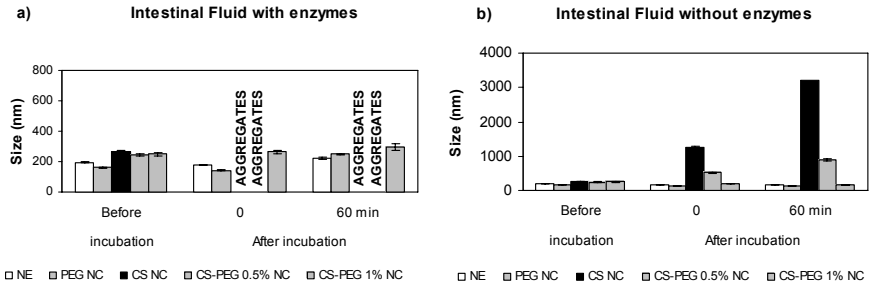


Figure 5: Stability of PEG nanocapsules and chitosan-PEG nanocapsules with 0.5% and 1% pegylation degrees, in simulated intestinal fluid with enzymes (a) and without enzymes (b). (Mean ± s.d.; n=3).

Consequently, from these studies, we can conclude that the chemical modification of chitosan with PEG helps to maintain the stability of the nanocapsules in either the gastric or the intestinal medium. Moreover, the degree of pegylation particularly affects the stability of the nanocarrier in the intestinal medium containing enzymes.

More specifically, we noticed that chitosan-PEG nanocapsules with the lowest pegylation degree (0.5%) suffered a certain aggregation, whereas those with 1% PEG were totally stable. An explanation of these results could be that PEG grafted to chitosan would reduce the interaction of the nanocarrier with the intestinal enzymes probably by a steric stabilization effect.

Cytotoxicity studies in Caco-2 cells

Previous studies in Caco-2 cells have made it clear that the toxicity of chitosan depends on its physico-chemical properties but mainly on the concentration of the polymer exposed to the epithelium (28, 29). Additionally, we have reported that chitosan nanocapsules have a dose-dependent cytotoxicity, the 50% lethal concentration (LC50) being around 1 mg/ml (13). In this study, our purpose was to determine whether or not the pegylation of chitosan could help in improving the biocompatibility of the nanocarrier with the Caco-2 cells. Figure 6 shows the effect of chitosan-PEG nanocapsules with different chitosan pegylation degrees on the cellular viability of Caco-2 cells. Interestingly, the results showed that chitosan-PEG nanocapsules have a very good biocompatibility with the monolayers. More specifically, the LC50 for chitosan-PEG nanocapsules was between 10-20 mg/ml. This indicates that the cytotoxicity inherent to chitosan nanocapsules was reduced by 10-20 times thanks to the pegylation of chitosan.

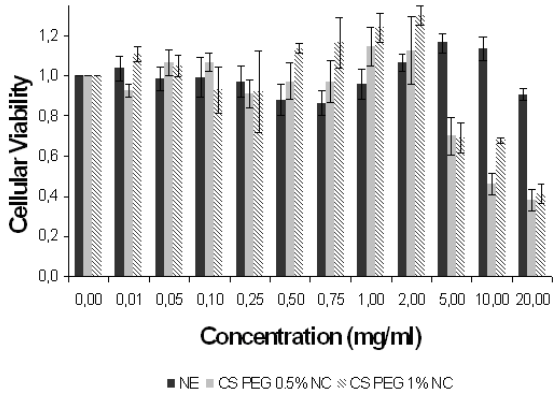


Figure 6: Cytotoxicity study: fraction of NADH+ activity measured in Caco-2 as a function of the concentration of the nanoemulsion and chitosan-PEG 0.5 and 1% nanocapsules. (Mean ±SD, n=4-8).

Transepithelial electrical resistance studies

The ability of chitosan to decrease the TEER values across the Caco-2 cell monolayers has been thoroughly investigated (30-32). Recently, we also evaluated

the TEER of the Caco-2 monolayer exposed to chitosan nanocapsules and found that a significant decrease in the original TEER value was only noted for a relatively high concentration of nanocapsules (13). In the present work, we found it important to evaluate if the pegylation of chitosan, could affect to the interaction of the nanocarrier with the tight junctions of the monolayer. To perform this experiment, we have selected chitosan-PEG 0.5% nanocapsules, which were applied to the monolayer at a concentration of 80 μg/cm^2. The results in Figure 7 show that chitosan-PEG 0.5% nanocapsules caused a slight but significant reduction in TEER values at 100 minutes after exposure to the nanocapsules. It was also noted that the original TEER value of monolayers was recuperated after the removal of the nanocapsules. Consequently, these results indicate that chitosan-PEG 0.5% nanocapsules have a minor effect on the TEER of the monolayer; an effect that was comparable to that previously observed for chitosan nanocapsules (13)..

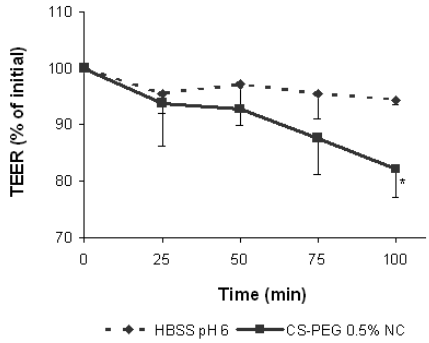


Figure 7: Transepithelial electric resistance (TEER) of Caco-2 monolayers exposed to 250 μg/ml of chitosan-PEG 0.5% nanocapsules (solid black line) and its respective control of HBSS pH 6 (dotted black line). (Mean ± SD, n=3).
* Significantly different from HBSS pH6 ($p < 0.05$).

In vivo efficacy of chitosan nanocapsules

In a previous work, we have shown the ability of chitosan nanocapsules to increase the intestinal absorption of salmon calcitonin (13, 14). These positive results were mainly attributed to the special role of chitosan at improving the

interaction of the nanocapsules with the absorptive epithelium. In the present study, our objective was to investigate if the pegylation of chitosan, which was found to affect very positively the cytotoxicity and the stability of the nanocapsules in biological fluids, could also influence the efficacy of the nanocapsules as carriers for oral peptide administration. With this idea in mind, we tested the ability of chitosan-PEG nanocapsules in promoting the absorption of salmon calcitonin and, hence improving its pharmacological response, using rats as an animal model.

Figure 8 shows the profiles of the calcium levels achieved following oral administration of both chitosan-PEG formulations assayed as well as the controls, the nanoemulsion and a salmon calcitonin aqueous solution. The first observation is that, as expected, the controls showed a negligible hypocalcemic effect. On the contrary, following oral administration of chitosan-PEG nanocapsules we observed a significant reduction in the serum calcium levels and this important response was maintained for at least 24 hours. In addition, it should be noted that the response elicited by the two types of chitosan-PEG nanocapsules varied depending on the pegylation degree of chitosan. Indeed, the most important response was obtained for the nanocapsules prepared with the lowest degree of substitution of chitosan (0.5%), this response being similar to the one corresponding to chitosan nanocapsules (13).

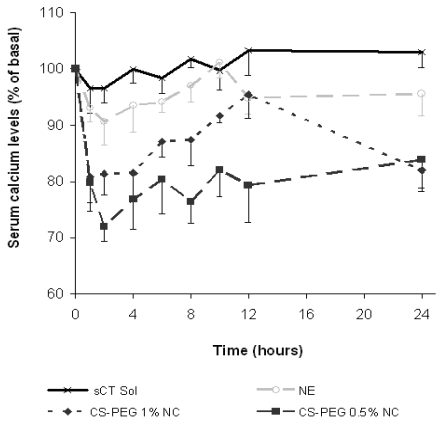


Figure 8: Hypocalcemic effect after oral administration in rats of chitosan-PEG 0.5 and 1% nanocapsules as well as the control nanoemulsion (NE) and the aqueous solution of salmon calcitonin (sCT Sol). Dose of salmon calcitonin: 500 IU (Mean ± s.e.; n=6).

Finally, the comparison of areas above the hypocalcemic effect curve (AAC $_{0\text{-}24h}$) obtained following administration of the different formulations (Figure 9) confirms the efficacy of chitosan-PEG nanocapsules in comparison to the controls. In fact, the $AAC_{0\text{-}24h}$ of the serum calcium levels-time curve after oral administration of both chitosan-PEG nanocapsules, 0.5 and 1%, was significantly higher than that of the nanoemulsion and salmon calcitonin aqueous solution. In addition, it can be noted that the AAC values of both types of chitosan-PEG nanocapsules were statistically different.

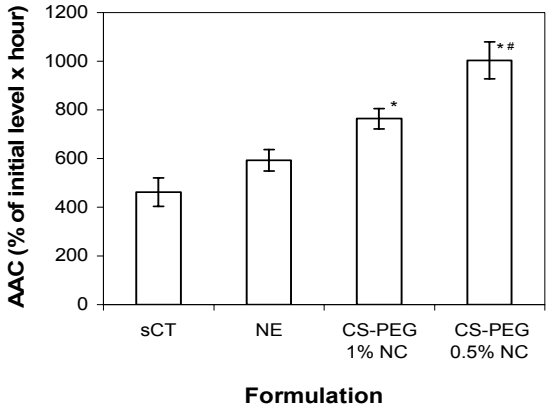


Figure 9: Area above hypocalcemic effect curve (AAC $_{0\text{-}24\ hr}$) after oral administration to rats of salmon calcitonin in solution or incorporated in the nanoemulsions and chitosan-PEG 0.5 and 1% nanocapsules. Dose of salmon calcitonin: 500 IU.
* Significantly different from salmon calcitonin solution and the control nanoemulsion ($p < 0.05$).
# Significantly different from chitosan-PEG 1% nanocapsules ($p < 0.05$).

As in the case of chitosan nanocapsules (13, 14), the explanation of the efficacy of chitosan-PEG nanocapsules could be found in the efficient interaction of the nanocapsules with the intestinal mucosa, forming a depot from which the peptide can be slowly released. This efficient interaction is possibly due to the small size of the nanocapsules but also due to the mucoadhesive properties of chitosan. In this sense, and given the fact that chitosan-PEG nanocapsules are very stable in the gastrointestinal fluids, one could argue that the reduction of their efficacy with the

increase in the pegylation degree could be related to the partial mask of the chitosan mucoadhesive properties. Indeed, it is plausible that PEG partially hides the positive charge of the amino groups, thereby altering their interaction with the negatively charged mucosa. On the other hand, although we can not discard the possible decrease in the TEER of the intestinal epithelium, the slight reduction observed *in vitro* under quite drastic conditions, suggests that this effect is not responsible for the efficacy of these new nanocarriers.

Overall, these results highlight the crucial role of the modulation of chitosan with PEG at stabilizing nanocapsules, maintaining the oral absorption of salmon calcitonin.

Conclusions

In this paper we have evaluated the efficacy of a novel nanocarrier consisting of chitosan-PEG nanocapsules, for the oral administration of the model peptide salmon calcitonin. Additionally, we compared the behaviour of these comb polymer nanocapsules with that of chitosan nanocapsules and PEG nanocapsules. The results showed an improvement of the *in vitro* stability of the nanocarriers when PEG is present in the formulations. Moreover, the pegylation of chitosan increased the cellular viability of Caco-2 cells. Finally, after oral administration of chitosan-PEG nanocapsules to rats, a great pharmacological response was obtained. Therefore, chitosan-PEG nanocapsules have a great potential as carrier for improving the oral absorption of peptide drugs.

Acknowledgments

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Parte 4

Estudio del mecanismo de interacción de las nanocápsulas de quitosano con células Caco-2 y con células goblet (HT29-M6).

Artículo 6

Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery

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Sometido a evaluación

Abstract

We have previously shown that high molecular weight (Mw>100kDa) chitosan nanocapsules are efficient vehicles for improving the oral absorption of salmon calcitonin (sCT). In the present work, our goal was to further investigate the potential of these nanocapsules by studying the effect of different variables, such as the use of chitosan olygomers, the amount of lecithin used to prepare the nanocapsules and the dose of sCT, on the response elicited by the peptide-loaded chitosan nanocapsules administered orally to rats. In addition, in this work we aimed at elucidating the mechanism of action of chitosan nanocapsules as carriers for oral peptide delivery.

The different formulations of chitosan nanocapsules, prepared by the solvent displacement technique, presented a particle size in nanometer range, a positive surface charge and the ability to encapsulate efficiently sCT. Following oral administration to rats, all formulations of sCT-loaded chitosan nanocapsules exhibited the ability to reduce the calcemia levels, however, the intensity of the response varied depending on the variables investigated. An increment on the amount of lecithin did not modify significantly the extension of the hypocalcemic effect which lasted for at least 24 hours. However, the use of chitosan olygomers reduced the duration of the response up to 4 hours post-administration. Finally, the reduction of the peptide dose led to a reduction of the intensity, but not the duration, of the hypocalcemic effect.

The mechanism of action of chitosan nanocapsules was investigated by confocal fluorescence microscopy, after incubation of the nanocapsules with the Caco-2 cell model or in the co-culture of Caco-2 with HT29-M6 cells. The confocal xz images evidenced that chitosan nanocapsules are associated and remain in the apical side of both model cell cultures. Moreover, no fluorescence associated to the nanocapsules was observed either in the paracellular region or in the basolateral side of cells monolayer. In the co-culture, the association pattern of chitosan nanocapsules was not equal for both cell types, showing a preferable association to the mucus secreting cells (HT29-M6). Therefore, these results indicate that the

mucoadhesive character of chitosan nanocapsules may be taken as responsible for the enhancement of the intestinal absorption of peptides.

Keywords: chitosan, nanocapsules, oral peptide delivery, mucoadhesion

Introduction

Peptide drugs are poorly absorbed after oral administration because of their susceptibility to enzymatic degradation and their low permeability across the intestinal epithelium. Being conscious of these important biopharmaceutical limitations, many pharmaceutical scientists have taken the challenge of designing new delivery strategies intended to enhance the oral absorption of these macromolecules. Among them, the encapsulation of macromolecular drugs in nanoparticles/nanocapsules is considered a promising approach towards overcoming the mentioned barriers. Indeed, nowadays it is known that these nanosystems can protect sensitive molecules against degradation in the gastrointestinal environment (1, 2) and also favour the interaction of the associated biomolecule with the intestinal epithelium (3-5). In addition, with respect to the factors that affect this interaction, it has been clearly demonstrated that the size of the particles has a critical role in their interaction with the intestinal barrier (6-8). Knowing this, recently, the challenge has been oriented towards elucidating how the surface properties and composition affects the interaction of the nanosystems with the intestinal epithelium (9).

Mucoadhesive polymers represent one class of biomaterials with an interesting potential for the design of transmucosal nanoparticulate carriers. These polymers offer the possibility to facilitate the interaction of the nanocarrier with the intestinal mucosa and, hence, its access to the underlying epithelium. Indeed, this mechanistic principle has been adopted to explain the efficacy of particles made of acrylic polymers (10, 11), polyanhydrides (12, 13) and chitosan (14, 15) as carriers for the transmucosal delivery of peptides. More specifically, it has been suggested that, because of their composition, these particles are targeted to the mucus covering the intestinal epithelium. Once the particles reach the mucus blanket, they are able to diffuse through it and reach the underlying epithelium, where they may stay for extended periods of time (16). Interestingly, the behaviour of these particles is quite different compared to that of polymer solutions or large polymer devices.

In recent years, we have particularly focused our work on designing and evaluating the potential of nanosystems based on the mucoadhesive polysaccharide chitosan. More specifically, we have observed that chitosan nanocapsules are able to enhance and prolong the systemic absorption of the model peptide, sCT administered orally (17). Additionally, recent experiments have indicated that the modification of chitosan molecular weight (within the range of 160-450 kDa) or the chitosan salt form (glutamate vs. hydrochloride) does not affect the efficacy of the nanocapsules as carriers for sCT (18). These nanocapsules consist of an oily core, conveniently stabilized with lecithin, and surrounded by a chitosan coating. The formation of this coating is mediated by the interaction between the negatively charged phospholipids and the positively charged chitosan molecules. Taking this previous information into account, we found it important to further evaluate the determinants of the in vivo performance of these nanocapsules by analyzing other experimental factors such as: (i) the use of chitosan olygomers (Mw <10kDa), (ii) the use of high lecithin concentration, which might affect the amount of chitosan associated to the surface of the nanocapsules and (iii) the reduction of the dose of nanocapsules containing sCT administered.

Another important goal of the present work was to elucidate the mechanism of action of chitosan nanocapsules. In previous studies in Caco-2 cells we have observed that the association of chitosan nanocapsules to the monolayer is low and similar to that observed for the control non-coated nanoemulsion (17). This lack of evidence of the role of the chitosan coating led us to consider the necessity of selecting a different model cell line in order to investigate the mechanism of action of chitosan nanocapsules. Indeed, Caco-2 cells undergo spontaneous differentiation forming a monolayer of polarized enterocytes that possess morphologic and functional similarities to the small intestine (19). However, a disadvantage of this well-establish cell culture model is the lack of the mucus layer. Thus, in this study we analyzed and compared the behaviour of chitosan nanocapsules upon incubation with the Caco-2 monolayer and also with the co-culture of Caco-2 and mucus secreting cells (HT29-M6).

Therefore, the aims of the present work were, first to investigate further the determinants of the efficacy of chitosan nanocapsules as carriers for the oral administration of peptides, and, second, to elucidate the mechanistic events of chitosan nanocapsules responsible for the enhancement of the oral peptide delivery.

Materials and Methods

Materials

sCT was kindly donated by Almirall Prodesfarma, S.A. (Spain). Miglyol 812®, a triglyceride formed from medium chain fatty acids was supplied by Lemmel (Spain). The surfactant soybean L-α-lecithin and Poloxamer 188 (Pluronic F-68®) were supplied from Sigma-Aldrich (Spain). Chitosan chloride with a deacetylation degree of 85% and viscosity of 16 mPa was purchased from FMC Biopolymer/ Novamatrix with the name of Protasan® Cl 113 (Norway). N- (fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt was purchased from Molecular Probes Europe BV (The Netherlands).

Preparation of chitosan olygomers

Chitosan olygomers were prepared from medium molecular weight chitosan (medium Mw chitosan) by oxidative degradation using sodium nitrite ($NaNO_2$) according to the procedure described by Janes et al (20). For this propose, 0.1 ml of $NaNO_2$ (0.1 M) were added to 2 ml of a chitosan solution (1% w/v) at room temperature under magnetic stirring. The reaction was left overnight in order to ensure completion of the degradation. Finally, the resulting chitosan solution was freeze-dried.

The molecular weight of chitosan was estimated from the viscosity values, measured in a capillary viscosimeter (Automated Microviscometer, Anton Para

GMBH, Austria) at 25ºC. The capillary diameter used was 1.6 mm. To measure the viscosity of the polymer, chitosan solutions were prepared at concentrations lower than 1% (w/v). The solvents used were 2% acetic acid with 0.2 M sodium acetate. The intrinsic viscosity was determined by linear extrapolation to infinite dilution and used with the appropriate Mark-Houwink parameters (21) in order to obtain the molecular weight of the original (medium Mw) and the resulting chitosan olygomers.

Preparation of chitosan nanocapsules

Chitosan nanocapsules were prepared according to the procedure previously described by our group (17). First, we prepared a lecithin-stabilized nanoemulsion (22, 23) as a reference formulation by solvent displacement technique. Briefly, an organic phase composed of 125 µl de Miglyol, 40 mg of lecithin dissolved in 0.5ml of ethanol, and 9.5 ml of acetone was added to 20 ml of an aqueous phase containing Poloxamer 188 (0.25% w/v), under magnetic stirring. The mixture turned milky immediately as a result of the formation of the nanoemulsion. Then, the solvents were evaporated under vacuum until a final volume of 10 ml. In a second step, this nanoemulsion was coated with chitosan by simple incubation in the polymer solutions. More specifically, 4 ml of the control nanoemulsion were incubated with 1 ml of chitosan aqueous solution (chitosan olygomers or medium molecular weight chitosan, Cl113) (0.5% w/v) for 1 h, leading to the formation of chitosan nanocapsules.

In a different experiment, chitosan nanocapsules were prepared according to the conditions indicated above but increasing the amount of lecithin up to 120 mg.

sCT-loaded chitosan nanocapsules were obtained following the procedure described above but adding 50 µl of an aqueous solution of sCT (20 mg/ml) to the organic phase containing the oil.

For the *in vitro* cell culture studies, fluorescent nanostructures were prepared. In this case, 0.5 mg of fluorescein-phosphoetanolamine (λ_{ex}496/λ_{em}519

nm) were added to the organic phase and, then, the procedure was followed as indicated above.

Characterization of chitosan nanocapsules

The size and polydispersion index of the nanocapsules were analyzed by photon correlation spectroscopy (PCS), following appropriate dilution with ultrapure water. Each analysis was performed in triplicate at 25ºC with an angle detection of 90º. The zeta potential was determined by laser Doppler anemometry (LDA). For the determination of the electrophoretic mobility, samples were diluted with KCl 1mM and placed in the electrophoretic cell where a potential of ±150 mV was established. The PCS and LDA analysis were performed using a Zetasizer® 3000 HS (Malvern Instruments, Malvern, UK). Each batch was analyzed in triplicate.

The morphological analysis was performed by transmission electron microscopy (TEM), (Philips CM12, Eindhoven, Netherlands). Samples were stained with phosphotungstic acid solution (2% w/v) for 1 minute and, then dried overnight.

Salmon calcitonin encapsulation efficiency and in vitro release studies

sCT was associated to low molecular weight chitosan nanocapsules. The amount of peptide encapsulated was determined indirectly in the supernatant, following separation of nanocapsules from aqueous medium by a combined ultrafiltration- centrifugation technique (Centricon YM-100, Millipore, USA) at 1000 x g for 1h. The supernatant was diluted with acetate buffer pH=4 and assayed for sCT content by HPLC at 220 nm (Agilent Technologies, Germany), as described in the British Pharmacopoeia, 1998 (column: Vidac 218TP). A calibration curve was made with solutions of sCT in acetate buffer, pH=4.0, at concentrations ranging from 5 to 100 µg/ml. Each sample was assayed in triplicate.

The *in vitro* release studies of sCT from chitosan nanocapsules were performed by incubating the loaded nanocapsules in acetate buffer (pH=4), under horizontal stirring at 37°C. At appropriate time intervals, individual samples were ultrafiltered at 1000 x g for 1 h. The sCT released was determined by HPLC.

In vivo efficacy of chitosan nanocapsules

To perform the *in vivo* studies, male Sprague-Dawley rats (225- 275 g), from the Central Animals House of the University of Santiago de Compostela (Spain), were fasted for 12 h before experiments, but rats were allowed access to water *ad libitum*. Animals were kept conscious during the experiments. All animal experiments were approved by the Ethical Committee of the Faculty of Medicine of the University of Santiago de Compostela.

