A mi mejor amigo,

Pedro Valle

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

FACULTADE DE FARMACIA

Departamento de Farmacia e Tecnoloxía Farmacéutica

NANOSISTEMAS PARA EL TRATAMIENTO DE LA DIABETES MELLITUS POR VIA TRANSMUCOSAL.

Angela Valle Gallego

Santiago de Compostela, 2010

"Es de bien nacido ser agradecido"

Esta tesis no se habría podido llevar a cabo sin la colaboración de diferentes personas y organizaciones a quienes les quiero expresar mi agradecimiento.

En primer lugar, a mi director Francisco Goycoolea, quien me ha acompañado con calidez y compañerismo en cada paso, compartiendo inquietudes, éxitos y fracasos durante la realización de la tesis. Gracias por el altruismo, el apoyo, el aliento, por la constante dedicación y la pasión por la investigación. A María José Alonso por ofrecerme la oportunidad y los medios para dedicarme a la investigación, sin ella esta tesis no habría sido posible. Agradecer también sus valiosos, prácticos y experimentados consejos, y el rigor de su trabajo. A Federico Mallo, por sus puntos de vista, acertadas sugerencias y apoyo financiero.

A Lucas González por sus ideas, explicaciones y aportaciones en este trabajo. A Josefina Oliver y Encarnación de Miguel por sus colaboraciones en los estudios de histología. A Cristina Taboada por el material prestado y sus recomendaciones.

A las entidades financiadoras de este trabajo, el proyecto europeo "Nanobiosaccharides", a la Red Gallega para el estudio de la Obesidad, al proyecto "Desenvolvemento de novos sistemas de liberación de fármacos e material xenético " y al Instituto Nacional de Empleo (INEM).

A todos mis compañeros, excompañeros y profesores del Departamento de Farmacia y Tecnología Farmacéutica de la Universidad de Santiago de Compostela, especialmente al grupo

Nanobiofar. Agradecer sus consejos y compañía: Yolanda P., Edison S., Ana G., Teresa G., Ivana D., Cecilia P., Bruno S., Pablo H., Manuela R., Jenny P., Patrizia P., Sara V., Felipe O., Celina V., Manuel S., Victoria L., Giovanna L., Sonia A., Gustavo R., Giovanni K., José Vicente G., Ester P., Rita G., Desiré T., María A., Marcos G., Noemi C., Carmen R., Dolores T., Begoña S., Alejandro S. También al resto de compañeros del departamento, Felipe, Manolo, Nano, Lourdes, Laura, Álvaro, Ángel, Carmen, Fran, Mariana, Ramón… Quiero resaltar la labor facilitada por Rafael R. durante los experimentos in vivo.

A mis compañeros del Laboratorio de Endocrinología de la Universidad de Vigo, especialmente destacar a dos grandes amigos Mayte C. y Manuel G. por su paciencia y ayuda para transmitirme todos sus conocimientos sobre manipulación animal. A Soledad F., Marina R., Eva V., Darío A., Verónica C., Vanesa y Alba. También mencionar a los compañeros del laboratorio de Inmunología, especialmente a Daniel P.

En los agradecimientos personales quiero destacar a mi familia por apoyarme siempre a pesar de la incomprensión. Especialmente quiero mencionar a mi madre, mi tía Isolina y mi sobrino David. A todos mis amigos que se han interesado por mi, me han escuchado pacientemente durante estos años. Gracias por vuestro apoy: Isabel L., Fran L., Guillermo E., Ana S., Pedro B., Marta P., Ana F., Alicia C., Minia A. y en general a la “tropa de Café Sur”. Por último, pero no por ello menos importante, a mis compañeros de Tai Chi con los que tanto me río: Carmen T., Mercedes C. y Manchi.