In this study, we analyzed the effect of some experimental variables on the efficacy of chitosan nanocapsules to induce a pharmacological effect, after oral administration: (i) the effect of the nature of the coating, using chitosan olygomers (10 kDa *vs.* 100 kDa); (ii) the effect of the nature of the coating, using a high amount of the surfactant lecithin (120 mg *vs.* 40 mg); (iii) the effect of the dose of sCT-containing nanocapsules (250 *vs.* 500 IU).

Blood samples were collected from the tail vein 30 min prior to the oral administration of the different formulations, in order to establish the baseline calcium levels, and also at different times after dosing. The serum was separated by centrifugation at 3000 xg for 5 min. Hypocalcemic effect was determined in serum samples by a colorimetric method at 570 nm (Kit OR-cresolphtalein v/v, Spinreact, Spain).

Cell culture experiments

Caco-2 (enterocyte-like cells) and HT29-M6 (mucus secreting cells) were cultivated in 80 cm^2 flasks (Nunc, Denmark) using Minimum Eagle Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% non-essential amino acids and penicillin/ streptomycin solution. Cells were maintained in a controlled atmosphere at 37ºC with 95% of relative humidity and 5% CO_2. The culture medium was changed every second day for approximately 5-6 days until cells reached approximately 80-90% of confluence. After the passage operation, cells were seeded approximately at 2.5 x 10^5 cells per flask. For the experiments, Caco-2 cells at passage number 67 and HT29-M6 at passage number 11 were used.

Qualitative study of the interaction of chitosan nanocapsules with the Caco-2 and HT29-M6 cells

The study of the interaction of chitosan nanocapsules with the cell cultures was performed on Transwell^{TM} filter inserts (0.4 µm pore size diameter, area 0.33 cm^2). For this purpose, Caco-2 (enterocyte like cells) and HT29-M6 (mucus secreting cells) models cell lines were seeded with a density of 6 x 10^4 cells/cm^2, either as a single cell type (Caco-2) or as co-culture (Caco-2:HT29-M6 as 1:1 ratio). Cells were left to grow over 21 days until differentiation and the barrier integrity was checked.

Prior to the experiment, cells were rinsed with Hank´s balanced salt solution (HBSS) pH 6.5-5% glucose and allowed to equilibrate at 37ºC. After this time, the apical medium was removed and cells were incubated with fluorescent chitosan Cl113 nanocapsules in HBSS-5% glucose pH 6.5 for 1 h. After incubation, the suspensions were removed and the epithelium was thoroughly washed with PBS-CaMg. The samples were fixed with paraformaldehyde 3% in MTSB buffer and paraformaldehyde 3% with 0.2% saponin for 5 min. After permeabilization, the cells were washed and the intercellular protein, E-cadherin, was stained using the

immunofluorescence method. The cells were incubated with the primary antibody for 1 h and then, after extensive washing with PBS, with the secondary antibody, an anti-mouse IgG antibody, covalently attached to Alexa 546 (λ_{ex}550/λ_{em}580nm) together with Hoechst reagent (λ_{ex}350/λ_{em}461nm) at 1µg/ml for nuclear labelling for 45 min. After labelling, the cells were thoroughly washed with PBS, filters were cut out and directly embedded in one drop of the mounting medium Dako®. Preparations were examined under a confocal laser scanning microscope (Leica TCS-SP2, Leica Microsystems). The same protocol was followed for the experiments in Caco-2 cells and in the co-culture (Caco-2 and HT29-M6 cells).

Results and discussion

As indicated in the introduction, we have previously shown that chitosan nanocapsules are able to enhance the oral absorption of the peptide sCT, leading to an important and long-lasting hypocalcemic effect (17, 18). Interestingly, the uncoated formulation, a nanoemulsion, led to an insignificant response; a result that evidenced the important role of the chitosan coating for the successful delivery of the associated peptide. In the present work, our first goal was to evaluate the effect of some technological factors inherent to the formation of the chitosan nanocapsules, i.e. the use of chitosan olygomers and the amount of lecithin used to prepare nanocapsules, as well as the effect of the dose of sCT-loaded nanocapsules on their *in vivo* efficacy. In the second part of this work, our aim was to elucidate the mechanism of action of chitosan nanocapsules as potential nanocarriers for oral peptide delivery.

Production and characterization of low molecular weight chitosan

Chitosan is commercially available with a molecular weight (Mw) of around 100 kDa and higher. Therefore, in order to obtain chitosan olygomers, an initial step of this work was to depolymerize chitosan by oxidative degradation using sodium nitrite. Using this procedure, the degree of depolymerization of chitosan can

be accurately controlled, since the number of glycosidic linkages broken is stoichiometric with the moles of sodium nitrite added. After incubation of the polymer solutions with sodium nitrite and using the parameters previously reported (21), straight-line fits were obtained for chitosan olygomers and chitosan commercially available (Protasan® Cl 113) (R^2 > 0.9). The molecular weight calculated for chitosan olygomers and the undepolymerized chitosan Cl 113 gave estimated values of 10 kDa and 100 kDa respectively.

Production and characterization of chitosan nanocapsules

Chitosan nanocapsules were obtained by attaching chitosan onto preformed nanoemulsions. The attachment of chitosan was mediated by the ionic interaction between the negatively charged phospholipids and the positively charged chitosan molecules. The evidence of the coating was obtained from the analysis of the size and zeta potential of the different formulations (Table 1). The results show that the size of chitosan nanocapsules is significantly larger than that of the control nanoemulsion and also that it decreases with the molecular weight of chitosan. These results could be well explained by the fact that the thickness of the chitosan coating is expected to be dependent on the chain length of chitosan molecules. These differences in the coating were not translated into differences in the surface charge. Indeed, both formulations of nanocapsules exhibited very similar zeta potential values, thus confirming the effective coating of the nanoemulsion irrespective of the chitosan Mw. On the other hand, with respect to the influence of the amount of lecithin, it was found that an increase in this amount from 40 to 120 mg, led to a significant enlargement of the nanocapsules (from 266 to 333 nm, which was also accompanied by a reduction of the surface charge from +35 to +28 mV (23). These results suggest the formation of a larger coating caused by the interaction of lecithin and chitosan. Additionally, the presence of a greater amount of lecithin at the inter-phase would justify the reduction of the zeta potential.

Table 1: Physicochemical properties and encapsulation efficiency of different nanocarriers (mean ± s.d.; n=3).

Nanocarrier	Polymer Mw (kDa)	Lecithin (%) w/v	Size (nm)	ζ Potential (mV)	Encapsulation efficiency (%)
Nanoemulsion	---	0.4	195.8±1.1	-52.0±1.1	>90
Medium Mw Chitosan nanocapsules	100	1.2	333.0±6.1	+28.3±2.1	51.9±1.9
Medium Mw Chitosan nanocapsules	100	0.4	266.6±7.6	+34.8±0.6	44.12±3.24
Olygomer Chitosan nanocapsules	10	0.4	202.1±1.3	+30.7±1.9	60±2.65

The morphological appearance of the nanocapsules prepared with low and high Mw chitosan are shown in Fig. 1. Both formulations presented a spherical morphology, however, their appearance was drastically different: high Mw chitosan nanocapsules exhibit a thick coating, whereas low Mw chitosan nanocapsules do not show an apparent coating. These results agree with those of the particle size, which indicated that the thickness of the coating is greatly dependent on the chitosan Mw.

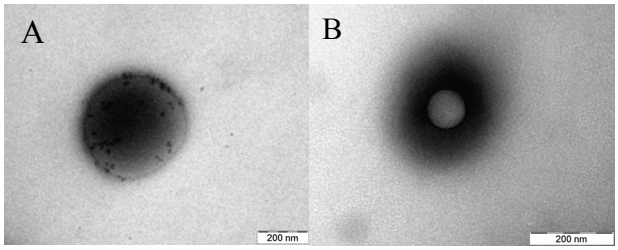

Figure 1: Transmission electron micrographs of A) chitosan olygomers nanocapsules and B) chitosan medium molecular weight nanocapsules.

On the other hand, the preparation of fluorescent nanocapsules, using FITC-phosphoethanolamine as a marker, for *in vitro* cell culture experiments did not lead to any change in the physicochemical properties of the nanostructures as compared to the blank formulations.

Encapsulation efficiency and in vitro release studies of salmon calcitonin

We have already shown that the encapsulation efficiency of sCT into chitosan nanocapsules was reduced when compared to that attained in the non-coated nanoemulsion (17). This effect was attributed to a competition between the peptide and the chitosan, both positively charged in their association to the surface of the nanoemulsion. In the present study, we observed that the encapsulation efficiency was also dependent on the chitosan molecular weight. In fact, as shown in Table 1, olygomer chitosan nanocapsules showed slightly higher encapsulation efficiencies (60%) than medium Mw chitosan nanocapsules (44%). This indicates that the olygomer chitosan coating was not able to displace sCT in the same extension than medium Mw chitosan coating. This result could be an indirect indication of the reduced amount of chitosan olygomers forming the coating as compared to that corresponding to the medium Mw chitosan. Unfortunately, the experimental approaches aimed at quantifying the chitosan coating were unsuccessful. However, the indirect observation of the lower amount of chitosan olygomers forming the coating is in agreement with the smaller size of the resulting chitosan nanocapsules.

The results of the *in vitro* release studies of sCT from olygomer chitosan nanocapsules indicated that the amount of peptide released from the carrier was negligible for up 6 hours. These results are comparable to those obtained for the control nanoemulsion; however, they differ from those observed for medium Mw chitosan nanocapsules. In fact, this latter formulation released 20% of the encapsulated sCT very rapidly (17). This fast release was assigned to the amount of peptide that was expelled from the oily cores due to the competition with chitosan molecules. Consequently, the lack of this fast release phase in the olygomer chitosan nanocapsules could be, as suggested above, in relation with the reduced amount of chitosan on the coating of these nanocapsules.

In vivo efficacy of chitosan nanocapsules

We have previously shown that chitosan nanocapsules are able to enhance the oral absorption of sCT (17, 18). In the present work, one of the modifications we have introduced into the formulation is an increase in the amount of lecithin from 0.4% to 1.2% w/v. The idea behind this modification was that the use of a greater amount of lecithin would probably lead to a denser coating and, as a consequence, to a different *in vivo* behaviour. This was assumed based on the mechanism of coating formation due to the complexation of chitosan and lecithin at the interphase of the emulsion. In agreement with this hypothesis, we observed an increase in the size and a reduction in the zeta potential of the nanocapsules (as previously discussed). However, as shown in Figure 2, this change in the formulation did not have a consequence on the *in vivo* performance of the nanocapsules. Indeed, the response elicited by the new formulation was not significantly different of the one corresponding to the chitosan nanocapsules already described (17). In both cases, an important and long-lasting reduction of the serum calcium levels was also observed after oral administration of the sCT-loaded nanocapsules.

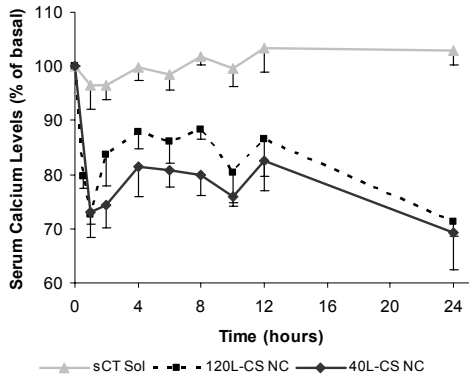

Figure 2: Hypocalcemic effect after oral administration to rats of chitosan nanocapsules prepared with 0.4% and 1.2% w/v of lecithin (0.4L-CS NC and 1.2L-CS NC, respectively) and a sCT aqueous solution (sCT Sol). The dose administered in all the cases was 500IU. (Mean ± s.e.; n=6).

An alternative for modifying the coating of the nanocapsules was based upon the use of chitosan olygomers. Fig. 3 depicts the pharmacological response attained after oral administration of olygomer chitosan nanocapsules (Mw=10 kDa) and medium Mw chitosan nanocapsules (Mw=100 kDa). The results indicate that olygomer chitosan nanocapsules led to an enhancement of the hypocalcemic effect when compared to the peptide solution. However, the improvement of the response associated to this formulation only lasted 4 hours, whereas that corresponding to medium Mw chitosan nanocapsules was maintained for at least 24 hours. This reduction in the efficacy of the formulation could be related with the limited amount of chitosan around these nanocapsules, as suggested above. In fact, in a previous work we observed that the performance of chitosan nanoparticles as nasal vaccine carriers was not significantly affected by the molecular weight of chitosan (24).

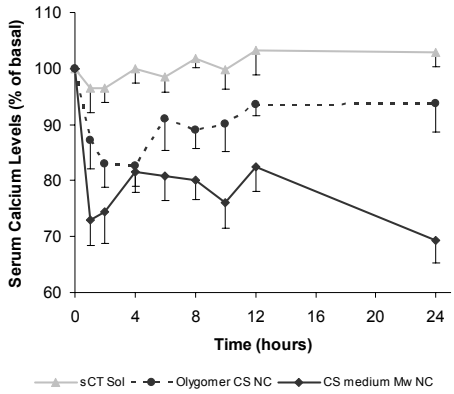

Figure 3: Hypocalcemic effect after oral administration to rats of chitosan olygomers nanocapsules (olygomer CS NC), chitosan medium molecular weight nanocapsules (CS medium Mw NC) and a sCT aqueous solution. The dose of sCT was 500IU. (Mean ± s.e.; n=6).

Finally, we found it important to determine the relationship between the dose of sCT (250 IU vs. 500 IU), and consequently of chitosan nanocapsules, administered orally and the resulting pharmacological response. As shown in Fig. 4, there was a clear effect of this parameter, the response being greatly reduced for the

lowest dose of sCT (250 IU). This was expected since most of the oral administration studies performed with this peptide have used doses of 500 IU or higher. However, the fact that the low dose led to a pulsatile pharmacological profile, characterized by one peak at 1-2 hours and a secondary response between 12-24 hours was surprising. This kind of profile could be related to the release behaviour of the nanocapsules. We could speculate that there is a certain amount of sCT retained in the chitosan coating which would be available for a fast release, whereas most of the well-encapsulated peptide will require a certain time to be released from the carrier and then, absorbed. This long-term controlled delivery hypothesis would only be acceptable if we could assume that the nanocapsules are associated to the intestinal mucosa. The following experiments were intended to clarify this.

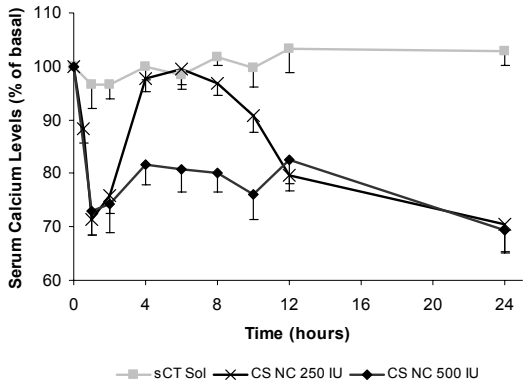

Figure 4: Hypocalcemic effect after oral administration to rats of chitosan nanocapsules prepared with 0.4% w/v of lecithin with two different doses of sCT: 250 UI (CS NC 250IU) and 500 IU (CS NC 500IU). As a control, we have included a sCT aqueous solution of 500IU (sCT Sol). (Mean ± s.e.; n=6).

Qualitative study of the interaction of chitosan nanocapsules with the Caco-2 and HT29-M6 cells

We have previously performed some mechanistic studies in Caco-2 cells, aimed at understanding the greater *in vivo* performance of chitosan nanocapsules

compared to the control nanoemulsion (17). An observation from this previous study was that chitosan nanocapsules are able to open the tight junctions only when applied at a very high dose. Therefore, we concluded that, such a situation is not likely to happen *in vivo* and, consequently, the slight increase in the permeability can not be taken as a unique mechanism in explaining the *in vivo* efficacy of the nanocapsules. On the other hand, we performed a quantitative analysis association of fluorescent nanocarriers to the Caco-2 monolayer and observed a very similar association chitosan nanocapsules and the control nanoemulsion (17). Thus, we concluded that the chitosan coating did not affect the association of the nanocarrier with the cells.

Taking these previous results into account, in this study, our goal was to further elucidate the mechanism of action of chitosan nanocapsules by comparing their qualitative interaction with the Caco-2 cell monolayer and also with a co-culture of enterocytes and mucus-secreting cells (Caco-2:HT29-M6). Using confocal laser scanning microscopy, we could visualize the intercellular unions of cells after immunostaining of the intercellular protein E-cadherin, as well as the nucleus labelled with Hoechst. The localization of the nanocapsules was possible after labelling the formulations with phosphoethanolamine covalently attached to fluorescein. The incorporation of the marker to the nanocapsules did not modify their physicochemical properties.

The results of the study with the Caco-2 cell monolayer indicated that chitosan nanocapsules (green spots) were able to interact with the cells, showing a random distribution (Fig. 5A1). In addition, the images of consecutive cross-sections suggest that the nanocapsules are able to enter the cell by a transcellular pathway. In fact, the lack of co-localization of the nanocapsules (green) with the E-cadherin marker (red), led us to exclude the presence of the nanocapsules in the paracellular region (which should appear in yellow). Furthermore, the analysis of the xz section of the monolayer indicated that, after one hour of incubation, the nanosystems have not crossed the monolayer, but they rather remained at the apical side of the monolayer (Fig. 5A2). These observations corroborate with those from our previous studies which suggested the intracellular presence of chitosan nanocapsules (17) and

also those reported about the interaction of chitosan nanoparticles with Caco-2 cells (4, 25). Nevertheless, an additional conclusion from this study is that, despite their apparent intracellular localization, the nanocapsules do not exhibit a capacity to cross the epithelium. The reason for their permanence at the upper level of the monolayer is still unknown and requires further investigation..

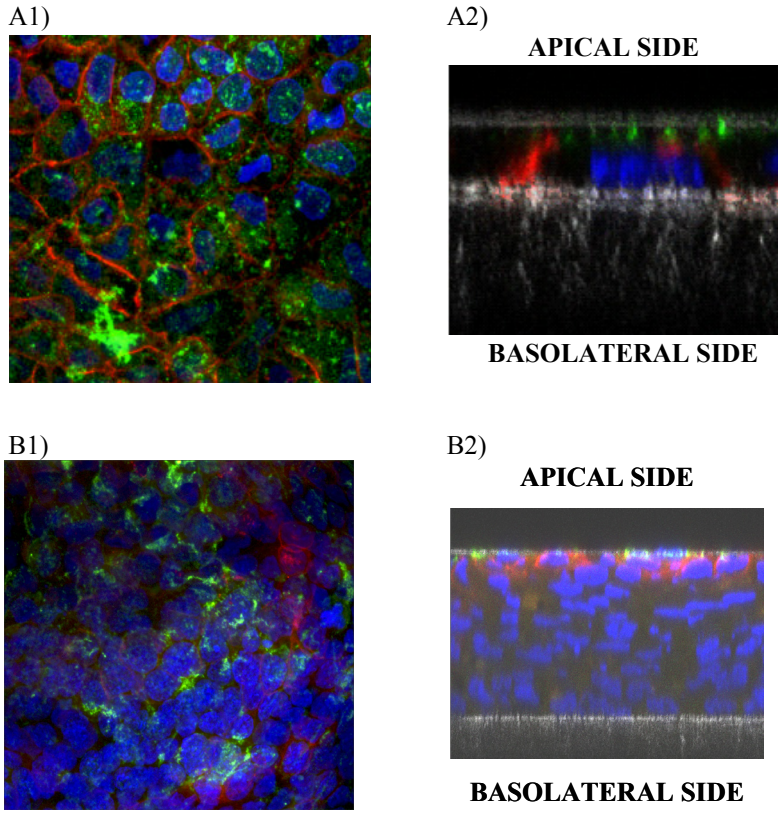

Figure 5: Confocal scanning microscopy images showing the association of fluorescent nanocapsules (green) to Caco-2 cells and to the co-culture Caco-2:HT29-M6 (E-cadherin in red and nucleus in blue).
Caco-2: A1) Montage of 24 horizontal cross sections illustrating the interaction of fluorescent chitosan nanocapsules to the cells (step size in z-axis of 0.5 μm) and A2) Confocal xz section showing the accumulation of fluorescent chitosan nanocapsules in the apical side of the monolayer.
Co-culture Caco-2:HT29-M6: B1) Montage of 16 horizontal cross sections showing the association of fluorescent chitosan nanocapsules to HT29-M6 cells (step size in z-axis of 0.5 μm). B2) Confocal scanning microscopy xz section showing the accumulation of fluorescent chitosan nanocapsules (green) in the apical side of the HT29-M6 cells.

Interestingly, the images obtained upon exposure of the formulations with the co-culture Caco-2:HT29-M6 are slightly different. First, we have evidenced a great interaction of chitosan nanocapsules with the cells (Fig. 5B1), which was not seen for the nanoemulsion control. In addition, we have observed that the association pattern of chitosan nanocapsules to both cell types was quite different. Indeed, there was a remarkable and preferable association of the nanocapsules with one cell type, HT29-M6 cells. Fig. 5B2 shows a xz-image of a region consisting of a multilayer of HT29-M6 cells (nuclei labelled in blue). As in the case of the Caco-2 monolayer, the nanocapsules (green) remained associated to the apical side of the co-culture and the paracellular transport of the carriers was excluded. Unfortunately, it was not feasible to take a xz confocal image showing the Caco-2 cells in alternation with the HT29-M6 cells due to their different organizational depth (Caco-2 in monolayer and HT29-M6 in multilayer). However, it was possible to observe the surface of the co-culture by fluorescent microscopy (Fig. 6B). This image shows an important number of fluorescent chitosan nanocapsules specifically located on the top of Goblet islets, thus evidencing the mucoadhesive character of the nanocapsules.

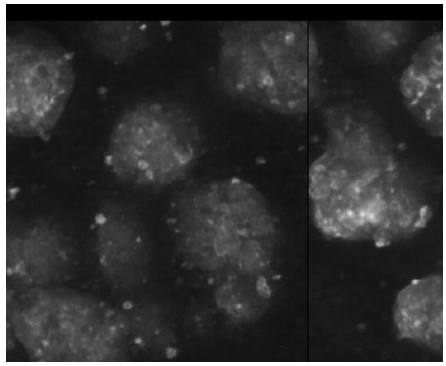

Figure 6: Fluorescent microscopy image of the co-culture Caco-2:HT29-M6 showing chitosan nanocapsules labelling specifically on top of goblet islets.

Consequently, from this study, we can infer that the mucoadhesive character of chitosan nanocapsules is determinant for their ability to interact with the

intestinal mucosa and facilitate the intestinal absorption of sCT. Theoretically, this mechanistic behaviour would also be applicable to other chitosan-coated nanostructures such as lipid nanoparticles (26) or PLGA nanoparticles (14). In fact, Kawashima *et al* (14), using the everted rat intestinal sac model, found that the mucoadhesive properties of chitosan-coated PLGA nanoparticles were responsible for the intimate contact of the nanosystem with the intestine and, thus, for the improvement on the peptide absorption.

These mucoadhesive properties apply not only to chitosan nanocarriers but also to chitosan solutions. However, it has been indicated that the ability of chitosan to enhance the absorption of drugs is greatly reduced in mucus-covered cultures and that the concentration of both chitosan and the drug into a nanoparticulate form could help favouring the absorption of the drug (27). Moreover, regarding the oral administration of chitosan as such, it should be kept in mind that it precipitates upon reaching the intestinal region and, consequently, it is not expected to enhance drug absorption.

Finally, it should be emphasized that despite this first evidence of the mucoadhesive properties of chitosan nanocapsules, further studies are required in order to fully elucidate the mechanism of action of chitosan nanocapsules.

Conclusions

In the present report we observed that the *in vivo* efficacy of chitosan nanocapsules, as carriers for sCT, can be modulated by adjusting the formulation parameters. In addition, the mechanistic studies performed in Caco-2 cells and in a co-culture with mucus secreting cells have revealed that the mucoadhesive properties of chitosan nanocapsules may represent a key factor for their ability in improving the peptide absorption after oral administration.

Acknowledgements

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DISCUSIÓN GENERAL

Discusión General

Entre los distintos nanosistemas desarrollados con el fin de mejorar la absorción de biofármacos de naturaleza peptídica a través de mucosas, las nanocápsulas, compuestas por un núcleo oleoso y un recubrimiento polimérico se presentan como una opción atractiva[1, 2]. Las ventajas que ofrecen estos sistemas coloidales residen en la capacidad del núcleo oleoso para proteger la molécula encapsulada frente a la degradación enzimática[3, 4] y, al mismo tiempo, promover la absorción de fármacos a través del epitelio[5]. Por otro lado, el recubrimiento polimérico del núcleo oleoso ofrece interesantes ventajas inherentes al material

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seleccionado. Entre los distintos polímeros empleados, el polisacárido quitosano se presenta como una atractiva alternativa debido a sus propiedades mucoadhesivas[6] y promotoras de la absorción[7]. De hecho se ha comprobado que su presencia en los sistemas nanoparticulares, ya sea como material constituyente de los mismos [8, 9, 10, 11, 12], o como material de recubrimiento [13, 14] ejerce un papel fundamental en su capacidad para mejorar la absorción de fármacos y vacunas administradas por vías mucosas. Además, es importante destacar que se trata de un polímero biocompatible [15, 16] que presenta una muy baja toxicidad por vía oral [17].

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Por otro lado, se sabe que la peguilación de los polímeros, como el PLA, no sólo permite modificar las propiedades superficiales de los mismos sino también mejorar la estabilidad de los nanosistemas en contacto con los fluidos biológicos [18]. Por tanto, presumiblemente, la peguilación del quitosano (quitosano-PEG) podría aportar una mejor estabilidad a las nanocápsulas en contacto con los fluidos gastrointestinales.

En la presente tesis hemos estudiado el potencial de las nanocápsulas de quitosano como vehículos para la administración de péptidos a través de la mucosa nasal e intestinal.

Desarrollo y caracterización de nanocápsulas de quitosano

La elaboración de nanocápsulas de quitosano se llevó a cabo mediante la técnica de desplazamiento del disolvente, previamente desarrollada en nuestro laboratorio[19]. En una etapa inicial del trabajo experimental, se procedió a la optimización del proceso de obtención de las nanocápsulas de quitosano, evaluándose distintos factores: la concentración de lecitina, la concentración de quitosano y la etapa del proceso en la que el quitosano es incorporado. Esta incorporación puede producirse en la fase acuosa sobre la que se adiciona la fase orgánica o mediante incubación de una nanoemulsión previamente formada en una solución de quitosano. Para la caracterización de las nanocápsulas de quitosano, se determinó el tamaño de partícula y la carga eléctrica superficial. Asimismo, se visualizó la morfología de los sistemas mediante microscopía de transmisión electrónica.

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El análisis del tamaño de partícula de las nanocápsulas de quitosano, realizado mediante espectroscopía de correlación fotónica, mostró que los sistemas se encuentra en rango nanométrico que oscila entre 200 y 570 nm, dependiendo de las variables estudiadas. Además, se observó que, independientemente del momento de incorporación del polímero, tanto la lecitina como el quitosano ejercieron un efecto estadísticamente significativo sobre el tamaño de partícula, siendo la concentración de quitosano el principal factor que afecta a dicho parámetro.

Con respecto a la influencia de la concentración de quitosano, se apreció que, independientemente del método de preparación, la incorporación de pequeñas cantidades de quitosano no se traducía en cambios significativos en el tamaño (Fig. 1). Sin embargo, para la concentración más alta de quitosano (0.3% p/v), el tamaño de partícula sufrió un importante aumento que fue atribuido al depósito de cantidades crecientes de quitosano sobre la superficie del núcleo oleoso. De hecho, el aumento de tamaño lleva consigo una progresiva inversión de la carga superficial del sistema, hecho igualmente observado en otros estudios al aumentar la cantidad de quitosano [20] o el peso molecular del polímero [21].

En cuanto a la influencia de la concentración de lecitina, se observaron distintos comportamientos dependiendo del método de preparación empleado en la elaboración del sistema. Así pues, como se muestra en la Fig 1A, cuando el quitosano se incorpora en la fase acuosa sobre la que se adiciona la fase orgánica, el tamaño de partícula disminuye significativamente al aumentar la concentración de lecitina, especialmente con la concentración más alta de quitosano (0.3%). Este hecho podría atribuirse a la presencia de una segunda población de nanocomplejos de la lecitina-quitosano acompañando a las nanocápsulas. En consecuencia, de este estudio se pudo concluir que tanto la concentración de lecitina como de quitosano poseen un valor crítico en la formación de nanocápsulas de quitosano.

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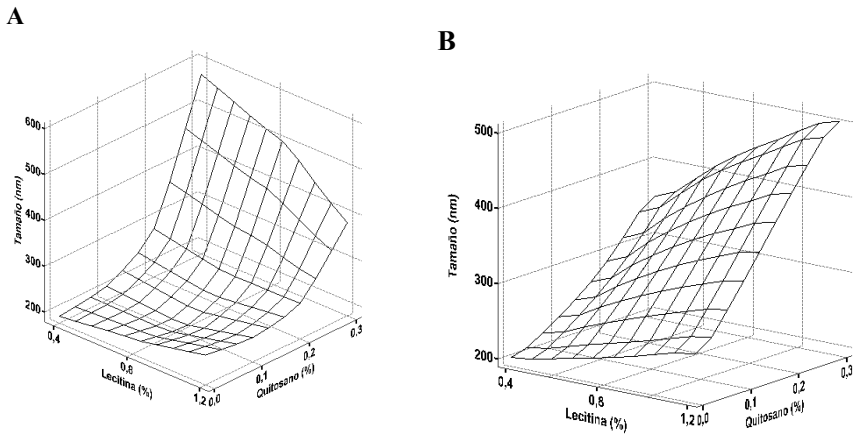


Fig. 1: Superficies de respuesta que muestran el efecto de la concentración de lecitina y quitosano sobre el tamaño de las nanocápsulas, en las que el quitosano se incorporó en la fase acuosa de las nanoemulsiones (A) o tras la incubación de las nanoemulsiones en soluciones de quitosano (B).

Por otro lado, cuando la incorporación del quitosano re realizó mediante incubación de una nanoemulsión previamente formada en una solución de quitosano (Fig. 1B), se pudo constatar un aumento de tamaño del nanosistema. Además dicho aumento se hizo más apreciable al aumentar la concentración de lecitina; resultado que fue hipotéticamente atribuido a un mayor depósito del quitosano.

La evidencia de la presencia de la cubierta de quitosano fue corroborada mediante la medida de la carga superficial de las nanocápsulas. En efecto, la inversión del potencial zeta desde valores altamente negativos, en el caso de la nanoemulsión, a valores positivos, en el caso de las nanocápsulas de quitosano, permitió confirmar el recubrimiento de los núcleos oleosos con quitosano (Fig. 2). No se observó una influencia de la concentración de lecitina en la carga superficial de los sistemas. No obstante, el quitosano ejerció un efecto sobre dicho parámetro, aumentando el valor del potencial zeta al incrementar la concentración del polisacárido.

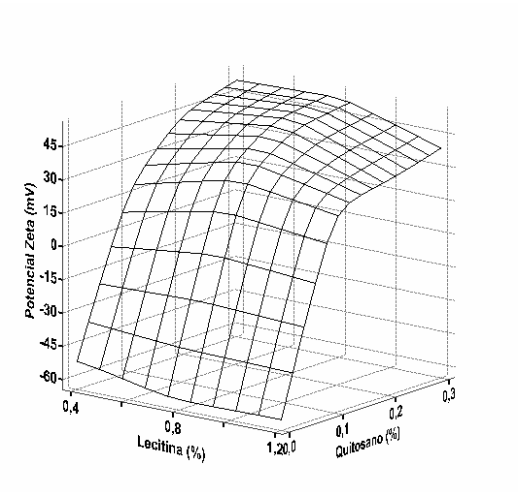


Fig. 2: Superficie de respuesta que muestra el efecto de la concentración de lecitina y quitosano sobre el potencial ζ de las nanocápsulas preparadas tras la incubación de las nanoemulsiones en soluciones de quitosano.

Una vez evaluados los distintos factores que afectan al proceso de preparación de las nanocápsulas de quitosano, se seleccionó la formulación preparada mediante incubación de la nanoemulsión con quitosano, utilizando una concentración de lecitina de 0.4% p/v y de quitosano de 0.1% p/v.

A continuación, se procedió a evaluar el efecto del peso molecular del quitosano y del tipo de sal sobre las características del sistema (Tabla 1). Los resultados mostraron un aumento del tamaño de partícula en función del peso molecular; sin embargo, no se observó efecto del tipo de sal de quitosano sobre el tamaño de las nanocápsulas resultantes. Además de las variedades de quitosano disponibles comercialmente, en el presente estudio se utilizaron oligómeros de quitosano. Para la obtención de dichos oligómeros se procedió a la despolimerización o fraccionamiento del quitosano mediante la adición de nitrito sódico a una solución acuosa de quitosano Cl110 (PM~140 kDa), obteniendo oligómeros de quitosano cuyo peso molecular (PM~10 kDa) se determinó a partir de medidas de viscosidad. En cuanto a la carga superficial de los sistemas, no se observó efecto del peso molecular de polímero sobre el potencial zeta de las nanocápsulas de quitosano. No obstante, el hecho de que ambos sistemas presenten

carga superficial positiva, confirma un recubrimiento de la nanoemulsión por ambos tipos de quitosano.

Tabla 1: Propiedades del quitosano empleado en la preparación de nanocápsulas de quitosano y características de los sistemas desarrollados.

Sistema	Tipo de sal del quitosano	Peso molecular del quitosano (kDa)	Tamaño de partícula (nm)	Potencial ζ (mV)
NE	----	----	195.8±1.1	-52.0±1.1
NC CS Cl 110	Clorhidrato	140	266.6±7.6	+34.8±0.6
NC CS Cl 213	Clorhidrato	270	344.3±15.0	+32.0±0.8
NC CS G 113	Glutamato	160	250.5±7.9	+34.4±0.7
NC CS G 213	Glutamato	450	330.7±5.8	+31.3±1.6
NC CS bajo PM	Clorhidrato	10*	202.1±1.3	+ 30.7±1.9

***** Obtenido a patir de medidas de viscosidad.

Por último se investigó el efecto de la peguilación del quitosano en las características del sistema (Tabla 2). Se estudiaron dos grados de peguilación, 0.5 y 1% del clorhidrato de quitosano de peso molecular 140 kDa (CS Cl110). Esto significa que el 0.5 ó 1% de los grupos amino del quitosano se encuentran covalentemente unidos a una cadena de PEG de 5000 Da. Tras la caracterización de los sistemas, se observó que tanto las medidas de tamaño de partícula como de carga superficial de las nanocápsulas peguiladas al 0.5 y 1%, fueron muy similares a las características de las nanocápsulas sin peguilar.

Tabla 2: Propiedades de las nanocápsulas de quitosano y quitosano-PEG con dos grados de peguilación.

Sistema	Tamaño de partícula (nm)	Potencial ζ (mV)
NC CS	266±7	+34.8±0.6
NC CS-PEG 0.5%	247±7	+32.8±0.5
NC CS-PEG 1%	251±17	+29.7±3.1

Para finalizar la caracterización de los sistemas, se realizó un estudio morfológico de los vehículos previa tinción de los mismos con ácido fosfotúngstico (Fig. 3). La visualización mediante microscopía de transmisión electrónica reflejó la esfericidad y el rango nanométrico de los sistemas. Para las nanocápsulas de quitosano Cl110 fue posible la identificación del recubrimiento de los núcleos oleosos con el polímero. Desafortunadamente, ni el recubrimiento con oligómeros de quitosano ni con quitosano-PEG pudo ser identificado.

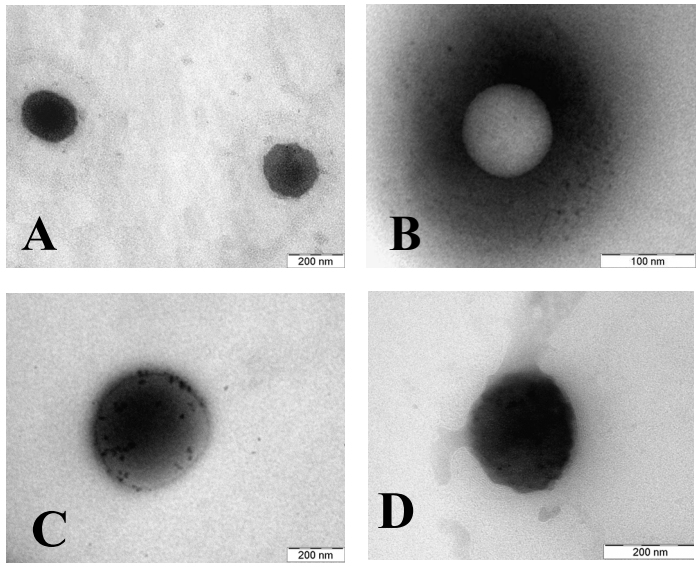


Fig. 3: Fotografías de microscopía de transmisión electrónica: A) nanoemulsión, B) nanocápsulas de quitosano Cl110, C) nanocápsulas de oligómeros de quitosano y D) nanocápsulas de quitosano-PEG 0.5%.

Eficacia de encapsulación y liberación de calcitonina a partir de las nanocápsulas

La incorporación de la calcitonina a las nanocápsulas se realizó en la fase orgánica previa disolución del péptido en agua. A continuación, se determinó la encapsulación de la calcitonina en los sistemas indirectamente mediante el análisis del péptido libre (no encapsulado), utilizando cromatografía líquida de alta resolución. Los resultados mostraron que la eficacia de encapsulación era

dependiente de la composición del sistema. Así pues, en el caso de la nanoemulsión se obtuvo una eficacia de encapsulación del 100%, resultado que fue atribuido a la interacción electrostática entre la calcitonina (pI=10.4) y los fosfolípidos cargados negativamente, así como también a la interacción de las regiones hidrofóbicas de la calcitonina con los componentes lipídicos[22]. Por otro lado, se observó que la asociación de calcitonina a las nanocápsulas de quitosano era del 40%. Esta reducción de la eficacia de asociación fue atribuida a una competición entre el péptido y el quitosano, ambos cargados positivamente, en su interacción con los glóbulos de la nanoemulsión. Cabe destacar que las propiedades estructurales del quitosano no ejercieron ningún efecto sobre la cantidad de fármaco asociado al sistema, a excepción de los oligómeros de quitosano cuya eficacia de encapsulación fue del 60%.

En cuanto al efecto de la peguilación del polímero sobre la eficacia de asociación de la calcitonina, se observó un pequeño incremento en la cantidad de fármaco encapsulado en las nanocápsulas de quitosano-PEG (52%) con respecto a las nanocápsulas de quitosano sin peguilar (40%). Las diferencias en la eficacia de asociación de la calcitonina a las distintas formulaciones de nanocápsulas podrían guardar relación con las distintas cantidades de quitosano presentes en la superficie de las nanocápsulas.

La liberación de la calcitonina a partir de las nanocápsulas de quitosano y de las nanocápsulas de quitosano-PEG mostró un perfil bifásico, caracterizado por una liberación inicial ("burst effect") seguida de una ausencia de liberación durante 6 horas (Fig. 4). Cabe mencionar que se observaron ciertas diferencias en la cantidad de fármaco liberado en la fase inicial dependiendo del las características del polímero: peso molecular, tipo de sal y grado de peguilación. Asimismo, en el caso de las nanocápsulas de oligómeros de quitosano y la nanoemulsión control, no hubo una liberación apreciable de calcitonina durante el tiempo que duró el estudio. Curiosamente, al igual que se observó en los estudios de asociación, la intensidad de

[22] Epand R.M., Epand R.F., Orlowski R.C., Schlueter R.J., Boni L.T., Hui S.W. Amphipathic helix and its relationship to the interaction of calcitonin with phospholipids. Byochemistry (1983) 22: 5074-5084.

la fase de liberación inicial fue superior para las formulaciones elaboradas con quitosano de alto peso molecular. Este hecho podría atribuirse al desplazamiento de las moléculas de calcitonina debido a la presencia de quitosano en la superficie de las nanocápsulas.

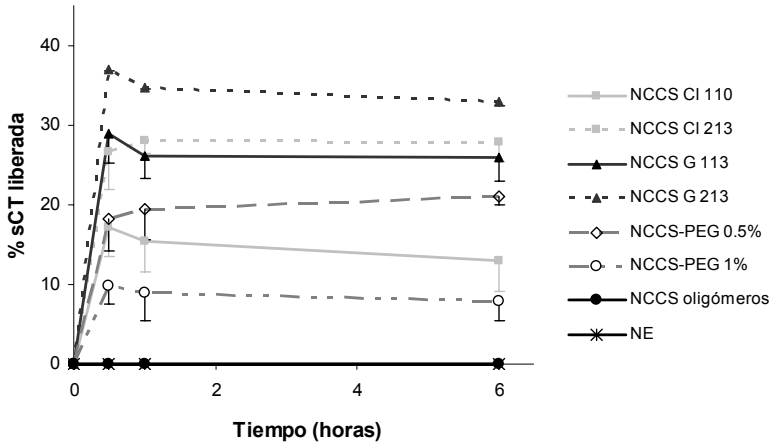


Fig. 4: Perfil de liberación de calcitonina de salmón a partir de nanocápsulas con distintos recubrimientos.

Estabilidad de las nanocápsulas en fluidos biológicos

Teniendo en cuenta que los sistemas coloidales son susceptibles a la agregación en medios de elevada fuerza iónica conteniendo enzimas, en el presente trabajo se consideró importante investigar el comportamiento de los sistemas nanocapsulares tras su incubación en fluidos biológicos (gástrico e intestinal). Con el fin de evaluar el efecto de la cubierta polimérica en la estabilidad de los sistemas se incluyeron en ele estudio las nanocápsulas de quitosano Cl110 y ambos tipos de nanocápsulas de quitosano-PEG (0.5 y 1% de peguilación) así como el sistema control, la nanoemulsión. Adicionalmente, en este estudio se incluyó una formulación conteniendo únicamente PEG en la cubierta, denominadas nanocápsulas de PEG (tamaño 161 nm y carga superficial -45 mV).

La evaluación de la estabilidad de estos sistemas se realizó siguiendo la evolución del tamaño de partícula tras su incubación en los fluidos biológicos simulados (USP XVII), observándose distintos comportamientos dependiendo de las características del nanosistema y también de las del medio de incubación (fluido gástrico o intestinal). Más concretamente, en fluido gástrico simulado (Fig. 5), la nanoemulsión sufrió un proceso de agregación, mientras que la presencia de una cubierta polimérica ya sea de quitosano, PEG o quitosano-PEG estabilizó el sistema en dicho medio (Fig. 5a). El incremento de tamaño observado en la nanoemulsión se atribuyó a la presencia de pepsina ya que en ausencia del enzima, el sistema se mantuvo estable. La explicación de este hecho está relacionada con la adsorción del enzima a la superficie de la nanoemulsión cargada negativamente, si se tiene en cuenta que el pI de la pepsina es 3.5 [23] y el pH del fluido gástrico es 1.2. Sin embargo una cubierta con PEG, a pesar de no modificar la carga superficial del sistema, proporciona una estabilización estérica que impide la adsorción del enzima. Un efecto similar fue el observado por Tobio y col. [24], quienes encontraron que un recubrimiento de las nanopartículas de PLA con PEG reduce la interacción con los enzimas. En cuanto a la capacidad de quitosano para prevenir la desestabilización de los sistemas, estudios previos mostraron el efecto positivo de este polisacárido para prevenir la agregación de las nanocápsulas de poli-ε-caprolactona en presencia de lisozima[25]. Para corroborar que el incremento de tamaño se debe a la presencia de enzimas, se estudió la estabilidad de los sistemas en ausencia del enzima (Fig. 5b), observando el mantenimiento del tamaño de la nanoemulsión. En el caso de las nanocápsulas, como era de esperar, no se observó cambio alguno en su granulometría.

[23] Chaiyasut C., Tsuda T. Isoelectric points estimation of proteins by electroosmotic flow: pH relationship using physically adsorbed proteins. Chromatography. (2001) 22: 91-95.

[24] Tobio y col.; 2000. *ibid* pag 213.

[25] Calvo P., Vila-Jato J.L., Alonso M.J. Effect of lysozyme on the stability of polyester nanocapsules and nanoparticles: stabilization approaches. Biomaterials (1997) 18: 1305-1310.

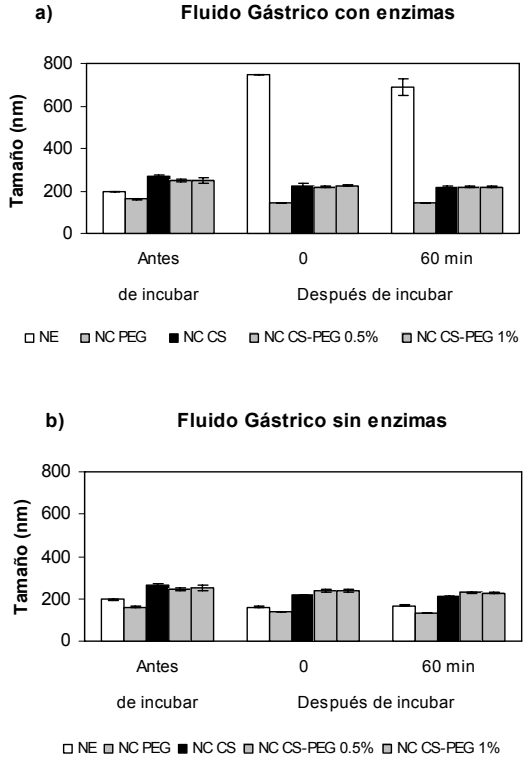


Fig. 5: Estabilidad en fluido gástrico con enzimas (A) y sin enzimas (B) de los distintos sistemas nanocapsulares desarrollados: nanoemulsión (NE), nanocápsulas de PEG (NC PEG), nanocápsulas de quitosano, nanocápsulas de quitosano-PEG 0.5% (NC CS-PEG 0.5%) y nanocápsulas de quitosano-PEG 1% (NC CS-PEG 1%).