Resumen

A pesar de los avances científicos en la búsqueda de nuevas formas farmaceuticas, el desafío sobre la administración de péptidos terapéuticos por vías no parenterales continua presente. Por ello, el principal objetivo de esta tesis ha sido el desarrollo de sistemas biocompatibles de tamaño nanométrico capaces de vehiculizar insulina y exendina-4 a través de la mucosa oral y/o nasal, para el tratamiento de la diabetes mellitus. Con este fin, se investigaron sistemas específicos para cada molécula. Para la administración de insulina por vía oral, los nanocomplejos de lecitina-insulina lograron reducir los niveles de glucosa en rata diabética. Aunque el mayor efecto sobre la glucemia se alcanzó con la incorporación de quitosano al sistema, originando nanopartículas de lecitina-quitosano. Por otra parte, las nanocápsulas a base de lecitina-quitosano para la vehiculización de exendina-4, demostraron ser una herramienta eficaz para la administración del péptido por vía nasal

INDICE PAG

INTRODUCCIÓN

1. DIABETES MELLITUS: Definición y clasificación

Según la Organización Mundial de la Salud (OMS) "la diabetes es una enfermedad crónica que aparece cuando el páncreas no produce suficiente insulina o cuando el organismo no utiliza eficazmente la insulina que produce (resistencia insulínica). El efecto de la diabetes no controlada es el aumento de los niveles de glucosa en sangre (hiperglucemia)".

La Diabetes Mellitus (DM) es una enfermedad metabólica crónica, encuadrada dentro de las enfermedades crónicas no transmisibles que son las responsables de la pérdida de la mayor cantidad de años potenciales de vida. Esta patología se considera responsable de generar la mayor discapacidad y mortalidad mundial (5 %), ocupando gran parte de los recursos sanitarios de todos los países. Por esta razón, el 80% de la mortalidad se producen en países con ingresos bajos o medios. La OMS estima que en el mundo hay más de 220 millones de personas con diabetes, causada por la evolución del estilo de vida, sedentarismo e incremento de grasas/azucares en la dieta. Probablemente, de no mediar intervención alguna, para el año 2030 esta cifra se habrá duplicado.

El diagnostico de la enfermedad es un punto clave para su evolución. Según la Asociación Americana de Diabetes (ADA, año 2000), los criterios para el diagnóstico son:

1. Síntomas de diabetes mellitus (polidipsia, poliuria, polifagia, pérdida de peso) + glucemia casual ≥ 200 mg/dl (11.1mmol/dl). Casual es definido como en cualquier momento del día sin respetar el tiempo desde la última ingesta.

2. Glucemia en ayunas (8 h) ≥ 116 mg/dl (7 mmol/l). El ayuno es definido como la no ingesta calórica de por lo menos 8 horas.
3. Glucemia a las 2 h de sobrecarga oral de glucosa (75 g de glucosa anhidra disuelta en agua) ≥ 200 mg/dl (11.1 mmol/l).

DM de tipo 1 (juvenil o de inicio en la infancia). Se caracteriza por una producción muy deficiente o ausencia de insulina debido a la destrucción de las células β del páncreas generalmente por un mecanismo autoinmune o idiopático.

- Sus síntomas consisten, entre otros, en excreción excesiva de orina (poliuria), sed (polidipsia), hambre constante (polifagia), pérdida de peso, trastornos visuales, cansancio y tendencia a producir cuerpos cetónicos.
- Se produce en individuos genéticamente predispuestos que presentan 90% HLA DR3 o HLA DR4 asociado a un factor ambiental.

DM de tipo 2 (de inicio en la edad adulta). Se debe a una utilización ineficaz de la insulina (resistencia) o a una producción insuficiente. Este tipo representa el 90% de los casos mundiales y se debe en gran medida a sobrepeso y a la inactividad física, asociado a un factor genético.

- Los síntomas pueden ser similares a los de la diabetes de tipo 1, pero a menudo menos intensos. En consecuencia, la enfermedad puede diagnosticarse sólo cuando ya tiene varios años de evolución y han aparecido complicaciones.
- La mayor incidencia es en adultos, sin embargo, en la actualidad también se está incrementando en adolescentes e incluso preadolescentes con obesidad.

- Existe una relación entre la hiperinsulinemia y el síndrome X o síndrome de metabólico que incluye hipertensión, dislipemia e hipercoagulación [1]

Como consecuencia de los elevados niveles de glucosa prolongados en el tiempo, se producen complicaciones crónicas que se dividen en tres categorías: microangiopatía, macroangiopatía y neuropatía, responsables de enfermedad cardiovascular, retinopatía diabética, nefropatía y mal perforante plantar (pie diabético). En líneas generales, la diabetes es una de las principales causas de ceguera adquirida a nivel mundial y la mayor causa de transplante renal y amputaciones no-traumáticas. El 75% de los pacientes diabéticos fallecen en un episodio cardiovascular [2].