Por el contrario, la estabilidad de los sistemas en fluido intestinal simulado fue variable dependiendo de su composición (Fig. 6). La nanoemulsión y las nanocápsulas de PEG permanecieron estables, sin embargo, las nanocápsulas de quitosano sufrieron una agregación importante al cabo de 1 hora de incubación (Fig. 6a). La utilización del quitosano-PEG 0.5 % permitió reducir la agregación del sistema, aunque no de forma importante. Sin embargo, al aumentar el grado de peguilación del quitosano al 1% se obtuvo una estabilización completa del sistema, mostrando una vez más la importancia de la composición de la cubierta.

Con el fin de elucidar si el proceso de agregación de las nanocápsulas de quitosano y quitosano-PEG 0.5% se debe, al pH y fuerza iónica del medio o a la presencia de enzimas, se evaluó la estabilidad en ausencia de enzimas (Fig. 6b). En dicho medio se observó un aumento significativo del tamaño de las nanocápsulas de quitosano, si bien no se apreció un proceso de agregación masiva. Asimismo, como era de esperar, el tamaño de las nanocápsulas de quitosano-PEG 1%, así como el de la nanoemulsión permanecieron inalterados. Por tanto, de dicho estudio se pudo concluir que el principal factor responsable del proceso de agregación fue la presencia de enzimas. Además, el PEG ejerció un efecto estabilizante, siendo necesario una peguilación del 1% para mantener la estabilidad del vehículo en este medio.

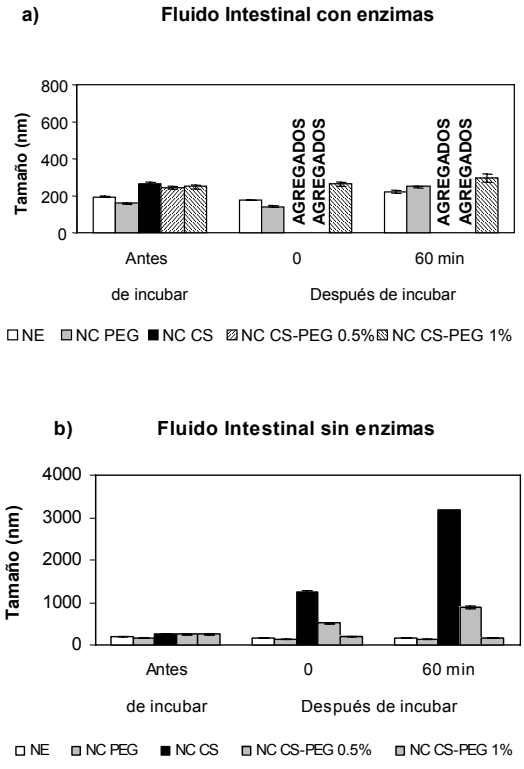


Fig. 6: Estabilidad en fluido intestinal con enzimas (A) y sin enzimas (B) de los distintos sistemas nanocapsulares desarrollados: nanoemulsión (NE), nanocápsulas de PEG (NC PEG), nanocápsulas de quitosano, nanocápsulas de quitosano-PEG 0.5% (NC CS-PEG 0.5%) y nanocápsulas de quitosano-PEG 1% (NC CS-PEG 1%).

Estudios en cultivos celulares

Un objetivo importante del presente trabajo fue el de dilucidar el mecanismo de interacción de los nanosistemas con los epitelios y con las mucosas. Para ello, se seleccionó el cultivo celular Caco-2, línea celular proveniente de adenocarcinoma de colon, que presenta gran similitud morfológica y bioquímica con los enterocitos[26], por lo que es ampliamente utilizada en la evaluación de la permeabilidad de fármacos y sistemas de liberación. Los estudios realizados en estos cultivos incluyen la determinación de la citotoxicidad, la medida de resistencia transepitelial y el análisis de la asociación de los nanosistemas a la monocapa, tanto desde un punto de vista cuantitativo como cualitativo.

Estudios de citotoxicidad en células Caco-2

Hasta el momento se sabe que la viabilidad de las células Caco-2 tras su exposición a soluciones de quitosano, es dosis-dependiente[27, 28]. No obstante, también se sabe que, además de la dosis, las propiedades del quitosano tales como el peso molecular, el grado de deacetilación o el tipo de sal, juegan un papel importante en la toxicidad celular[29, 30, 31]. Lamentablemente, la comparación rigurosa de los resultados obtenidos en los diferentes estudios publicados resulta

[26] Artursson P. Cell cultures as models for drug absorption across the intestinal mucosa. Crit. Rev. Ther. Drug Carrier System. (1991) 8: 305-330.

[27] Dodane V., Khan M.A., Merwin J.R. Effect of chitosan on epithelial permeability and structure. Int. J. Pharm. (1999) 182: 21-32.

[28] Smith J., Wood E., Dornish M. Effect of chitosan on epithelial cell tight junctions. Pharm. Res. (2004) 21: 43-49.

[29] Schipper N.G.M., Varum K.M., Artursson P. Chitosans as absorption enhancers for poorly absorbable drugs.1: Influence of the molecular weight and the degree of acetylation on drug transport across human intestinal epithelium (Caco-2) cells Pharm. Res. (1996) 13: 1686-1692.

[30] Carreno-Gomez B., Duncan R. Evaluation of the biological properties of soluble chitosan and chitosan microspheres. Int. J. Pharm. (1997) 148: 231-240.

[31] Huang M., Khor E., Lim L. Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. Pharm. Res. (2004) 21: 344-353.

difícil debido a las distintas condiciones experimentales empleadas y también a la carencia de información minuciosa relativa a dichas condiciones.

En el presente trabajo decidimos evaluar la toxicidad de los sistemas desarrollados, determinando para ello la actividad mitocondrial de las células tras su exposición a los distintos tipos de nanocápsulas. Más concretamente, las nanocápsulas difieren en cuanto al peso molecular y tipo de sal del quitosano, así como en la peguilación del mismo.

La nanoemulsión presentó una muy baja citotoxicidad, no alcanzándose la dosis letal 50 (DL50) en las condiciones experimentales de este estudio. Por otro lado, las nanocápsulas de quitosano, mostraron una citotoxicidad que fue dependiente de las propiedades del quitosano. Más concretamente, se pudo apreciar una dependencia del tipo de sal, siendo el glutamato de quitosano la variedad menos tóxica. Por el contrario, el peso molecular del polímero no mostró una influencia clara en la viabilidad celular (Fig. 7). De hecho la DL50 tras la exposición de las nanocápsulas de quitosano fue de 606 μg/cm^2 (1 mg/ml) para ambos tipos de nanocápsulas de clorhidrato de quitosano y en torno a de 1212 μg/cm^2 (2 mg/ml) para las nanocápsulas de glutamato de quitosano.

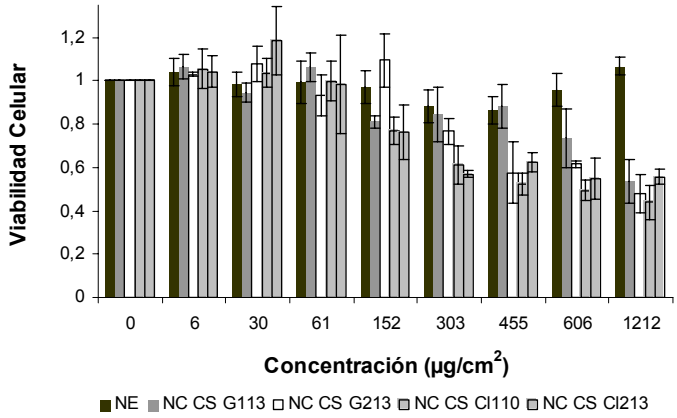


Fig 7: Efecto de diferentes formulaciones de nanocápsulas de quitosano (NC CS G113 y NC CS G213: nanocápsulas de glutamato de bajo y alto peso molecular, respectivamente; NC CS Cl110 y NC CS Cl213: nanocápsulas de clorhidrato de bajo y alto peso molecular, respectivamente), así como de la nanoemulsion (NE), sobre la viabilidad celular en cultivos Caco-2.

Por otro lado, como puede observarse en la figura 8, la peguilación del quitosano, mostró un efecto positivo en la viabilidad celular. Más concretamente, tras exponer el cultivo celular a las nanocápsulas de quitosano-PEG 0.5 % y 1% se observó una viabilidad del 100% hasta concentraciones superiores a 2mg/ml, siendo la DL50 de 6 mg/cm^2 (10 mg/ml) para el 0.5 % de peguilación y entre 6 y 12 mg/cm^2 (10 y 20 mg/ml) para el 1%.

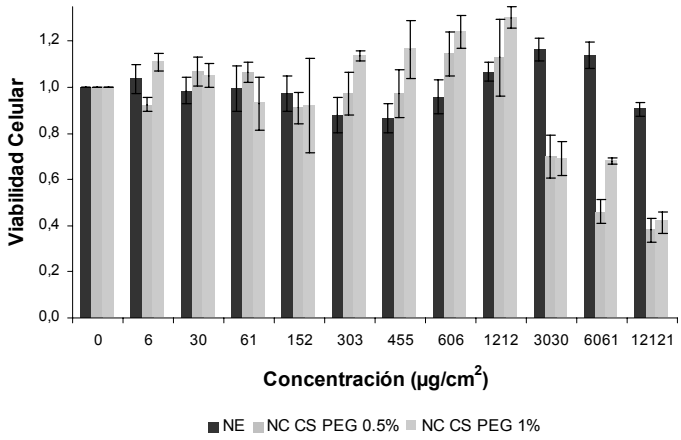


Fig. 8: Efecto de diferentes formulaciones de nanocápsulas de quitosano-PEG NC CS PEG 0.5% y NC CS PEG 1% (nanocápsulas de quitosano-PEG con un grado de peguilación del 0.5 y 1% respectivamente), así como de la nanoemulsion (NE), sobre la viabilidad celular en cultivos Caco-2.

Por tanto, de este estudio se puede concluir que las nanocápsulas de quitosano presentan una baja toxicidad celular y que dicha citotoxicidad se reduce como consecuencia de la peguilación del quitosano.

Estudios de interacción con células Caco-2: medida de la resistencia transepitelial

Diversos estudios han puesto de relieve la capacidad del quitosano para alterar las uniones estrechas existentes entre las células, dando lugar a una

disminución acusada de la resistencia transepitelial[32, 33]. Con el objeto de comprobar si esta propiedad inherente al quitosano se mantiene cuando forma parte de estructuras nanocapsulares, se evaluó la modificación de la resistencia transepitelial de la monocapa celular Caco-2 tras su exposición a las diferentes formulaciones nanocapsulares desarrolladas. Asimismo, en dicho experimento se analizó el comportamiento de la nanoemulsion sin recubrir (Fig. 9). Los resultados obtenidos mostraron que, mientras que la nanoemulsión no dio lugar a ninguna variación en la resistencia transepitelial, las nanocápsulas de quitosano provocaron un descenso en dicho parámetro cuya extensión resultó ser dosis-dependiente. De hecho, la reducción de la resistencia transepitelial fue marcada únicamente tras la exposición a dosis de nanocápsulas relativamente elevadas (204.5 $\mu g/cm^2$), estando dicha dosis asociada a un descenso de la viabilidad celular. Si comparamos estos resultados con los obtenidos en experimentos independientes, para otros sistemas a base de quitosano, nos encontramos con que el efecto permeabilizante (reducción de la resistencia transepitelial) fue superior para las nanopartículas lipídicas recubiertas de quitosano [34] e inapreciable para las nanopartículas de quitosano[35]. La explicación a estos diferentes comportamientos se puede atribuir a que, en este último caso, el quitosano forma parte de una partícula sólida y, sin embargo, tanto en las nanocápsulas como en las nanopartículas lipídicas, el quitosano se encuentra como un recubrimiento soluble. Asimismo el mayor efecto observado para las nanopartículas lipídicas, con respecto a las nanocápsulas, podría guardar relación con la naturaleza del lípido.

[32] Artursson y col.; 1994. *ibid* pag 212.

[33] Dodane y col.; 1999. *ibid* pag 224.

[34] Garcia-Fuentes M., Prego C., Torres D., Alonso M.J. A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly (ethylene glycol) as carriers for oral calcitonin delivery. Eur J Pharm Sci. (2005) 25: 133-143.

[35] Behrens I., Vila-Pena A.I., Alonso M.J., Kissel T. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. Pharm. Res. (2002) 19: 1185-1193.

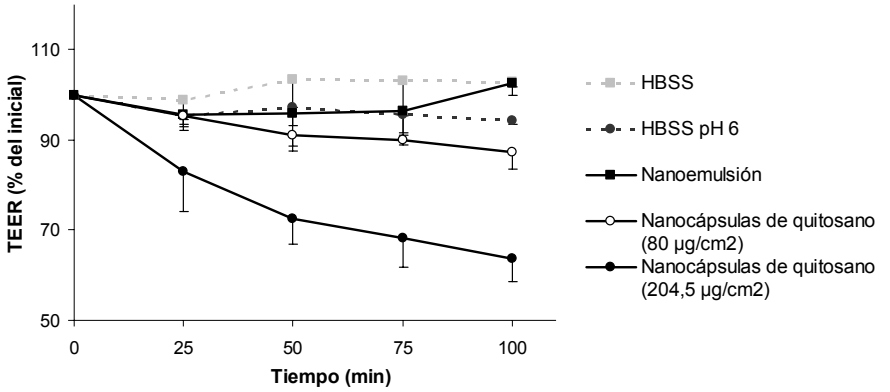


Fig. 9: Resistencia transepitelial (TEER) del cultivo celular Caco-2 expuesto a la nanoemulsión y a distintas dosis por área de superficie de nanocápsulas de quitosano (CS Cl110), así como a los controles, HBSS y HBSS pH 6.

Por otro lado, como se muestra en la Fig. 10, no se constató una influencia del peso molecular ni del tipo de sal de quitosano en la capacidad permeabilizante de las nanocápsulas. Estos resultados concuerdan con los estudios realizados por Schipper y col[36], en los cuales se demuestra que el peso molecular del polímero no afecta su capacidad permeabilizante. Por otro lado, en la Fig. 10 también puede observarse que la peguilación no dio lugar a una variación significativa en la resistencia transepitelial con respecto al sistema sin peguilar, para la concentración estudiada (80 $\mu g/cm^2$). Por último, es importante destacar que con todos los sistemas ensayados, se recobraron los valores normales de resistencia transepitelial a las 24 horas.

En su conjunto, estos resultados sugieren que la capacidad permeabilizante de las nanocápsulas es limitada y que dicha capacidad no se ve influenciada por el peso molecular o la peguilación del quitosano.

[36] Schipper y col.; 1996. *ibid* pag 224.

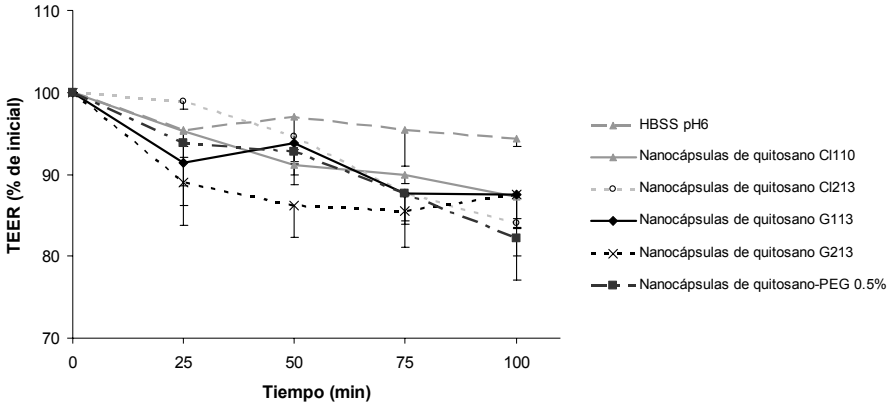


Fig. 10: Resistencia transepitelial (TEER) del cultivo celular Caco-2 expuesto a nanocápsulas de quitosano de distintos pesos moleculares (Cl 110, Cl 213, G113 y G213) y a nanocápsulas de quitosano-PEG 0.5%, así como a HBSS pH 6. La dosis de nanocápsulas fue de 80μg/cm^2 en todos los casos.

Estudios de interacción con células Caco-2: análisis cuantitativo y cualitativo

En una primera fase, la cuantificación de la interacción de las nanocápsulas de quitosano con las células Caco-2 se realizó previo marcaje de las mismas con rodamina. De este modo, tras la incubación de distintas concentraciones de los nanosistemas, se determinó la concentración de rodamina asociada a las células. Los resultados de este estudio mostraron una relación lineal entre la concentración de nanocápsulas en el medio de incubación y la cantidad de nanocápsulas asociadas a las células. Estos resultados sugieren que, en el rango de concentraciones ensayado, no se alcanza la saturación de dicho proceso (Fig. 11). Asimismo, cabe destacar que la asociación a las células fue muy similar para ambos tipos de sistemas, no apreciándose una influencia de la cubierta de quitosano. Estos resultados contrastan con los obtenidos para las nanopartículas lipídicas recubiertas de quitosano[37], para las cuales se observó una asociación muy superior, aunque también se mostró

[37] Garcia-Fuentes y col.; 2005. *ibid* pag 227.

independiente de la presencia de quitosano en la cubierta. No obstante, dada la no influencia de la cubierta de quitosano, la importante asociación del nanosistema se debe relacionar con la naturaleza sólida del núcleo lipídico. Asimismo, se ha de destacar que la baja asociación observada en este estudio para las nanocápsulas de quitosano fue comparable a la observada previamente para las nanopartículas de quitosano [38]. Por tanto, cabe concluir que no solo la composición, sino también la disposición de los diferentes componentes de los nanosistemas, juegan un papel muy importante en su interacción con las células.

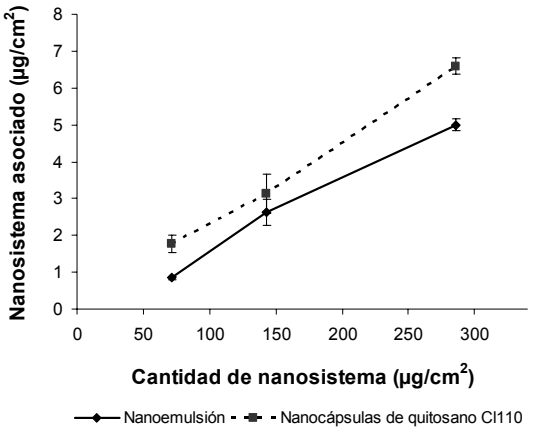


Fig. 11: Cantidad de nanocápsulas de quitosano y nanoemulsión fluorescentes asociadas a células Caco-2 tras 1 hora de incubación en relación a la concentración incubada

Una vez observado que tanto la nanoemulsión como las nanocápsulas de quitosano se asocian en cuantías similares a las células Caco-2, decidimos evaluar cualitativamente la interacción de las nanocápsulas de quitosano mediante el análisis por microscopía confocal. Para ello se marcaron los vehículos con fluoresceína (verde) y para las células se utilizó un doble marcaje: la E-cadherina, proteína intercelular que se marcó con Alexa 546 (rojo) y los núcleos con Hoescht (azul). El

[38] Behrens y col.; 2002. *ibid* pag 227.

análisis del comportamiento de las nanocápsulas de quitosano tras la incubación con las células Caco-2, aparece reflejado en la figura 12A.

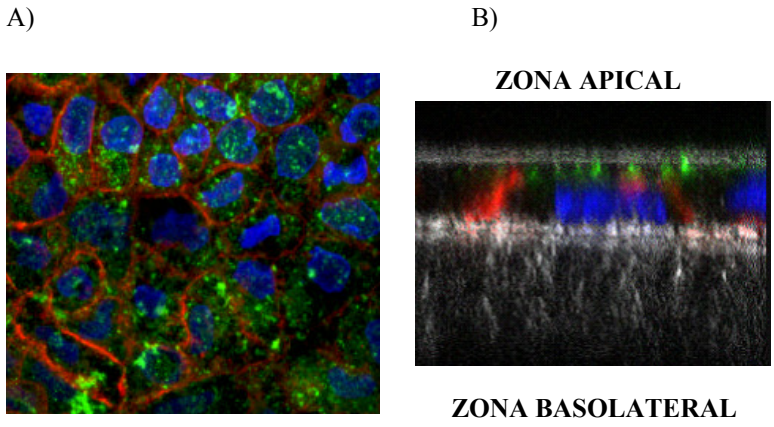


Fig. 12: Imágenes de microscopía confocal que muestran la asociación de las nanocápsulas de quitosano fluorescentes (verde) a las células Caco-2 (E-cadherina en rojo y núcleos en azul). A) Montaje de 24 secciones horizontales que ilustra la interacción de las nanocápsulas de quitosano con las células Caco-2. B) Sección xz que muestra la acumulación de nanocápsulas de quitosano en la zona apical de la monocapa de células.

En dicha figura puede apreciarse una asociación importante de las nanocápsulas a las células, situándose preferentemente en la superficie e interior de las células y no en las regiones paracelulares, dada la ausencia de co-localización de los dos marcadores.

Por otro lado, como se desprende de la figura 13, las nanocápsulas asociadas permanecieron en el borde apical de la monocapa, no habiéndose detectado la presencia de las mismas en la zona basolateral. Por tanto, esta observación lleva a concluir que las nanocápsulas de quitosano no atraviesan la monocapa de células (Fig. 12B).

Con el fin de profundizar en el análisis de la interacción de ambos sistemas con modelos celulares intestinales, se investigó el comportamiento de las nanocápsulas de quitosano y de la nanoemulsión control con el co-cultivo Caco-

2:HT29-M6 (células secretoras de mucus). Las imágenes de microscopía evidenciaron una asociación muy importante en el caso de las nanocápsulas de quitosano, mientras que la asociación de la nanoemulsión fue muy débil. Además, cabe destacar que la interacción de las nanocápsulas no fue igual en los dos tipos celulares, siendo particularmente remarcable en las células secretoras de mucus (Fig. 13). Estos resultados ponen claramente de manifiesto las propiedades mucoadhesivas de las nanocápsulas de quitosano.

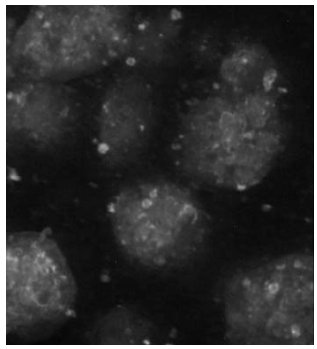
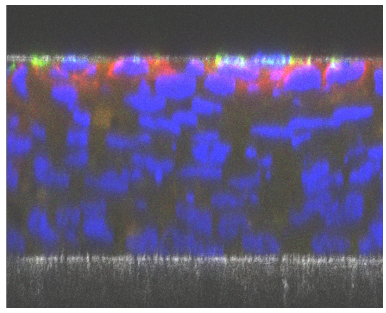


Fig. 13: Imagen de microscopía de fluorescencia que muestra las nanocápsulas de quitosano fluorescentes marcando específicamente los islotes mucosecretores.

Por último, igual que sucedía en el monocultivo de células Caco-2, el sistema permaneció en la zona apical de las células, no observándose el transporte de los mismos al compartimento basolateral (Fig. 14).

ZONA APICAL



ZONA BASOLATERAL

Fig. 14: Sección xz de microscopía confocal que muestra la acumulación de nanocápsulas de quitosano (verde) en la zona apical de las células HT29-M6 (E-cadherina en rojo y núcleos en azul).

Evaluación in vivo de las nanocápsulas

Respuesta farmacológica tras la administración nasal de calcitonina encapsulada en nanocápsulas de quitosano

En los últimos años, la administración nasal ha recibido una atención considerable, especialmente para conseguir un efecto sistémico de fármacos peptídicos con dificultad para ser administrados por otras vías no parenterales como es la vía oral. Esto se debe a las características diferenciales de la cavidad nasal con respecto al tracto gastrointestinal, como son la escasa actividad enzimática, el pH y la ausencia de efecto de primer paso hepático. No obstante, a pesar de las ventajas que ofrece esta modalidad de administración, la biodisponibilidad nasal de fármacos peptídicos es aún baja[39], por lo que la búsqueda de vehículos para aumentar la absorción nasal de péptidos continúa siendo necesaria.

En el presente trabajo se exploró el potencial de las nanocápsulas como vehículos para mejorar la biodisponibilidad de la calcitonina por vía nasal. Se investigó la eficacia in vivo de las nanocápsulas de quitosano, así como de la nanoemulsión y de una solución acuosa de calcitonina en presencia y ausencia de quitosano. Tras la administración por vía nasal se evaluó la respuesta hipocalcémica mediante la medida del nivel de calcio sérico.

Como se desprende de la figura 15, tanto las nanocápsulas de quitosano como la nanoemulsión dieron lugar a disminuciones significativas de la calcemia, mientras que la calcitonina en solución, ya sea en presencia o no de quitosano, no mejoró apreciablemente la absorción del péptido. La reducción del nivel de calcio observada en el caso de la nanoemulsión pudo estar relacionada con un efecto estabilizador y/o promotor de la absorción atribuido a los lípidos. En efecto, Mitra y col. [40] observaron que las gotículas de aceite de una emulsión o/w juegan un papel

[39] Lee W.A., Ennis R.D., Longenecker J.P., Bengtsson P. The bioavailability of intranasal salmon calcitonin in healthy volunteers with and without a permeation enhancer, Pharm. Res. (1994) 11: 747-750.

[40] Mitra R., Pezron I., Chuw A., Mitra A.K. Lipid emulsions as vehicles for enhanced nasal delivery of insulin. Int. J. Pharm. (2000) 205: 127-134.

importante en la absorción de insulina por vía nasal. Además de esta capacidad promotora, los lípidos han mostrado la facultad de ofrecer protección frente a la degradación enzimática [41, 42]. No obstante, como se demuestra en este estudio, el recubrimiento de la nanoemulsión con quitosano permite intensificar y prolongar el efecto hipocalcémico de la calcitonina. Estos resultados concuerdan con los obtenidos para las nanopartículas de quitosano conteniendo insulina[43]. De este modo, mientras que las soluciones de quitosano parecen actuar significativamente sobre las uniones intercelulares, los sistemas particulados no parecen ejercer este efecto de forma apreciable. Por otro lado, si bien las soluciones y sistemas particulados exhiben un carácter mucoadhesivo, el resultado de esta mucoadhesión es distinto. En efecto, el quitosano en solución interacciona con el moco dando lugar a la formación de un gel viscoso[44], mientras que las nanocápsulas interaccionan favorablemente con el moco, pero siguen manteniendo su forma particulada, no causando una modificación apreciable en la viscosidad de la mucina (resultados no mostrados).

[41] Damgé C., Michel C., Aprahamian M., Couvreur P., Devissaguet J.P. Nanocapsules as carriers for oral peptide delivery. J.Control. Release (1990) 13: 233-239.

[42] Lowe P.J., Temple C.S. Calcitonin and insulin in Isobutylcyanoacrylate nanocapsules: Protection against proteases and effect on intestinal absorption in rats. J. Pharm. Pharmacol. (1994) 46: 547-552.

[43] Fernández-Urrusuno R., Calvo P., Remuñán-López C., Vila-Jato J.L., Alonso M.J. Enhancement of nasal absorption of insulin using chitosan nanoparticles. Pharm. Res. (1999) 16: 1576-1581.

[44] Illum L., Farraj N.F., Davis S.S. Chitosan as a novel nasal delivery system for peptide drugs. Pharm. Res. (1994) 11: 1186-1189.

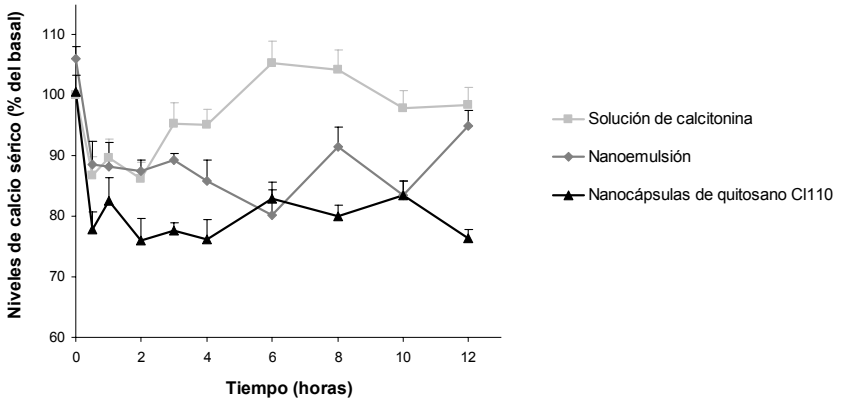


Fig. 15: Efecto hipocalcémico tras la administración nasal a ratas de nanocápsulas de quitosano Cl 110, nanoemulsión y una solución acuosa de calcitonina.

Respuesta farmacológica tras la administración oral de calcitonina encapsulada en nanocápsulas

Teniendo en cuenta los resultados positivos obtenidos tras la administración por vía nasal de la calcitonina encapsulada en las nanocápsulas de quitosano, decidimos ahondar en el estudio de dichos vehículos, retando la administración por vía oral de péptidos.

Inicialmente investigamos la eficacia de las nanocápsulas de quitosano Cl110 utilizando como control una solución acuosa de calcitonina y la nanoemulsión. A continuación procedimos a evaluar las siguientes variables de formulación: peso molecular y tipo de sal del quitosano, concentración de lecitina, dosis de calcitonina y, por último, la influencia de la peguilación del polímero.

A diferencia de lo ocurrido tras la administración por vía nasal de la nanoemulsión conteniendo calcitonina, este vehículo no logró inducir una reducción de los niveles de calcio séricos tras su administración oral. Sin embargo, el recubrimiento con quitosano -nanocápsulas de quitosano- dio lugar a una eficacia farmacológica acentuada, obteniéndose una reducción del nivel de calcio sérico del

25-30% desde la primera hora hasta al menos 24 horas post-administración (Fig. 16). Estos resultados ponen de manifiesto, una vez más, la capacidad del quitosano para interaccionar con el epitelio y promover la absorción del fármaco encapsulado.

Las diferencias encontradas entre las nanocápsulas de quitosano y la nanoemulsión se explican fácilmente si se tienen en cuenta los resultados obtenidos en cultivos celulares, Caco-2:HT29-M6, donde la presencia de mucus favoreció la interacción con las células de las nanocápsulas de quitosano. Por otro lado, estos resultados están en concordancia con los obtenidos en estudios realizados con otros nanosistemas, tales como liposomas y nanopartículas de poliésteres, recubiertos de quitosano[45, 46, 47]. Asimismo, la falta de respuesta inducida por la solución de quitosano[45] pone de manifiesto la importancia de que el quitosano se presente en forma de sistema particulado.

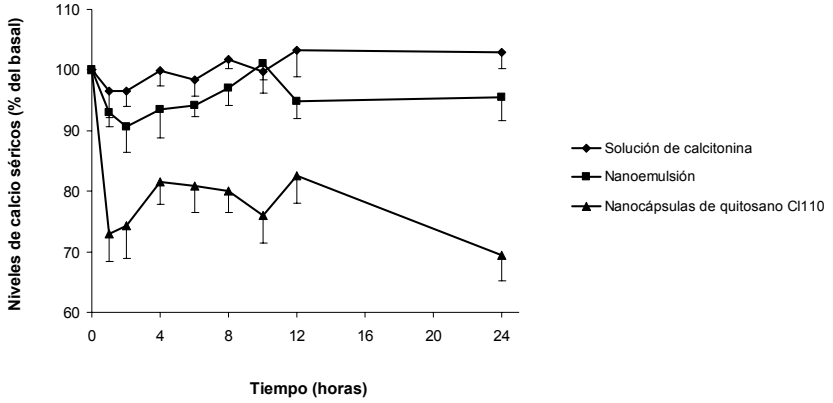


Fig. 16: Efecto hipocalcémico tras la administración oral a ratas de nanocápsulas de quitosano Cl 110, nanoemulsión y una solución acuosa de calcitonina.

[45] Takeuchi H., Yamamoto H., Niwa T., Kawashima Y. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. Pharm. Res. (1996) 13: 896-901.

[46] Kawashima Y., Yamamoto H., Takeuchi H., Kuno Y. Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin. Pharm. Dev. Technol. (2000) 5: 77-85.

[47] Takeuchi H., Matsui Y., Yamamoto H., Kawashima Y. Mucoadhesive properties of carbopol and chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. J. Control. Release (2003) 86: 235-242.

En cuanto a las variables de formulación evaluadas, no se observó influencia ni del tipo de sal del quitosano ni de la concentración de lecitina en la eficacia de las nanocápsulas de quitosano. Con todas ellos se consiguieron resultados satisfactorios, sin observar diferencias significativas en los perfiles de calcio obtenidos (Fig. 17). Por otro lado, la variación del peso molecular del quitosano no se tradujo en un cambio significativo en la respuesta salvo para los oligómeros, en cuyo caso se logró una reducción de los niveles de calcio sérico que se prolongó solamente hasta las cuatro horas post-administración. La explicación a estos resultados es difícil de precisar ya que los resultados hallados en la bibliografía hasta el momento no han revelado una influencia clara de este parámetro. De hecho mientras Chae y col[48] observaron que la reducción del peso molecular del quitosano para un grado de deacetilación >85%, reduce la interacción del polímero con las células, Schipper y col[49] mostraron que, para quitosano con un grado de deacetilación >85%, tanto el quitosano de bajo peso molecular como de alto peso molecular poseen una capacidad permeabilizante muy similar.

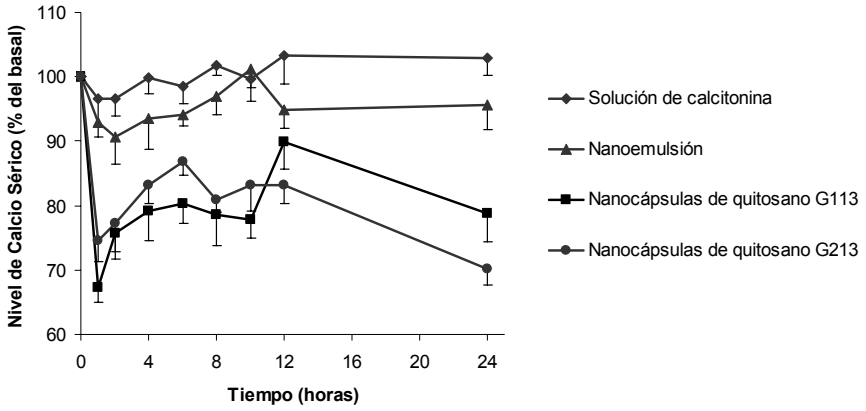


Fig. 17: Efecto hipocalcémico tras la administración oral a ratas de nanocápsulas de quitosano de distintos pesos moleculares, así como la nanoemulsión y una solución acuosa de calcitonina.

[48] Chae S.Y., Jang M-K., Nah J-W. Influence of molecular weight on oral absorption of water soluble chitosans. J. Control. Release. (2005) 102: 383-394.

[49] Schipper y col.; 1996. *ibid* pag 224.

Con respecto al efecto de la reducción de la dosis de calcitonina administrada (250 UI *vs.* 500UI), se apreció una importante reducción del nivel de calcio sérico durante las dos primeras horas post-administración. No obstante, sorprendentemente, a partir de las 12 horas se vuelve a apreciar un importante efecto hipocalcémico que se mantiene al menos hasta las 24 post-administración.

Por último, según muestra la figura 18, la peguilación del quitosano también mostró un efecto en la respuesta hipocalcémica. La eficacia farmacológica mejoró para nanocápsulas de quitosano-PEG con respecto a la nanoemulsión y a la solución de calcitonina. Sin embargo, se observó un comportamiento diferente de estas formulaciones dependiendo del grado de peguilación. Mas concretamente, el efecto hipocalcémico resultó más pronunciado para las nanocápsulas de quitosano-PEG 0.5%, siendo dicho efecto similar al observado para las nanocápsulas sin peguilar. Las diferencias observadas dependiendo del grado de peguilación del quitosano pueden estar relacionadas con una reducción del efecto mucoadhesivo o promotor de la absorción del polisacárido. En efecto, es probable que el PEG cause un efecto estérico que, aunque contribuye a la estabilidad del sistema en los fluidos gastrointestinales, pudiera ocultar parcialmente la carga positiva de los grupos amino del quitosano, alterando así la interacción del sistema con la carga negativa del mucus.

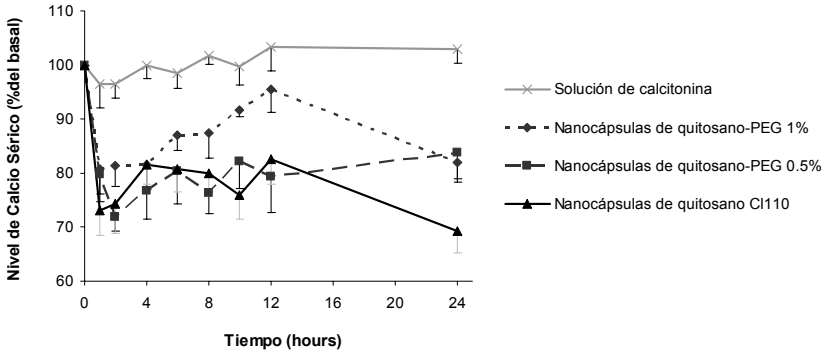


Fig. 18: Efecto hipocalcémico tras la administración oral a ratas de nanocápsulas de quitosano-PEG al 0.5 y 1% de peguilación así como las nanocápsulas de quitosano, la nanoemulsión y una solución acuosa de calcitonina.

CONCLUSIONES

Conclusiones

Los resultados obtenidos en el trabajo experimental que se describe en esta memoria ponen de manifiesto el potencial de las nanocápsulas de quitosano como vehículos destinados a la administración de péptidos por vías no parenterales como son la vía nasal y la vía oral. De un modo más detallado, se han extraído las siguientes conclusiones:

1. La técnica de desplazamiento del disolvente ha permitido la obtención de nanocápsulas constituidas por un núcleo oleoso y una cubierta de quitosano o de quitosano-PEG. Dichas nanocápsulas han sido obtenidas utilizando quitosano de diferente peso molecular (desde menos de 10 kDa hasta más de 200 kDa) y en forma de distintas sales (clorhidrato y glutamato de quitosano). Las nanocápsulas resultantes tiene un tamaño nanométrico (entre 200 y 570 nm), dependiente de ciertas variables de formulación y una carga superficial positiva (en torno a +30 mV) e independiente de las variables de formulación ensayadas. Asimismo, se ha logrado encapsular eficazmente el péptido calcitonina en los distintos tipos de nanocápsulas obtenidas.

2. La cubierta polimérica de las nanocápsulas, ya sea de quitosano o quitosano-PEG estabiliza los núcleos oleosos en fluido gástrico. En fluido intestinal se observó un importante efecto de la peguilación del quitosano en la estabilidad de las nanocápsulas.

3. Los estudios de citotoxicidad llevados a cabo en cultivo celular Caco-2 reflejaron una baja toxicidad de las nanocápsulas de quitosano, estando la DL50% comprendida entre 606 $\mu g/cm^2$ (1 mg/ml) y 1212 $\mu g/cm^2$ (2 mg/ml). Además, se observó una reducción de dicha toxicidad como consecuencia de la peguilación del quitosano.

4. La facultad de las nanocápsulas de quitosano para interaccionar con el cultivo celular Caco-2, determinada mediante medidas de resistencia eléctrica transepitelial, reflejó una capacidad permeabilizante reducida e independiente del peso molecular y del tipo de sal del quitosano, así como de su grado de peguilación.

5. La asociación de las nanocápsulas de quitosano a células Caco-2 fue baja y muy similar a la de la nanoemulsión control. Las imágenes obtenidas mediante microscopía de confocal de fluorescencia han permitido localizar las nanocápsulas de quitosano únicamente en la zona apical de las células y no en las uniones intercelulares ni en la zona basolateral, lo que ha llevado a descartar la capacidad de las mismas para atravesar la monocapa. Asimismo, las imágenes correspondientes a la interacción con el cocultivo Caco-2:HT29-M6 (enterocitos y células secretoras de mucus) pusieron de manifiesto una importante asociación de las nanocápsulas de quitosano a las células secretoras de mucus, constatando así sus propiedades mucoadhesivas.

6. Los resultados de los niveles de calcemia obtenidos tras la administración de las formulaciones de calcitonina por vía nasal revelaron la capacidad de las nanocápsulas de quitosano para aumentar la absorción de la calcitonina. Además dicha respuesta fue significativamente superior a la conseguida tras la administración de una solución de quitosano y

calcitonina, lo que demuestra la importancia de que el quitosano se presente formando una estructura coloidal.

7. Los resultados de la eficacia farmacológica obtenidos tras la administración de las formulaciones de calcitonina por vía oral permitieron concluir el potencial de las nanocápsulas de quitosano para la administración oral de calcitonina. Más concretamente, las nanocápsulas dieron lugar a una reducción significativa de los niveles de calcemia (en torno al 30%), respuesta que se prolongó durante al menos 24 horas. Asimismo, se observó que modulando el grado de peguilación del quitosano es posible obtener un sistema estable en los fluidos gastrointestinales, de toxicidad muy reducida y con capacidad para promover la absorción oral de péptidos.

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ANEXO: Trabajo Científico Relacionado

A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery

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Abstract

We have previously reported the formation and characterization of poly(ethylene glycol) (PEG)-coated and chitosan (CS)-coated lipid nanoparticles. In the present work our goal was to study the interaction of these surface-modified lipid nanoparticles with Caco-2 cells and to evaluate the potential of these nanostructures as oral delivery systems for salmon calcitonin (sCT).

The interaction of rhodamine®-loaded nanoparticles with the Caco-2 cell monolayers was evaluated quantitatively and qualitatively by confocal laser scanning microscopy and fluorimetry, respectively. The ability of these nanoparticles to reversibly enhance the transport of hydrophilic macromolecules through the monolayers was investigated by measuring the transepithelial electric resistance and the permeability to Texas Red®-dextran. Finally, in vivo studies of the response to sCT-loaded nanoparticles were performed in rats.

The results showed that the association of rhodamine®-loaded nanoparticles to the Caco-2 cell monolayer was independent of the surface coating of the nanoparticles (CS-coated versus PEG-coated nanoparticles). However, while PEG-coated nanoparticles did not affect the permeability of Caco-2 monolayers, CS-coated nanoparticles produced a dose-dependent reduction in the transepithelial electric resistance, simultaneously to an enhanced dextran transport. The results obtained following oral administration of sCT-loaded CS-coated nanoparticles to rats showed a significant and prolonged reduction in the serum calcium levels as compared to those obtained for control (sCT solution). In contrast, the hypocalcemic response of sCT-loaded PEG-coated nanoparticles was not significantly different of that provided by the control (sCT solution). Therefore, these results indicate that the surface composition of the particles is a key factor in the improvement of the efficiency of oral sCT formulations. Moreover, the encouraging results obtained for CS-coated nanoparticles underline their potential as carriers for peptide delivery.

Keywords: Nanoparticles; Chitosan; PEG; Peptide delivery; Transepithelial transport.

Introduction

The low transepithelial transport together with the instability in the gastrointestinal tract, are the main obstacles for making the oral administration of peptides feasible. Among the alternatives explored so far to overcome these limitations, the design of submicrometric carriers appears to be a promising approach (Chen and Langer, 1998). For example, it has already been shown that the nanoencapsulation of proteins in colloidal particles protects them against the harsh environment of the gastrointestinal tract (Lowe and Temple, 1994), and enhances their transmucosal transport (Mathiowitz et al., 1997 and Tobío et al., 1998). This ability of the colloidal carriers to enhance the transport of the associated macromolecules has been attributed to different mechanisms depending on the nanocarrier composition. These mechanisms are (i) mucoadhesion, (ii) particle internalization phenomenon and (iii) permeation enhancing effect.

The adhesion of a carrier system to the mucus may improve the residence time and contact of the drug with the underlying epithelium, thus increasing the drug concentration in the site of absorption (Mikos et al., 1991). Such mechanism of transport enhancement has led to the idea of modifying the surface of colloids in order to optimize their mucoadhesion. Hydrophilic polymers such as polyacrylate derivatives and chitosan are examples of mucoadhesive materials used to coat colloidal carriers. For example, polyacrylate derivatives in the form of nanoparticles with mucoadhesive characteristics have recently been proposed for the oral administration of sCT (Torres-Lugo et al., 2002). Similarly, Sakuma et al. (1997) reported an enhancement of the oral absorption of sCT following its association to poly(N-isopropylacrylamide) nanoparticles. Moreover, the formation of a mucoadhesive CS coating around PLGA nanoparticles has shown a positive effect in improving the oral absorption of sCT (Kawashima et al., 2000). Moreover, in our group, we have shown that CS-based colloidal particles, in which CS is in the form of a coating or a nanomatrice, are able to improve the transport of drugs across the nasal (Vila et al., 2002) and the ocular mucosa (Calvo et al., 1997 and De Campos et al., 2003).