2. TRATAMIENTO FARMACOLÓGICO DE LA DIABETES MELLITUS

La historia de la diabetes mellitus cambió radicalmente tras el descubrimiento de la insulina. En 1921, Banting y Best descubrieron que tras la administración intravenosa de extractos pancreáticos de perro se producía una disminución en los niveles de glucosa. Actualmente, la terapia de la DM tipo 1 continua basándose en la administración exógena de insulina. Sin embargo, en general el tratamiento farmacológico para la DM podría resumirse de la siguiente forma:

1. Aporte exógeno de **insulina** (humana o animal)
2. Aumento de la sensibilidad a la insulina endógena, bien con biguanidas o tiazolidinas.
3. Aumento de la secreción/liberación de insulina endógena: sulfonilureas, metiglinidas y análogos, o bien con incretino-

miméticos (análogos del GLP-1 y los inhibidores de la DPP-4): **Exenatida**

4. Reducción de la resistencia periférica a la acción de la insulina: metformina.
5. Reducción de la absorción digestiva de glucosa, mediante inhibidores de la α-glucosidasas o fibras vegetales y derivados [1].

2.1. Insulina

Es una hormona polipeptídica de 51 residuos de aminoácidos (masa molecular ≈ 5807.69 Da) en su forma activa, promueve de forma directa la incorporación de monosacáridos a las células y consecuentemente, el almacenamiento y metabolismo de carbohidratos, proteínas y grasas en la mayoría de los tejidos. La insulina es un dímero formado por dos cadenas, cadena A (21aa) y cadena B (30aa), unidas por dos puentes de disulfuro (Figura 1).

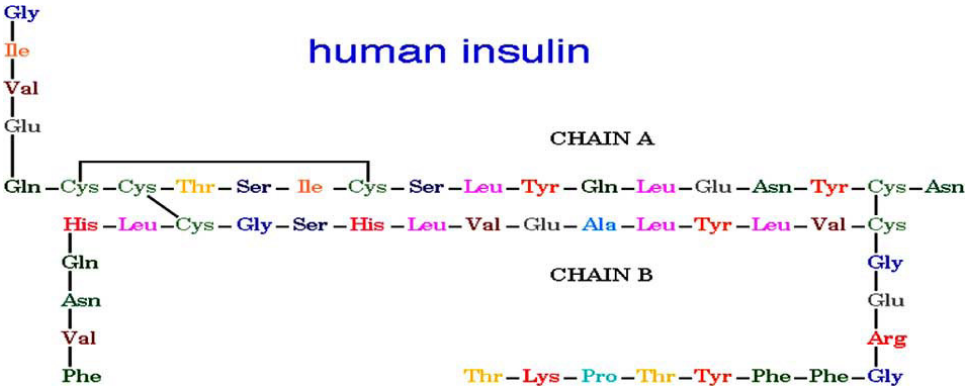


Figura 1: estructura de la insulina humana [3]

La secreción de la insulina se produce en el páncreas, más concretamente en las células β de los islotes de Langerhans, en forma de precursor inactivo llamado proinsulina. En el aparato de Golgi, se

almacena en forma de gránulos y es liberada por medio de un proceso de exocitosis. Ahí, la proinsulina pierde el péptido C, transformándose en insulina activa (Figura 2).

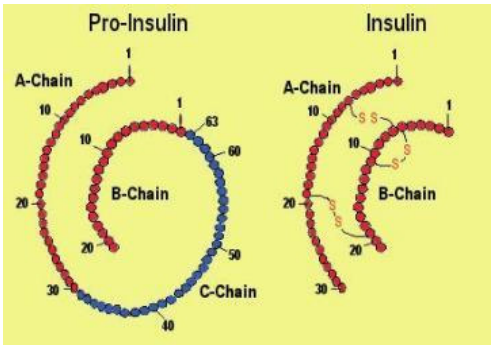


Figura 2. Proceso de formación de la insulina endógena

Las cadenas de insulina humana han sido preservadas de cambios evolutivos substanciales, presentando similitudes con insulinas de otras especies. Dentro de los vertebrados, la insulina bovina difiere únicamente en 3 residuos de aminoácidos, mientras que la insulina porcina presenta diferencia en uno (ver tabla 1). El cambio de aminoácidos provoca cambios de hidrofilia/hidrofobia causando variaciones en la solubilidad de la insulina.