In contrast with the well-defined role of mucoadhesion in drug absorption, the factors that affect the interaction and internalization of colloidal particles with epithelia remain unclear. In fact, despite the deep understanding achieved of the transport of non-degradable polystyrene particles (Florence and Hussain, 2001), little is still known about the behaviour of colloids of pharmaceutical interest. For a number of years it was broadly accepted that nanoparticles with hydrophobic surfaces are taken up more extensively by the intestinal epithelium than those with hydrophilic surfaces (Eldridge et al., 1990). However, some recent results have suggested that the presence of hydrophilic polymers such as PEG (Tobío et al., 2000) or CS (Vila et al., 2002) on the surface of nanoparticles can increase the transport of these systems through mucosal surfaces. Overall, these results indicated that the composition of the nanoparticle could affect not only the intensity but also the mechanism of transport. Indeed, mechanistic studies performed with different types of nanoparticles indicated that hydrophobic nanoparticles are preferentially transported through the gut associated lymphoid tissue, whereas particles with a more hydrophilic nature are transported across the regular enterocytes (Mathiowitz et al., 2000).

Finally, with respect to the permeation enhancing effect, this has been extensively investigated for polymer solutions, however, whether or not this effect remains unaltered when the polymer is in the form of nanoparticles or attached to them, needs to be further clarified. For example, in the case of poly(methacrylic) derivatives (Torres-Lugo et al., 2002), it has been shown that their typical permeation enhancing effect is maintained when they are in the form of nanogels. However, in the case of CS based colloidal systems, the maintenance of the inherent capacity of CS to open the tight junctions (Lueben et al., 1997) has not been fully identified (Behrens et al., 2002).

In the present work our goal was to investigate the in vitro and in vivo behaviour of two new drug nanocarriers consisting of a lipid nanoparticles coated by a hydrophilic coating (either PEG or CS). More specifically, we studied the interaction of polymer-coated lipid nanoparticles with the Caco-2 cell culture model and, then, we evaluated the pharmacological response of sCT associated to both

types of nanoparticles following oral administration to rats. The preparation of the lipid nanoparticles made of tripalmitin and coated with PEG or CS was previously reported by our group (Garcia-Fuentes et al., 2002; Garcia-Fuentes et al., in press). Moreover, we have already shown that these systems are stable in simulated gastrointestinal media and capable of effectively associating and releasing sCT. Therefore, the purpose of the studies reported here were to identify the role of the polymer coating on the efficacy and mechanism of action of the nanosystems as carriers for the oral administration of sCT.

Materials and methods

Materials

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, UK). Minimum Essential Medium Eagle (MEM), fetal bovine serum (FBS), non-essential amino acids, l-glutamine, 100 UI/ml penicillin/100 μg/ml streptomycin solution, 0.05% trypsin/0.02% EDTA solution, Dulbecco's phosphate buffered saline (DPBS) and Hank's balanced salt solution (HBSS) were purchased from Sigma (Sigma, Spain). Tripalmitin [Dynasan® 116] (Condea, Germany) was the lipid used for nanoparticle core formation. l-α-lecithin (Sigma, Spain), poloxamer 188 [Symperonic® F68] (ICI, Spain) and poly(ethylene glycol)-stearate (PEG, Mw≈2000 Da) [Simulsol® M52], a gift from Seppic (France), were used as surfactants. Chitosan (CS) (specifications: 75–85% of deacetylation degree, viscosity 20–200 cP at 1% in 1% acetic acid) in basic form was purchased from Aldrich (Spain). Rhodamine® was from Sigma (Spain) and Texas Red®-dextran and Bodipy® 650/665-Phalloidin from Molecular Probes Europe BV (The Netherlands). Salmon calcitonin (sCT) was a kind donation from Almirall Prodesfarma S.A. (Spain). Other reagents were analytical grade or better.

Nanoparticle preparation

Prior to the preparation of nanoparticles, CS was dissolved in 0.1 M hydrochloric acid and purified. More specifically, the CS solution was filtered and washed by dialysis for a period of three days. Then, the filtrate was centrifuged at 30,000×g for 1h to eliminate non-dissolved impurities. The supernatant from the centrifugation was freeze-dried, leading to a powder readily soluble in water.

CS-coated nanoparticles were prepared following a two-step procedure: first, tripalmitin nanoparticles, used as cores for their further coating with CS, were prepared by a double emulsion solvent evaporation method, as previously described (Garcia-Fuentes et al., 2002). This procedure was adopted because of its effectiveness for the encapsulation of peptides. Briefly, 50 µl of an aqueous solution of sCT (10 mg/ml) were added to a 0.5 ml solution of 25 mg of lecithin and 50 mg of tripalmitin in methylene chloride. A water-in-oil emulsion was formed upon 15 s of sonication (20 W). Then, 1 ml of water was added to the first emulsion, and sonicated again for 60 s (20 W). The solvent was eliminated by evaporation, first 30 min at room temperature and afterwards 30 min under vacuum. In the second step, tripalmitin nanoparticles at 1% (w/v) final concentration were incubated for 10 min in a 0.05% (w/v) CS/1.5% poloxamer 188 solution in order to allow the formation of a CS coating by simple interaction of the positively charged CS molecules and the negatively charged lipid cores. Nanoparticles were isolated by ultracentrifugation for 1 h at 50,000 × g and resuspended in purified water.

PEG-coated tripalmitin nanoparticles were prepared by an analogous procedure to that used to obtain tripalmitin nanoparticles, plus 25 mg of PEG-stearate added to the organic phase. Nanoparticles were isolated by ultracentrifugation for 1 h at 85,000 × g and then, resuspended in purified water.

For the preparation of rhodamine®-labeled nanoparticles, a 10 mg/ml methanol stock solution was prepared. Five microliter from this stock solution were diluted in the organic phase of the double emulsion method described.

Blank nanoparticles (non-loaded with sCT) were obtained following the procedure described above but substituting the aqueous solution of the peptide by water.

Physicochemical characterization of the nanoparticles and sCT association efficiency

The size and polydispersity of the nanoparticle suspensions were determined by Photon Correlation Spectroscopy (PCS) (Zetasizer® 3000HS, Malvern, UK), following appropriate dilution in purified water. The zeta potential of the nanoparticles was measured by Laser Doppler Anemometry (Zetasizer® 3000HS, Malvern, UK) upon dilution in a 1 mM NaCl solution.

The amount of rhodamine® encapsulated and released from the rhodamine®-loaded nanoparticles was determined from the supernatant after ultracentrifugation and measured by fluorimetry (λex = 525 nm; λem = 550 nm).

The association efficiency of sCT to the nanoparticles was calculated from the difference between the total amount of peptide added in the encapsulation process and quantity of non-encapsulated peptide. The separation of the nanoparticles from the non-encapsulated peptide was performed by ultrafiltration (Contricom® YM-100, Millipore, Spain) for PEG-coated nanoparticles or by ultracentrifugation for CS-coated nanoparticles (50,000 × g). Ultrafiltration was found to be more suitable to completely isolate sCT from PEG-coated nanoparticles, due to the high centrifugation speed required for their sedimentation. In contrast, CS-coated nanoparticles could be isolated by ultracentrifugation due to their adequate sedimentation characteristics. The concentration of sCT was determined by HPLC as described (British Pharmacopoeia (BP) 1998).

For the cell culture experiments, nanoparticles were isolated by ultracentrifugation and resuspended at proper concentrations in HBSS (pH 7.4). CS-coated nanoparticles were resuspended in HBSS with a pH value of 6.5 in order to

avoid particle aggregation. Control experiments for this formulation were also performed with the same HBSS solution.

Cell culture experiments

Caco-2 cells were cultivated on 80 cm^2 flasks (Nunc, Denmark) using MEM supplemented with 10% FBS, 1% l-glutamine, 1% nonessential amino acids and penicillin/streptomycin solution. Cells were maintained on a controlled atmosphere at 37ºC with 95% of relative humidity and 5% CO2. The culture medium was changed every other day for approximately 5–6 days until cells reached approximately 80–90% confluency. After the passage operation, cells were seeded approximately at 2.5×10^5 cells per flask. For the experiments, cells with passage numbers between 25 and 35 were used.

Cytotoxicity studies

Cells were cultivated on 96-well plates (0.33 cm^2/well) (Nunc, Denmark) with a cell density of 1.4×10^4cells/cm^2 for approximately 7 days until a homogeneous cell monolayer was obtained.

Cytotoxicity experiments were conducted for PEG- and CS-coated nanoparticles at concentrations between 0.1 and 20 mg/ml (0.2 ml/well). Monolayers were in contact with the suspension of nanoparticles for 2.5 h. After this time, the nanoparticle suspensions were removed and cell viability was determined using a colorimetric method (Cell Titer 96®, Promega, Madison, WI) where the reagents are bioreduced by the cells to a colored formazan product (readable at 490 nm) through a pathway where NADPH or NADH are involved. HBSS was used as a negative control, whereas a 2% sodium dodecyl sulfate (SDS) solution in HBSS as a positive control.

Results from the colorimetric assay were obtained by normalizing the absorbance of experimental wells with that of the controls: the absorbance value of the negative control was subtracted from the absorbance of positive controls and experimental wells. Then, these corrected observances were multiplied to equal the positive controls to one.

Qualitative analysis of the interaction of nanoparticles with the Caco-2 cells

Caco-2 cells were seeded with a density of 5.5×10^4 cells/cm^2 on sterile coverslips placed in 6-well plates and left to grow until differentiation (19–21 days). Cell membranes were incubated with rhodamine®-loaded nanoparticles formulations (0.25 mg/ml) in HBSS for 1 h. Then, nanoparticle suspensions were withdrawn and monolayers were rinsed five times with fresh DPBS. The samples were fixed for 10 min with 3.7% formaldehyde, and then permeabilized for 20 min with a 1% bovine serum albumin (BSA)/0.1% Triton X-100 solution in DPBS. After permeabilization, the cytoskeleton of the cells was stained with a 1% BSA/0.1% Triton X-100/5 UI/ml Bodipy® 650/665-Phalloidin solution for 20 min. After staining, the monolayers were thoroughly washed with DPBS, the coverslips separated from the wells, and the sample embedded in one drop of the mounting medium Fluorsave® (Carbiochem, US).

Samples were examined under a confocal laser scanning microscope (Leica TCS-SP2, Leica Microsystems). The excitation wavelengths used were 514 nm for rhodamine® and 633 nm for Bodipy® 650/665.

Qualitative analysis of the interaction of nanoparticles with the Caco-2 cells

Caco-2 cells were seeded with a density of 5.5×10^4 cells/cm^2 on 12-well plates and left to grow until full differentiation (19–21 days). Culture medium was

replaced by rhodamine®-loaded nanoparticles in HBSS at concentrations of 0, 0.25, 0.5 and 1 mg/ml and allowed for 1 h of contact. Then, the nanoparticle suspensions were removed and the cell monolayers washed thoroughly with Dulbecco's PBS (pH 5.0) in order to remove the nanoparticles which are not effectively associated to the cells. Finally, Caco-2 monolayers were dried overnight and dissolved in a 2% SDS/50 mM EDTA/DPBS at pH 6 ("lysis medium"). The remaining nanoparticles were quantified by fluorimetry. To calculate the amount of nanoparticles from the fluorescence, calibration curves (fluorescence versus nanoparticles concentration) were prepared in the same lysis medium. Before entering fluorescence values in the calibration curves, the fluorescence of monolayers without nanoparticles (0 mg/ml samples) in lysis medium was subtracted from the experimental values.

Measurement of the transepithelial electric resistance

Cells were seeded on 6-well Transwell® plates (4.71 cm^2/well, pore size 3 μm) (Costar®, Corning Inc., NY) with a cell density of 5.5×10^4 cells/cm^2. The plates were cultivated in MEM for 19–21 days. The integrity of cell monolayers was confirmed taking an initial transepithelial electric resistance (TEER) measurement. This initial value was recorded to follow the recuperation of the initial TEER of the monolayer. After this initial TEER measurement, cell monolayers were equilibrated with HBSS for 1 h prior the beginning of the experiment. For one set of experiments (test of the CS-coated nanoparticles and controls) the pH of the HPSS was adjusted to 6.5 in order to ensure the stability of the nanoparticles in the culture medium. For the other set of experiments (test of the PEG-coated nanoparticles and controls) the pH of the HBSS was 7.4. After the equilibration period, the apical solutions of the transwells were replaced by the nanoparticle suspensions (1 mg/ml of nanoparticles concentrations) or the control buffer (HBSS). Nanoparticle suspensions were kept in contact with the monolayers for 2.5 h and TEER measurements performed every 30 min. After this period of time, apical media were replaced again with MEM to check for recuperation of the control values. TEER values were monitored for the next 24 h and compared with the initial TEER measurement in order to check the recuperation of the membrane functionality. No significant differences in the

TEER/permeability experiments were found for the controls at pH 6.5 or 7.5. Therefore, for the sake of simplicity no mention to the pH of the HBSS solution will be made through the rest of the text.

TEER measurements were performed with a Millicell®-ERS connected to a pair of chopstick electrodes (Millipore, Spain). All values were corrected taking measurements from transwells with the same buffer/culture media but without cell monolayers.

Study of the transmembrane permeability

Cells were seeded on 6-well Transwell® plates (4.71 cm^2/well, pore size 3 μm) (Costar®, Corning Inc., Corning, NY) with a cell density of 5.5×10^4 cells/cm^2. The plates were cultivated in MEM for 19–21 days. Prior to the experiment, cells were pre-equilibrated for 1 h with their corresponding experimental buffers (HBSS at pH 6.5 or 7.5). Apical medium of experimental well was changed by a suspension of nanoparticles (1 mg/ml concentration) containing free Texas Red®-dextran (approximately 300 μg/ml). In the control wells, the same media without nanoparticles were used. In all cases, the basolateral medium used was HBSS pH 7.4. At the beginning of the experiment, samples from the apical media were collected to precisely calculate the total amount of Texas Red®-dextran present in the apical chamber. Samples were collected from the basolateral chamber and replaced by fresh HBSS every 30 min for up to 2.5 h.

The concentration of Texas Red®-dextran was measured by fluorimetry. The apparent permeability of the monolayers to the paracellular marker calculated from the following equation: $Papp(cm/s)=dQ/dt/(A\times C_0)$, where Papp is the apparent permeability coefficient, dQ/dt the rate of appearance of Texas Red®-dextran in the basolateral side, A the surface area of the monolayers and C_0 the initial concentration of Texas Red®-dextran in the apical side.

Oral administration of sCT loaded nanoparticles

All animal experiments were approved by the Ethical Committee of the Faculty of Medicine of the University of Santiago de Compostela. Male Sprague–Dawley rats (225–275 g) from the Central Animals House of the University of Santiago de Compostela (Spain) were fasted for 12 h before experiments but allowed water ad libitum. Animals were kept conscious during the experiments.

The following formulations were administered intragastrically to rats: (1) sCT aqueous solution, (2) PEG-coated nanoparticles and (3) CS-coated nanoparticles. In all cases the sCT dose administered was 500 UI/kg and the volumes of formulations used were 0.5 ml. Due to the different sCT loading of the particles, the dose of nanoparticles administered varied between 19.2 mg/kg for the PEG-coated nanoparticles and 70.8 mg/kg for the CS-coated nanoparticles. Blood samples were collected from the tail vein 30 min prior to the administration of the formulations in order to establish the baseline calcium level. Then, blood samples were collected at different time intervals for up to 24 h. The serum was separated by centrifugation at 3000×g for 5 min. Hypocalcemic effect was determined in serum samples by a colorimetric method at 570 nm (Kit OR-cresolphtalein v/v, Spinreact, Spain). Results are shown as the mean values of serum calcium levels (±S.E.M.) of six animals. An ANOVA test was performed on the data ($\alpha<0.01$) with the statistical software package SPSS 11.0.

Results and discussion

In the present report, the behaviour of two innovative colloidal systems, differing in their surface composition, in terms of their utility for oral peptide administration is discussed. These are PEG-coated (PEG-coated nanoparticles) and CS-coated lipid nanoparticles (CS-coated nanoparticles). As indicated in Section 1, the selection of the polymer coating was based on previous work performed in our laboratory, which underlined the positive role of these polymer coatings at improving the interaction of colloidal systems with mucosal surfaces (De Campos et

al., 2003, Tobío et al., 2000, Vila et al., 2002 and Vila et al., 2004). On the other hand, the selection of a lipid core was based on the recognized ability of lipids to protect macromolecules against degradation, as well as to enhance the permeability of some epithelia (Muranishi, 1985).

Characteristics of the surface-modified lipid nanoparticles

In the present study the solid lipid cores were prepared using a multiple emulsion solvent evaporation technique (Garcia-Fuentes et al., 2002). CS was simply added to the external aqueous phase of the suspension, as this polymer is expected to attach to the core particles due to the formation of an ionic complex between the negatively charged lipids (tripalmitin and lecithin) and the positively charged polymeric chains. In the case of PEG-coated nanoparticles, the formation of the hydrophilic coating was achieved by adding PEG-stearate to the organic solution containing the lipid mixture. It was assumed that the amphiphilic nature of this polymer would facilitate the orientation of the lipid part (stearate) towards the lipid core and the hydrophilic PEG towards the external aqueous medium of the emulsion.

The physicochemical characteristics of the polymer-coated and uncoated blank nanoparticles are displayed in Table 1. The first general observation is that both hydrophilic coatings (PEG and CS) led to a logical increment in the size of the nanoparticles. However, this increase was more pronounced for CS- than for PEG-coated nanoparticles. Simultaneously, it can be seen that the zeta potential (ζ), indicative of the surface characteristics, is greatly affected by the nature of the coating. Logically, tripalmitin nanoparticles presented a marked negative charge due to the presence of anionic lipids in their composition. This negative charge was reduced in the case of PEG-coated nanoparticles due to the extension in the plane of shear produced by the presence of this polymer on the surface (Garcia-Fuentes et al., 2002). On the other hand, an inversion of the zeta potential from negative to positive values was observed for the CS-coated nanoparticles, a change attributed to the attachment of the cationic polymer CS to the surface of the cores.

Table 1: Physicochemical characteristics of blank uncoated and PEG- and CS-coated nanoparticles and the association efficiency (A.E.) of sCT to the different nanoparticles (mean±S.D., n=3).

Formulation	Particle size (nm)	ζ potential (mV)	A.E. (%)
Uncoated nanoparticles	200±2	−50.3±1.8	>90
PEG-coated nanoparticles	226±7	−34.8±2.8	>90
CS-coated nanoparticles	537±16	+29.2±6.2	30.7±2.3

Both types of surface-modified nanoparticles were fluorescently labeled with rhodamine®. This fluorochrome showed an encapsulation efficiency of 75% and less than 0.1% release for a 12 h period. The retention of the fluorescence associated to the nanocarrier was important in order to use it as a tracer for the study of the internalization of the particles.

Finally, the three carriers exhibited a high capacity to associate sCT. As reported in a previous work (Garcia-Fuentes et al., in press) this high association was attributed to a facilitated interaction of the cationic peptide with the negatively charged lipids. This mechanism of association justifies the reduction of the association efficiency caused by the CS coating. Indeed, it has been shown that the attachment of CS leads to the partial displacement of the surface-associated sCT (Garcia-Fuentes et al., in press).

Studies in the Caco-2 model cell line

Recent studies performed in the Caco-2 model cell line have provided evidence on the ability of polymer nanoparticles to cross epithelia (Desai et al., 1997

and Behrens et al., 2002). On the other hand, indirect observations by Gasco and coworkers (Bargoni et al., 1998) have led to the conclusion that lipid nanoparticles are able to cross the intestinal epithelium. However, despite the value of this previous information, no studies have been reported so far aimed at investigating the factors that affect the interaction of lipid nanoparticles with the intestinal epithelium. Consequently, one of the primary goals of this work was to study the interaction of these lipid nanostructures with the Caco-2 cells and to investigate whether or not the polymer coating affects this interaction. Before these transport studies, the cellular toxicity of the different types of particles was also evaluated.

Cytotoxicity of the nanoparticles

The evaluation of the cytotoxicity of the nanoparticles had a double objective: (i) to determine the concentrations that could interfere with the cellular metabolism in further studies in Caco-2 and (ii) to obtain a preliminary estimation of the safety of these new formulations.

The toxicity of the lipid core was expected to be very low given the fact that tripalmitin is a physiological triglyceride. In addition, the low toxicity of lipid nanoparticles has already been shown in human promyelotic cells (HL60) and human granulocytes (Müller et al., 1997). Therefore, the evaluation of the toxicity of the lipid cores was excluded from this study. As shown in Fig. 1, nanoparticles coated with PEG, a polymer of very low toxicity, showed high viabilities even at concentrations as high as 20 mg/ml. On the other hand, CS-coated nanoparticles exhibited certain toxicity at concentrations above 1 mg/ml, having an IC50 of 3.3 mg/ml. These values, indicative of a toxic effect of the CS coating are in agreement with those previously reported for CS solutions (Schipper et al., 1996). Consequently, from these studies we concluded that PEG-coated nanoparticles showed an extremely low cytotoxicity while CS-coated nanoparticles appeared to have a low toxicity. Therefore, a concentration of 1 mg/ml was selected as the threshold nanoparticles concentration for further Caco-2 experiments.

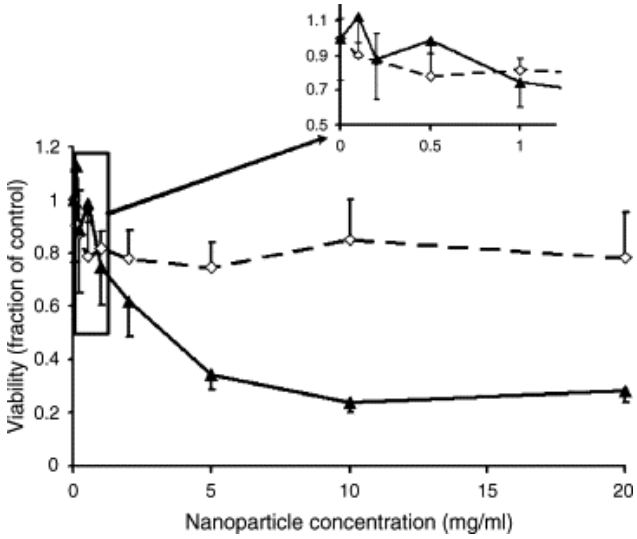

Fig. 1: Relative viability of Caco-2 cells following incubation with different concentrations of PEG-coated nanoparticles (◇) and CS-coated nanoparticles (▲) for 2.5 h (mean±S.D., n=8).

Qualitative and quantitative analysis of the interaction of nanoparticles with the Caco-2 cells

In order to visualize the limits of the cellular compartments and, thus, to help the localization of the nanoparticles associated to the monolayers we used Bodipy® 650/665-Phalloidin. This fluorescent dye binds selectively to F-actin, which is a major component of the cytoskeleton (Artursson et al., 1994). Control experiments showed that cells had low self-fluorescence and that no cross-fluorescence between the fluorochromes occurred.