Tabla 1: Aminoácidos de insulina humana, porcina y bovina [3]

Insulina	**A8**	**A10**	**B30**
Humana	Treonina	Isoleucina	Treonina
Porcina	Treonina	Isoleucina	Alanina
Bovina	Alanina	Valina	Alanina

A pesar de las diferencias en los residuos aminoacídicos de las diferentes especies, la actividad biológica de la insulina depende de la estructura helicoidal formada entre los aminoácidos A12-18, que se mantuvo entre las diferentes especies, mostrando prácticamente la misma actividad biológica. Por esta razón, durante años las insulinas de origen animal se emplearon como tratamiento de la DM. Sin embargo, actualmente las insulinas de origen bovino y porcino han desaparecido del mercado, desplazadas por las insulinas humanas recombinantes obtenidas por ingeniería genética [4].

Es importante conocer la conformación de la insulina porque es la responsable de la distribución del péptido en sangre y tejidos tras la administración, por tanto, se puede modular la duración del efecto variando la estructura tridimensional de la insulina. Los monómeros y los dímeros son estructuras que difunden fácilmente logrando un efecto rápido, mientras que los hexámeros tienen que disociarse previamente, logrando un comienzo de la acción más lento y prolongado.

En el caso de la insulina, los cambios de pH (*p.I* ~5.4) [5] además de modificar la carga neta, alteran la conformación del péptido. Del mismo modo que la concentración de la insulina también condiciona su disposición en el espacio. Por ello, cuando el pH está próximo a 7.4 o las concentraciones son muy elevadas, o bien, en presencia de iones Zn, la estructura de la insulina es hexamérica. Mientras que con valores de pH 3 la conformación es tetramérica, y en forma de dímero para valores muy ácidos (pH 1.6). Sin embargo, la estructura dimérica se mantiene para valores de pH entre 2-8 si la concentración

es ≤ 1.5 mg/dl, aunque para concentraciones > 1.5mg/dl la estructura es tetramérica [6, 7].

La semivida plasmática ($t_{1/2}$) del monómero de insulina varía entre 5-6 minutos. Su metabolización se produce en el hígado, riñón y músculo, aunque la mayor cantidad se lleva a cabo en el hígado (50%) y su excreción es renal. Sin embargo, la $t_{1/2}$ del péptido C es notablemente más larga (30 min). Ambos son secretados en concentraciones equimolares, por tanto, se puede inferir la cantidad de insulina liberada por el páncreas determinando la concentración de péptido C.

La corta vida plasmática de la insulina obligó al desarrollo de estrategias capaces de prolongar su acción. Entre ellas, unir la insulina a proteínas (p.e. protamina) o conseguir una cristalización controlada mediante adición de Zn (estructura hexamérica) y/o control del pH. Por el contrario, para conseguir insulinas de acción rápida se intercambian la posición de 2 aminoácidos que son claves para la formación de hexámeros obteniendo monómeros de insulina de acción rápida.

A pesar de no existir un acuerdo consensuado, las insulinas se clasifican según el comienzo y la duración del efecto en varios tipos [8] (Figura 3).

- Acción rápida son soluciones para administración intravenosa, mientras que las suspensiones son exclusivamente de aplicación subcutánea. El comienzo de la acción se produce a los 15 min y el efecto dura entre 3-5 h.

- Acción corta (glulisina, lispro, normal, aspart): el comienzo de la acción oscila entre 0.5-1 h con una duración entre 5- 8 h.
- Acción intermedias (lispro-protamina y NPH): la acción comienza entre 1-3 h y la duración será entre 18-24h.
- Acción lenta (detenir y glargina): la duración oscila entre 18-24 h pero el comienzo de la acción varía respecto a las insulinas de acción intermedia (6-8 h). Estas insulinas destacan porque producen una meseta en el perfil de liberación [3]. Este tipo de insulina reproduce la secreción basal de insulina del páncreas. Actualmente, se están llevando a cabo los estudios clínicos de fase 1 de la nueva generación de insulinas, que emplea un sistemas de nanopartículas formadas con poliaminoácidos auto-ensamblados, denominadas Medusa®. Los resultados ofrecen una substancial mejora sobre el control de la glucosa [9].

Con el fin de conseguir una acción prolongada con comienzo rápido, se desarrollaron las insulinas bifásicas. Se preparan mezclando dos tipos de insulinas, una de acción rápida (30%) con otra de acción intermedia (70%).