Confocal laser scanning microscopy images show that both, CS and PEG, surface-modified nanoparticles were able to penetrate through the Caco-2 monolayers (Figs. 2 and 3, supplementary material). More specifically, after 1 h of incubation, both nanoparticles formulations were seen in consecutive confocal sections of the monolayer. The disposition and appearance of the fluorescence spots varied depending on the depth of the section. In the apical side (top images of Fig. 2

and Fig. 3) the nanoparticles are randomly distributed. However, in the underlying sections, the nanoparticles are preferentially co-localized with Bodipy®-Phalloidin in the cytoskeleton. In addition, as the sections are taken deeper into the cell, the fluorescent spots appear less aggregated and with a smaller size. Under the conditions of the present study, the observed localisation of the nanoparticles, does not allow us to discriminate between paracellular and/or transcellular internalization. Therefore, we could hypothesize that nanoparticles can enter the epithelium by either or both paracellular and transcellular mechanisms of transport. Despite these possible routes, previous reports on the internalization of CS nanoparticles by the Caco-2 monolayers, led us to accept the transcellular internalization as the most plausible mechanism (Ma and Lim, 2003 and Behrens et al., 2002).

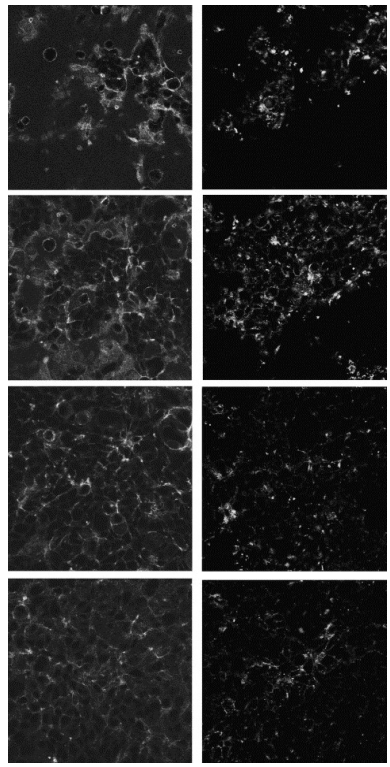

Fig. 2: Confocal scanning microscopy images of Caco-2 cell monolayers exposed to PEG-coated nanoparticles (0.25 mg/ml) and Bodipy® 650/665-Phalloidin for 1 h. The left column shows four sections collected at the Bodipy® 650/665-Phalloidin emission wavelength. The right column shows the same sections collected at the rhodamine® emission wavelength. The images are sections separated 3 μm each starting from the apical side (top).

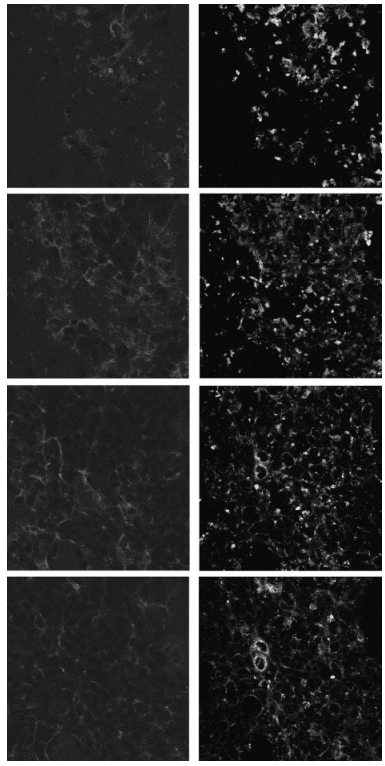

Fig. 3: Confocal scanning microscopy images of Caco-2 cell monolayers exposed to CS-coated nanoparticles (0.25 mg/ml) and Bodipy® 650/665-Phalloidin for 1 h. The left column shows four sections collected at the Bodipy® 650/665-Phalloidin emission wavelength. The right column shows the same sections collected at the rhodamine® emission wavelength. The images are sections separated 3 μm each starting from the apical side (top).

Therefore, the analysis of this previous information and the results of the present study led us to suggest that the polymer-coated lipid systems may enter the Caco-2 monolayer. Nevertheless, a more detailed study using different fluorescence markers would be necessary in order to understand the determinants of the mechanism of transport of the nanoparticles.

The quantitative analysis indicated that both nanoparticles formulations have an important interaction with the Caco-2 cells (Fig. 4). Interestingly, irrespective of the nature of the coating, the amount of nanoparticles bound to the cells increased linearly with the concentration of nanoparticles, reaching values of

around 15% of the dose. The similarity of the results obtained for CS-coated nanoparticles and PEG-coated nanoparticles was surprising for us, given the different nature of both coatings. In fact, the association values of PEG-coated PLA nanoparticles to the Caco-2 cells were much less important than those observed in the present work for PEG-coated lipid nanoparticles (Behrens et al., 2002). Consequently, more comparative studies are needed in order to identify the importance of the core and the coating in these mechanistic studies.

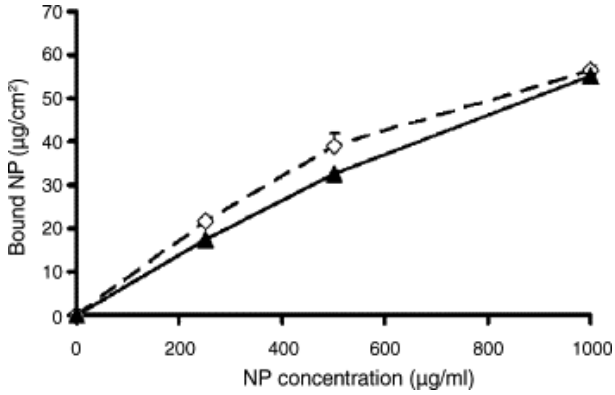

Fig. 4: Quantity of fluorescent nanoparticles (PEG-coated nanoparticles (◇), CS-coated nanoparticles (▲)) bound to Caco-2 monolayers after 1 h of incubation as a function of the concentration (mean±S.D., n=3).

Transepithelial electrical resistance and Texas Red®-dextran transport

A number of polymers, CS among them, have shown the ability to permeabilize the intestinal epithelium (Artursson et al., 1994). However, whether or not particles prepared from these polymers maintain their intrinsic capacity to modify the epithelial permeability remains unknown. Consequently, in the present work, we found it important to determine if this association has a consequence in the epithelial permeability.

The measurement of the transepithelial electrical resistance (TEER) is a common way to determine paracellular permeability to ions and their changes upon exposure to a permeation enhancer. This information is relevant as reductions in the

TEER can be correlated with partial disruption of the tight junction complex that restricts the paracellular route for hydrophilic compounds. In the present study, no significant changes were appreciated in the control wells during the experiment (Fig. 5). In addition, the TEER values of the monolayers exposed to PEG-coated nanoparticles exhibited a profile that was not significantly different than that of the control experiment: a small initial drop in TEER followed by a progressive recuperation of the initial TEER value. On the other hand, CS-coated nanoparticles presented a different behaviour compared to that of the corresponding control experiment. More specifically, the TEER values of the monolayers exposed to CS-coated nanoparticles decreased continuously during the experiment (2.5 h). Then, after removal of the nanoparticles formulations from the apical side, the monolayers were washed and incubated with MEM for the next 24 h. During this 24 h period, a slow recuperation of the TEER values was observed, reaching half of the initial TEER value after 6 h, and total recuperation in 24 h.

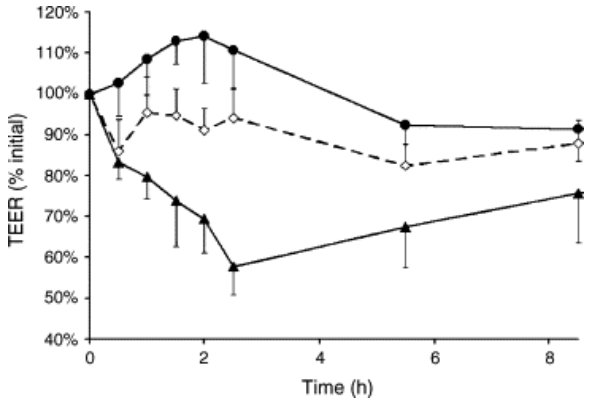

Fig. 5: Transepithelial electric resistance (TEER) of Caco-2 monolayers exposed to PEG-coated nanoparticles (1 mg/ml) (◊), CS-coated nanoparticles (1 mg/ml) (▲) or their respective controls (HBSS (□), HBSS pH 6.5 (●)) (mean±S.D., n= 3–6).

The apparent permeability (Papp) of the fluorescent marker Texas Red®-dextran (3000 Mw) was in good agreement with the TEER data (Table 2). The results showed that PEG-coated nanoparticles do not have a permeability enhancing effect. In contrast, CS-coated nanoparticles elicited a very marked transport

enhancing effect compared with their control. Indeed, the permeability values of the monolayers incubated with CS-coated nanoparticles were almost four-fold those of the controls. This transport enhancing effect is in good agreement with that previously reported for CS solutions (two- to four-fold enhance in manitol permeability with respect to the control) (Artursson et al., 1994 and Schipper et al., 1996).

Table 2. Apparent permeability coefficient (Papp) of Texas Red®-dextran (3000 Mw) determined in Caco-2 cell monolayers exposed to PEG-coated nanoparticles, CS-coated nanoparticles and their controls (mean±S.D., n= 3–6).

Treatment	P_{app} **(×10^{-7} cm/s)**	**Relative P_{app} (carrier/control)**
HBSS	1.70±0.86	–
PEG-coated nanoparticles	1.83±0.18	1.08
CS-coated nanoparticles	6.12±1.45	3.6

Overall, these results indicate that CS-coated nanoparticles produce a transient increase in the transepithelial permeability. This effect could be related to an opening of the paracellular route due to the interactions of CS with the proteins involved in the tight junctions between epithelial cells (Artursson et al., 1994 and Smith et al., 2004). Nevertheless, in the interpretation of these results we should be conscious of the fact that in these cell culture experiments an important amount of nanoparticles (0.333 mg/cm^2) is forced to be directly in contact with the cells, a situation that differs substantially from that expected following oral in vivo administration.

Oral administration of sCT-loaded surface modified nanoparticles

As a final step of the present work, and in order to elucidate the role of the polymer coating around the lipid nanoparticles in their ability to enhance the intestinal absorption of peptides, we determined the calcemia levels following the oral administration of the nanoparticles and the corresponding control (sCT solution). Two very distinct profiles were observed for PEG- and CS-coated nanoparticles (Fig. 6). PEG-coated nanoparticles did not produce any significant effect on serum calcium levels. In contrast, CS-coated nanoparticles showed a rapid and drastic reduction in serum calcium levels. Moreover, these low calcemia levels were maintained for at least 24 h. This hypocalcemic effect was significantly lower than that of the control for all the time points ($\alpha < 0.01$). This important extent of reduction of the serum calcium levels is similar to that previously reported for other systems, such as CS-coated poly(lactic-co-glycolide) nanoparticles and poly(N-isopropyl acrylamide)-coated polystyrene nanoparticles (Kawashima et al., 2000 and Sakuma et al., 2002). However, to our knowledge, the ability of CS-coated nanoparticles to prolong the pharmacological response is superior to that of the previously reported systems.

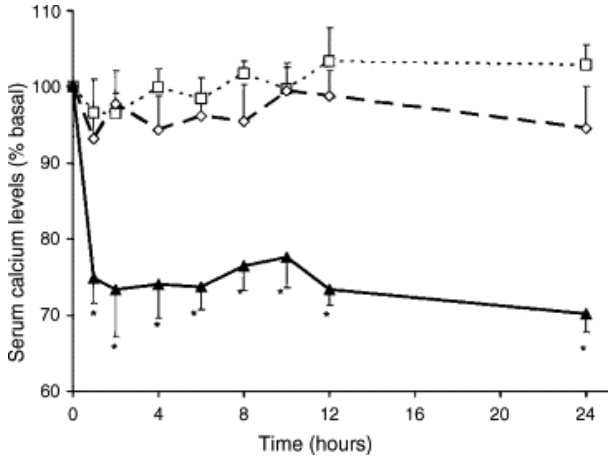

Fig. 6: Serum calcium levels after oral administration to conscious rats of sCT in solution (□), or associated to PEG-coated nanoparticles (◊) or CS-coated nanoparticles (▲) (mean±S.D., n= 6). *Significant differences from the sCT solution ($\alpha<0.01$).

At the present stage, our hypothesis to explain the success of the CS-coated nanoparticles is that they interact favourably with the mucus covering the intestinal mucosa (Takeuchi et al., 2001 and Behrens et al., 2002) and then, diffuse through this mucus layer reaching the underlying epithelium. These interactions would permit a site-specific delivery of sCT for prolonged times and, consequently, a prolonged pharmacological response. Moreover, it could be expected that CS, because of its ability to interact with the tight junctions, could enhance the paracellular transport of the peptide released at the epithelial level. In contrast, PEG-coated nanoparticles do not have a favourable interaction with the mucus layer covering the intestinal epithelium and, thus, have a limited access to the underlying epithelium. In addition, PEG-coated nanoparticles did not produce an enhancement of the epithelial permeability, which could also be responsible for the absorption of the peptide. These differences could justify the lack of pharmacological response observed for the PEG-coated nanoparticles despite their ability to enter the Caco-2 cells monolayer.

In summary, due to the extreme complexity inherent to the study of the particle uptake phenomenon and of the mechanisms of interaction with the epithelium (particularly for biodegradable particles), more detailed studies will have to be performed in order to fully understand the in vivo behaviour of these novel nanocarriers. Nevertheless, the results presented in this work are relevant as they show that particle uptake may not be the main mechanism behind the enhanced bioavailability of sCT in chitosan-coated nanoparticles.

Conclusions

Overall, the results of this work underline the importance of the surface composition of the nanoparticles in their ability to enhance the intestinal absorption of peptides. More specifically, the results showed that lipid nanoparticles coated with PEG or CS were able to enter the Caco-2 cell monolayers. However, only those coated with CS were able to open the tight junctions, thereby increasing the permeability of the model epithelium. Moreover, CS-coated nanoparticles were able

to enhance the oral absorption of the peptide sCT leading to a prolonged hypocalcemic response, whereas, PEG-coated nanoparticles were unsuccessful at increasing the absorption of the peptide. Consequently, the results suggest that the favourable interaction of the CS-coated nanoparticles with intestinal mucosa together with their permeation enhancing characteristics might be responsible for the improved oral absorption of the associated sCT.

Acknowledgements

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Polymer-coated lipid nanostructures as carriers for oral peptide delivery

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Abstract

Over the last decade we have assisted to a biotech revolution that is leading to the development of an important number of macromolecules with a great therapeutic potential. As a consequence, there is an increasing necessity to define specific formulation strategies for these delicate compounds. In our group, we have attempted to develop new nanocarriers for oral peptide administration. Among them, those consisting of a lipid core surrounded by a hydrophilic coating have resulted very promising. The lipid core was either liquid (Miglyol® 812 oil) or solid (tripalmitin) and the coating was a non-charged polymer such as polyethyleneglycol (PEG) or a positively charged polysaccharide i.e. chitosan. We first demonstrated that these polymer-coated nanostructures have an improved stability in the gastrointestinal fluids. Following this, we tested the ability of these new nanostructures to enhance the systemic absorption of the model peptide salmon calcitonin (sCT), after oral administration. The results indicated that, irrespective of the nature of the core, the chitosan-coated systems provided an enhanced and prolonged hypocalcemic response to sCT. In contrast, the PEG-coated lipid systems were not successful at increasing the response to sCT. The studies performed in the Caco-2 model cell line indicated that, irrespective of the coating, the nanostructures were internalized into the monolayer, however, only those coated with chitosan caused a significant reduction of the transepithelial resistance. Consequently, these results suggest that the superiority of the chitosan-coated nanostructures as compared to the PEG-coated nanostructures, could be related to inherent properties of chitosan, i.e. its favourable interaction with the mucus overlaying the intestinal mucosa and, possibly, its ability to open the tight junctions between epithelial cells.

Keywords: Nanoparticles, nanocapsules, lipid-based carriers, oral peptide delivery, chitosan, PEG.

Introduction

Overcoming the low bioavailability of drugs thathave low permeability through biological membranes is one of the most prominent pharmaceutical problems [1]. The advent of the biotech revolution has stressed even more this limitation as an increasing number of molecules with high therapeutic potential are dumped because of their poor biopharmaceutical properties [2]. Peptides and proteins are the molecules that best exemplify these biopharmaceutical properties: together with their poor permeability, thesemolecules are extremely labile to the action of proteolytic enzymes [3]. The combination of these two biopharmaceutical barriers (low permeability through the epithelia and degradation) constrainsthe oral bioavailability of peptides to values usually below 1%. One of the most appealing strategies to improve the oral absorption of these delicate macromolecules has been the association or inclusion of peptides in submicrometer-size carriers [4,5,6]. The challenge, however, has been the definition of the characteristics of the carrier that will lead to an optimized transport and, an adequate delivery of the associated peptide. Among the materials chosen for the design of these nanocarriers, the lipids have played an important role.

From lipid excipients to nanostructured lipid carriers

Lipids comprise a rather heterogeneous group of molecules that are characterized by the presence of aliphatic chains. Lipids have been used for pharmaceutical preparations due to their excellent biocompatibility and their wide spectrum of physicochemical characteristics, which make them useful for multiple applications [7]. To this date, waxes, mono-, di- and triglycerides, fatty acids, cholesterol derivatives or phospholipids are the most common classes of lipids used in traditional pharmaceutical formulations such as lipid emulsions, intramuscular depot injections, matrix tablets or suppositories.

In the last, a better understanding of the interactions of lipids at the cellular level has considerably increased the interest for these materials as drug transmucosal

carriers. In fact, electron spin resonance and NMR studies have shown that monoglycerides and fatty acids interact causing a disorder in the lipid membranesthat is supposed to be related to their capacity to permeate the epithelia [8]. Moreover, some lipidshave exhibited an affinity for the SH-proteins, which are known to play a role in transmembrane transport and tight junction permeability [9]. More recently, it has also been shown that some lipids can also interact with P glycoprotein and cytocrome P 450, thereby facilitating intracellular drug delivery [10].

However, maybe the most unique characteristic of lipid based formulations is their ability to be digested in the intestine, thus producing micelles that can enter the organism through the intestinal lipid transport system [7]. Drugs incorporated in the micelles resulting from lipid digestion may be internalized through this pathway [11]. The absorption enhancing properties of lipid excipients have already been tested for oral insulin delivery [12,13]. This early works provided some proof-ofconcept of the possibility to achieve higher oral bioavailability of peptides if administered with lipid promoters.

The new challenges faced by lipid formulations have stimulated the design of more advanced drug delivery systems, particularly submicrometric carriers, whose high surface/volume ratio maximizes the interactions with the biological environment. Moreover, some of these nanocarriers, i.e. liposomes and solid lipid nanoparticles, have shown their ability to cross the intestinal epithelium, probably though an endocytic pathway [14,15].

Liposomes in oral peptide delivery

The capacity of liposomes to encapsulate hydrophilic macromolecules has been suggested as a way to protect them in the gastrointestinalract [16]. However, in order to achieve this protective effect, it is critical to preserve the liposome integrity in this hazardous environment. Since the early works from Dapergolas et al. [17]

it is well known that liposomes made of phospholipids with a phase transition temperature above 37ºC are more stable in the GI tract than those composed of lipids in a liquidcrystalline state (such as natural phospholipids).

A step further in the development of oral liposomes has been based upon the optimization of their surface characteristics. For example, the modification of the surface of liposomes with protective coatings such as mucin or PEG resulted in enhanced oral insulin effect compared to uncoated liposomes. *In vitro* experiments confirmed that these liposome formulations were able to protect the peptide more efficiently against its degradation in intestinal fluids [16]. Nevertheless, the beneficial effect of these coatings should not only be attributed to its protective capability, as a subsequent study has also shown that these polymers increased the residence time of the liposomes in the GI tract. More concretely, mucin-coated liposomes displayed an extended gastric retention and PEGcoated liposomes an increase in the residence time in the small intestine [18]. Liposomes coated with other type of mucoadhesive polymers such as Carbopol® or chitosan have also shown to enhance the absorption of orally administered peptides [19,20].

The surface charge of liposomes also seems to have an important role in the capacity of liposome formulations to promote the oral peptide absorption, however, the optimal value of that parameter remains unclear. Some papers have indicated that the presence of anionic lipids improves the intestinal absorption of insulin [21,22], while others have reported the same effect, either for insulin or calcitonin for cationic liposomes [16,23].

Microemulsions and nanoemulsions in oral peptide delivery

Microemulsions and nanoemulsions are dispersed systems of two immiscible liquids. Microemulsions can also be defined as colloidal dispersions of liquids thermodynamically stabilized by a layer of surface-active molecules. Nanoemulsions are not thermodynamically stable, however, their coalescence can be hindered for prolonged periods of time if they are properly stabilized. With an

already marketed oral Cyclosporine A formulation (Sadimmune Neoral®), increased attention has been paid to the possibility of formulating peptides orally by including them in submicrometric emulsions. Nevertheless, up to date, the most successful results have been obtained with small peptides such as desmopressin [24], vasopressin [25] or thefibrinogen antagonist peptide SK&F 106760 [26]. This positive behaviour has been understood as a consequence of the lipid absorption promoting effect. This hypothesis has been further supported by the fact that composition, rather than emulsion droplet size was found to be the main factor responsible for the enhanced peptide absorption. Indeed, the presence of a polyoxyethylene derivative (Cremophor EL) was considered essential to achieve those high peptide oral bioavailabilities [27].

Despite the encouraging data obtained for Cyclosporine A and the SK&F 106760 peptide, the success of the submicron emulsion for oral delivery of high molecular weight peptides remains to be a challenge. Some improvements in the absorption of peptides, i.e. salmon calcitonin, have been observed for submicron emulsions which were coated with a mucoadhesive polymer such as Carbopol® [24]. This improved absorption of the associated peptide was attributed to the enzyme inhibitory action of this polymer [28]. More recent studies [29] have confirmed that the presence of Carbopol® as a coating was critical for the efficacy of the formulation.

Polymeric nanocapsules in oral peptide delivery

Nanocapsules are carriers comprising an inner lipid reservoir and an outer polymeric wall. Insulin-loaded polycyanoacrylate nanocapsules have render, so far, some of the most outstanding results in animal models. Significant reductions in the glycemia of diabetic rats were produced and maintained for prolonged periods of time, when that nanocapsule formulation was administered orally [30]. The extent and duration of the pharmacological effect was explained by the penetration of the nanocapsules through the mucosa and the further release of the peptide from the

internalized formulation [31]. Unfortunately, the results obtained in the dog model, although positive, were not as promising as those previously reported with rats [32].

New polymer-lipid nanostructures for oral peptide delivery

Although over the last years some breakthrough technologies have led to a new perspective of oral peptide delivery, renewed efforts are still needed to obtain a clinically useful carrier system. Lipid-based carrier systems are not an exception and still have to face important challenges to optimize their performance.

First, lipids are labile materials, very susceptible to enzymatic degradation in the intestine. As a consequence, lipid drug delivery systems are usually destroyed before reaching the adequate site for peptide absorption, thus loosing their ability to protect and enhance the transport of the peptide. Finally, it is generally accepted that the capacity of transport of lipid carriers, even though significant, may be insufficient at this stage to achieve a clinically useful effect.

In order to advance a further step in the development of a useful peptide delivery system for the oral route, we have designed new carrier systems based on a lipid core-polymer coat structure. The polymeric coating was intended to optimize the interaction of the delivery system with the biological environment present in the gastrointestinal tract. Hence, we selected polymer coatings that were supposed to prevent aggregation and destruction of the delivery systems, to confer mucoadhesive properties to the carriers or to enhance transepithelial permeability.

A schematic illustration of the nanostructures prepared is depicted in Figure 1. Since the composition of the core is supposed to affect drug incorporation and release [33], we have proposed three different cores for the nanostructures: a solid lipid (tripalmitin), a liquid lipid (Miglyol® 812) or a solid-liquid lipid mixture (tripalmitin/Miglyol® 812).

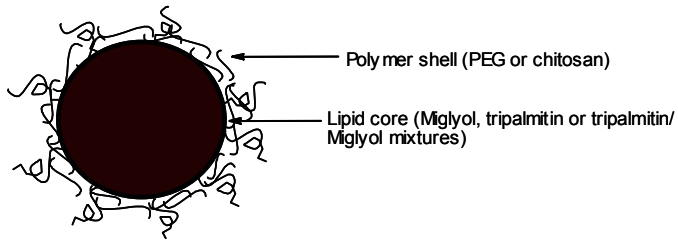

Figure 1: Illustration of the proposed architecture of the designed polymer-coated lipid nanostructures.

Regarding the polymer coating, two hydrophilic polymers have been selected: PEG and chitosan. The selection of PEG is based on the results of previous studies performed by our group, which have shown that a PEG coating around PLA nanoparticles greatly enhanced their stability in simulated gastrointestinal fluids [6]. Similarly, studies performed by Olbrich et al. have shown that a coating with ethylene oxide derivatives (Poloxamers) around the lipid nanoparticles protected them from degradation by pancreatic enzymes [34]. Moreover, it has been indicated that a PEG coating may favour the interaction of colloidal carriers with mucosal surfaces [18,35]. However, while the protective effect of PEG is well understood, its possible role in enhancing the interaction of nanosystems with epithelia remains unclear. Nevertheless, whether this improved interaction is simply a consequence of the enhanced stability of the nanoparticles in contact with biological fluids, or is additionally helped by some specific mechanisms, the clear observation is that the PEG-coated PLA nanoparticles are able to cross the nasal mucosa up to a more important extent than those non-coated [36,37].

On the other hand, the selection of the polysaccharide chitosan as a coating for transmucosal nanocarriers is justified by its interesting biopharmaceutical behaviour. From the point of view of drug delivery, some important features of this material are its mucoadhesiveness [38] and its capacity to increase the permeability of epithelia [39]. In addition, previous studies from our group have shown that chitosan-based nanosystems are able to interact with mucosal surfaces, thus facilitating the interaction and transport of the associated active compound across these surfaces. More specifically, chitosan nanoparticles were able to enhance the

transport of insulin across the rabbit nasal mucosa up to a much greater extent than chitosan solutions [40]. Similarly, low molecular weight chitosan nanoparticles elicited an interesting capacity for the transport of antigens across the nasal mucosa [41]. In addition, besides their favorable behaviour for the nasal transport of macromolecules, chitosan nanoparticles exhibit an affinity for the ocular mucosa. Recent studies aimed at studying the residence time of these nanoparticles in the ocular mucosa, following topical administration, indicated that the interaction of chitosan with the cornea and conjunctiva was enhanced and prolonged when presented in a nanoparticulate form [42]. All these studies provided evidence of the benefits of chitosan nanosystems as compared to other forms of presentation of chitosan.

Taking this previous information into account the aim of the present paper was not simply to review the state-of-the-art of the potential of lipid-based colloidal systems for oral peptide administration but to specifically emphasize the latest advances from our work regarding the design and evaluation of polymer-coated lipid nanosystems.

Preparation and characterization of polymer-coated lipid nanostructures

PEG-coated tripalmitin (or PEG-coated tripalmitin/Miglyol®) nanoparticles were prepared in a single step by a modified double emulsionsolvent evaporation method [43]. In this technique, hydrophilic drugs such as peptides may be incorporated to the nanoparticles in the inner aqueous phase. The PEG coating was formed due to the addition of the modified fatty acid PEG-stearate to the organic phase.

On the other hand, chitosan-coated nanocarriers have been prepared following different procedures depending on the nature of the core. Chitosan-coated solid lipid nanostructures were prepared by the double emulsion-solvent evaporation technique, indicated above [43]. When using this procedure, chitosan can be either added to the external aqueous phase of the double emulsion or once the lipid cores

were precipitated [44]. Alternatively, chitosan-coated submicron emulsions were prepared according to the solvent displacement-solvent evaporation technique, as previously reported [45]. As in the case of the solid cores, chitosan can be added before or after the solvent evaporation takes place. Irrespective of the technique, a critical ingredient for the formation of the chitosan-coated systems was the presence of lecithin, which was added to the organic phase in both procedures. Indeed, an ionic complex between lecithin and chitosan is formed at the interface of the colloidal system, thus facilitating the attachment of chitosan onto the surface of the system.

Lipid cores (non-coated) showed particle sizes around 200 nm upon preparation and strongly negative surfaces (Table 1). The coating of the tripalmitin cores with PEG produced a negligible effect on the particle size whereas the zeta potential shifted towards more neutral values [43]. As described for other PEG-coated systems, this change is a consequence of the extension of the shear plane of the colloidal system [46]. The formation of a chitosan coating around the lipid cores led to an increase in the particle size, accompanied by an inversion of the zeta potential from negative to positive values (Table 1) [47]. Both, the size increase and the inversion of the zeta potential, support the successful formation of a chitosan layer surrounding the lipid cores.

Table 1: Hydrodynamic diameter (analyzed by photon correlation spectroscopy) and zeta potential (analyzed by laser Doppler anemometry) of the studied nanostructures (Mean ± SD., n=3). Data from: [1]Prego et al. [54] and [2]García- Fuentes et al. [47].

Formulation	Diameter (nm)	Zeta Potential (mV)
Miglyol® cores[1]	195.8 ± 1.1	-52.0 ± 1.1
Chitosan Miglyol® nanocapsules[1]	266.6 ± 7.6	+34.8 ± 0.6
Tripalmitin cores[2]	200.0 ± 2.3	-50.3 ± 1.8
PEG-coated tripalmitin nanoparticles[2]	226.4 ± 7.5	-34.8 ± 2.8
PEG-coated tripalmitin/ Miglyol® nanoparticles[2]	207.4 ± 19.1	-36.6 ± 2.5
Chitosan-coated tripalmitin nanoparticles[2]	537 ± 16	+29.2 ± 6.2

The size and the morphology of the PEG- and chitosan-coated systems were visualized by transmission electron microscopy (Figure 2). All the nanostructures presented a spherical shape. In the case of PEG-coated nanoparticles, a difference in the staining was appreciated between the inner and the outer part of the nanoparticles, being this a possible indication of the core-coat structure of the systems. Unfortunately, this difference in staining could not be observed for chitosan-coated tripalmitin nanoparticles although the change in particle size was clearly visible in the micrographs. This dissimilarity between micrographs could be related not only to the different nature of the coating polymers but also to the high chitosan molecular weight as compared to that of PEG. This observation led us to suggest that the chitosan may be forming a more compact polymer layer compared to that of the PEG.

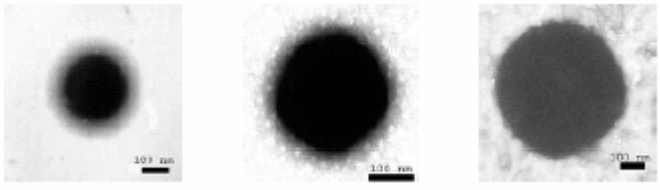

Figure 2: Transmission electron microscopy pictures of different lipid nanostructures: PEG-coated tripalmitin nanoparticles (left), PEG-coated tripalmitin/Miglyol® nanoparticles (center) and chitosan-coated tripalmitin nanoparticles (right).

NMR analysis has allowed us to confirm that the PEG-coated nanostructures are mainly composed of a triglyceride core and a small amount of surfactants and surface modifying molecules (i.e. lecithin, PEG-stearate) [48]. On the other hand, the core-coat model proposed for these structures was fully confirmed in the case of PEGtripalmitin nanoparticles. NMR experiments aimed to analyze the relaxation of the chemical groups of nanoparticle suspensions and their spin-population effects with the water protons confirmed the presence of PEG protruding towards the external phase and the presence of a solid lipid core comprising mainly the triglycerides. Moreover, quantitative NMR analysis has

confirmed the possibility of modulating the PEG coating density of these carriers by controlling the amount of PEGstearate added in the preparation procedure [48].

The NMR characterization of nanoparticles having a tripalmitin/Miglyol® core indicated that there were significant amounts of the oil incorporated within the nanoparticles matrix. Moreover, the presence of Miglyol® as separated liquid domains associated to the nanoparticles was confirmed by NMR relaxation analysis and the observed restricted diffusion dynamics of the entrapped oil. A further confirmation of this structure was obtained from differential scanning calorimetry (DSC) and x-ray diffraction spectroscopy [49].

Stability in simulated gastrointestinalfluids

Taking into account that the designed lipid nanostructures were intended for oral drug delivery, the assessment of their stability in gastrointestinal fluids was critical. Indeed, lipid nanoparticles have shown important particle aggregation in gastric media and a marked degradation in simulated intestinal fluid with enzymes [50,51]. Interestingly, the aggregation of the lipid cores in gastric medium has been found to be dependent on the mechanism of particle stabilization. Thus, we previously prepared lipid cores that were stabilized only with lecithin (uncoated cores), a surfactant that confers on the system a distinctly negative charge at neutral pH. At low pH, the surface charge of the lipid nanostructures was significantly reduced due to the less important ionization of the negatively charged phospholipids, thus leading to the aggregation of the nanoparticles in simulated gastric medium (Figure 3) [43]. The importance of the pH of gastric medium in the aggregation processes was also assessed by incubating the formulations in an inorganic acid solution (pH 1.2), without enzymes. As can be appreciated in Figure 3, the addition of a second ionic stabilizing surfactant (sodium cholate) did not enhance the stability of tripalmitin cores neither in inorganic medium nor in simulated gastric medium. On the contrary, by coating the lipid cores with polymers with steric stabilizing properties such as the PEG-stearate or the poloxamer 188, completely stabilized the formulations in both media [43,52]. As indicated before, previous experiments

performed in our group with polyester nanoparticles also support the key role of PEG in providing enhanced stability to the nanoparticles formulations incubated in simulated gastric media [6].

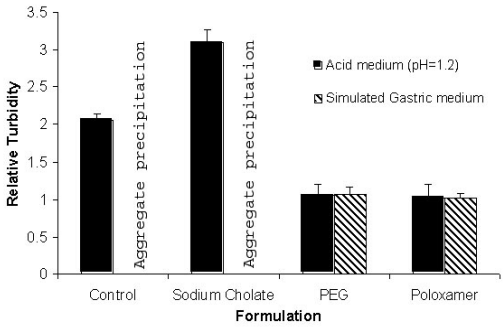

Figure 3: Relative turbidity (expresed as the ratio between the optical density value after 1 h of incubation with respect to the initial value) of tripalmitin cores (control) and tripalmitin cores stabilized with different surfactants in an inorganic acid medium and in simulated gastric medium (mean ±SD, n=3). Adapted with permission from García-Fuentes et al. (2002) [43]

The results of our experiments also showed that, as expected, the lipid cores undergo an important degradation in intestinal medium (Figure 4). In fact, the capacity of pancreatic lipase to degrade lipids is accelerated in these formulations as the lipid is highly dispersed and, therefore, presents an important surface area for interaction with enzymes. However, protection of the lipid cores by a hydrophilic polymer coating capable of hindering the interaction with lipolytic enzymes, such as that of provided by PEG-stearate or poloxamer 188, reduced significantly the degradation.

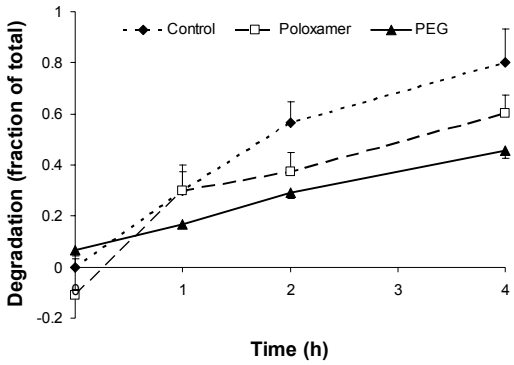

Figure 4: Degradation of tripalmitin cores (control) and PEG or poloxamer-coated tripalmitin nanoparticles in simulated intestinal medium (mean ±SD, n=3). Graph from García-Fuentes et al. (2002) [43].

Although the charge and solubility of chitosan are pH-dependent due to the presence of amine groups, our stability studies have evidenced that it may also enhance the stability of lipid nanostructures in gastrointestinal fluids [47,53]. More specifically, the results from our studies showed that chitosancoated lipid nanostructures are stable in simulated gastric fluids. Moreover, chitosan also protected the lipid cores against enzymatic degradation in simulated intestinal medium [47].

Peptide loading and release

A major goal in designing these lipid nanostructures was to achieve a sufficient loading of the peptide in the carriers while maintaining their active structure. Specific nanoparticle preparation methods were developed for this aim. In the case of nanocapsules, the peptide sCT was easily associated upon incorporation in the organic phase, using the nanoprecipitation technique. This smooth nanoencapsulation method resulted in high peptide encapsulation efficiencies (>98%) with preservation of peptide integrity [54]. On the other hand, peptides such as insulin or sCT were loaded in solid lipid (tripalmitin) nanoparticles using a

modified double emulsion method. When using this approach, the peptide was dissolved in the inner aqueous phase of the double emulsion. This method has allowed us to increase markedly the capacity of these solid lipid matrixes to incorporate peptides such as insulin (encapsulation efficiency>45%) [43] and sCT (encapsulation efficiency>90%) [47]. As in the case of the nanocapsules, no signs of peptide degradation were detected by reverse phase high performance liquid chromatography (Figure 5).

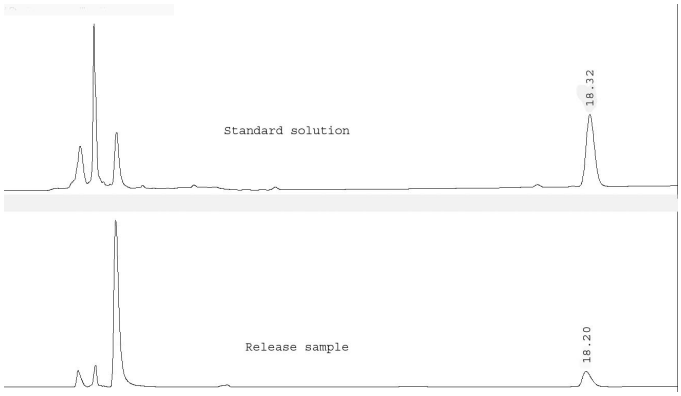

Figure 5: Reverse phase high performance liquid chromatograms of salmon calcitonin (retention time= 18-18.5 min) in a standard buffer solution (top) or after being released from a PEG-coated tripalmitin nanoparticle formulation (bottom).

This important association efficiency can be explained by the affinity of these peptides by the lipids. Indeed, it is known that phosphatidylglycerol, which present in our particles, shows a particularly strong interaction with this macromolecule [55]. On the other hand, insulin has already been shown to associate to lipid-based carriers such as polycyanoacrylate nanocapsules [30]. Moreover, as indicated in the introductory section, there is a significant number of references which have shown the efficient incorporation of peptides within lipid submicron emulsions [24,25]. Furthermore, in order to corroborate this, theoretically possible, interaction we have performed some adsorption experiments which have confirmed that our negatively charged nanostructures were able to adsorb high amounts of sCT following a Langmuir-type isotherm [47].

The release of the peptide sCT from the lipid nanostructures was slightly dependent on the lipid core. In the case of solid and solid-liquid mixtures, the release profile of sCT followed a biphasic pattern consisting of an initial burst (20% of the amount encapsulated) followed by a continuous and slow release [47]. The burst release was attributed to the surface-associated peptide whereas the second slow phase would correspond to the peptide entrapped in the lipid matrix. On the other hand, the burst release effect was reduced when the carriers were coated with the polymer chitosan (10% of the amount encapsulated) [47]. This reduction in the burst effect has been attributed to the lower amount of peptide on the surface of the nanostructures. In the case of the oily nanostructures, only the chitosan-coated systems showed a detectable release of sCT. The release process from these systems followed a biphasic pattern similar to that of the chitosan-coated solid nanostructures, although only negligible amounts of the peptide were released after the initial burst.

In conclusion, we have developed nanoencapsulation techniques that make feasible the association of peptides to lipid systems. By means of these techniques, lipid nanostructures are able to load peptides and release them in a controlled manner.

Studies in the Caco-2 cell model

Studies in the Caco-2 cell model were aimed at gaining information about the mechanism of interaction of the designed polymer-coated lipid nanostructures with the intestinal epithelium.

We have previously reported a study showing the capacity of PEG-coated polyester nanoparticles and chitosan nanoparticles to be internalized in the Caco-2 cell model [56]. The results from this work showed that the uptake of chitosan nanoparticles was higher than that of PEG-polyester nanoparticles, a result that was even more remarkable when both formulations were compared in the MTX-E12 cells, a model of mucus secreting cells. Interestingly, within the range of

concentrations studied (31.25- 1000 µg/ml), the association of chitosan nanoparticles to the monolayer was saturable and temperature dependent.

These results are different from those obtained for polymer-coated lipid nanostructures in the Caco-2 model cell line. Indeed, the amount of fluorescent particles associated to the cells was similar independently of the polymer coating [57]. On the other hand, when we compared the behaviour of the chitosan nanocapsules with that of the chitosan-coated tripalmitin nanoparticles, we observed an important difference in the amount of carrier associated to the cells, being it higher for the solid lipid cores [54,57]. However, these surprising results should be cautiously interpreted due to the different source of chitosan used to form the coating of the structures and also the fact that both systems were assayed separately.

We have also checked the capacity of our polymercoated nanostructures to modify the permeability of the cell monolayers. In this sense, it is known that chitosan has a capacity to cause a dosedependent decrease in the transepithelial electric resistance (TEER) [39] while PEG is supposed to be inert in terms of cellular interaction. In agreement with this, we observed that PEG-coated lipid cores did not cause a reduction in the TEER of Caco-2 cell monolayers in the range of concentrations investigated (220-330 µg/cm2). In contrast, chitosan-coated lipid nanostructures induced a dose-dependent drop in the TEER of Caco-2 monolayers reaching significant reductions for the range of concentrations (83-330 µg/cm2) [54,57]. The values of the reduction of the TEER values were additionally supported by the observation of enhanced paracellular transport of the macromolecular marker dextran-Texas Red® (Mw = 3000 Da). However, it should be added that these values were, in the case of nanocapsules, close to those that compromised cell viability (220 µg/cm2). Therefore, it could be expected that this change in the epithelium permeability could only be seen when an important amount of particles are accumulated onto the epithelium.

Reductions in the TEER similar to those of the chitosan-coated lipid nanostructures have been observed for chitosan solutions when the concentrations of the polymer were within the same range [58]. Interestingly, we observed that, as in the case of chitosan solutions, the normal TEER values were slowly recuperated

after the exposure to the chitosan-coated nanostructures. Indeed, values within 10% of the original value of TEER were achieved after 8 h of recuperation and after 24 h, all monolayers had returned to the initial resistance level [54,57].

In vivo efficacy of polymer-coated lipid nanosystems as oral peptide carriers

Keeping in mind that the main goal of our work was to design new peptide carriers for oral delivery, it was important for us to assess the ability of the new polymer-coated nanostructures to increase the transport of peptides across the intestinal mucosa. For this purpose, we chose sCT as a model compound [54,57]. A control experiment showed that sCT in solution was unable to reduce the serum calcium levels. Similarly, significant reductions in serum calcium levels could not be achieved after the oral administration of sCT included in the control nanoemulsion or in the PEG-coated tripalmitin nanoparticles. However, significant hypocalcemic responses were observed for sCT when included in the chitosan-coated lipid nanostructures. More importantly, this significant reduction in the serum calcium levels (25-27% reduction at 1 h post-administration for both chitosan-coated lipid nanostructures) was maintained for at least 24 h [54,57]. Interestingly, the positive behaviour of these new systems should not be simply attributed to the presence of chitosan but to their intrinsic nanostructural composition. In fact, Takeuchi et al. observed that the polymer itself in solution was ineffective at improving the intestinal calcitonin absorption [20]. In addition, we have recently evaluated *in vivo* the efficacy of chitosan microspheres (identical dose of chitosan and sCT) and found that they were unsuccessful at reducing the serum calcium levels [59]. Therefore, the general conclusion from these studies is that the efficacy of the new nanostructures presented in this report can not be assigned to their individual components, but to the way these components are nanostructured.

The mechanistic explanation for the positive behaviour of the chitosan-coated lipid nanostructures as compared to that of non-coated or PEG-coated lipid systems is not perfectly defined yet, although some preliminary hypothesis can be

established from the *in vitro* experiments. First, the enhanced stability provided by the polymeric coatings seems to be a relevant issue for peptide delivery. Chitosan, a polymer with well defined mucoadhesive properties, not only provides better colloid stability but possibly enhances the interaction of the nanostructures with the mucosal surfaces [19,38]. At this stage, increasing evidence points to the process of mucoadhesion as a way of gaining better access to the underlying epithelium [56]. Finally, taking into account the results from the Caco-2 experiments, one could attribute the success of the chitosan-coated lipid nanostructures to their ability to reduce the TEER. However, we should underline that sCT co-administered with chitosan solutions that show similar reduction in the TEER do not produce any significant hypocalcemic effect. In addition, it should be noted that the marked reductions in the TEER were observed for high doses of chitosan-coated lipid nanostructures while doses in animal where far below this limit. In conclusion, the positive behaviour of chitosan-coated lipid nanostructures seems to be determined by a combination of functions from the carrier that may include better peptide protection, improved carrier stability and enhanced interaction between drug and the mucosal surfaces.

Conclusions

This article reports the design and characterization of new polymer-coated lipid-based nanostructures for oral drug delivery. Among the systems prepared, chitosan-coated lipid nanostructures have shown the capacity to improve the oral efficacy of sCT. The positive behaviour of this system should be most probably attributed to a combination of factors that contribute to the protection of the peptide in the gastrointestinal tract and enhances its interaction with the epithelium of the intestine. Among them, the mucoadhesive properties of chitosan can have a major role at improving theefficacy of orally administered peptides.

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