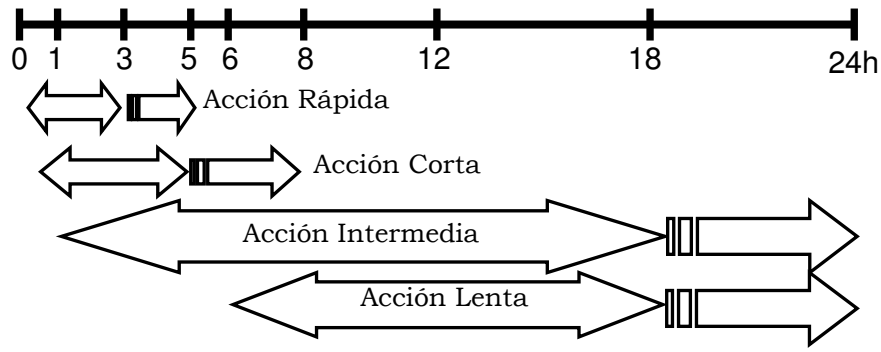
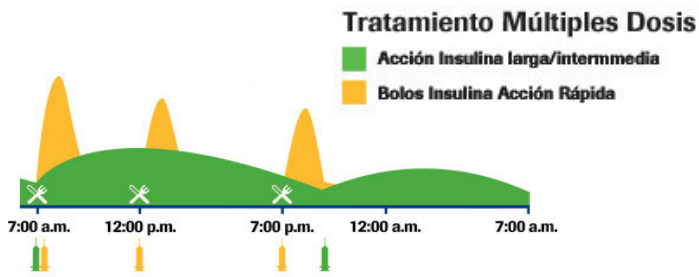


Figura 3: Clasificación de las insulinas

Para mantener adecuados niveles de glucosa es necesario que el aporte exógeno de insulina mimetice la secreción pancreática. Para ello, suspensiones de insulina con diferente acción se administran por vía subcutánea varias veces al día mediante **bolígrafos de insulina.** Estas jeringuillas especiales en forma de pluma estilográfica son el método más utilizado. Habitualmente se administra una insulina de acción intermedia o larga que intenta mimetizar la secreción basal del páncreas, combinada con la administración de insulinas de acción rápida o corta junto a las comidas principales (Figura 4a). Se estima que un paciente diabético se aplica ~80.000 inyecciones a lo largo de su vida (75 años). Otra forma de administración subcutánea menos común son las **bombas de insulina** que administran de forma continua una pequeña dosis de insulina de acción rápida (infusión basal) imitando la función del páncreas. Tras la ingesta de alimentos, la bomba se programa para infundir una dosis extra de insulina. Esta forma de infusión continua de insulina reduce el riesgo de hipoglucemia en un 70% (Figura 4b).

a)



b)

Figura 4: Perfiles de insulina suministrados por a) repetidas inyecciones subcutáneas b) el páncreas en individuos no diabéticos o por la bomba de insulina

Sin embargo, el tratamiento con bomba de insulina se asocia a numerosos inconvenientes: riesgo de infecciones, problemas en la higiene personal, problemas asociados al equipo y el coste del equipo, entre otros. Por ello, no está considerado el tratamiento de elección, y su uso se restringe a pacientes muy motivados (Figura 5).

Figura 5. Dispositivo de una bomba de inyección.

2.1.1. Detección de insulina

Para determinar la concentración de insulina *in vitro* se emplean métodos directos cromatográficos (HPLC) [10] o colorimétricos (mBCA) [11]. Sin embargo, la detección de insulina en muestras de plasma puede realizarse a través de métodos directos o indirectos. Con la determinación directa se deduce la cantidad total de insulina (endógena y/o exógena) mediante radio inmuno ensayo (RIA) [12]. Mientras que con los métodos indirectos se pueden determinar variaciones en la concentración de péptido C (procedimiento válido exclusivamente para determinaciones de insulina endógena, RIA) [13].

2.2. Exenatida (Byetta®) / Exendina-4

La Exenatida, producto sintético (AC2993) derivado de la Exendina-4 (Ex4), recientemente aprobada en Europa (2007) y USA (2005) para el tratamiento de la diabetes tipo 2 por vía subcutánea. Se administra en combinación con otros antidiabéticos orales (metformina o sulfonilureas) en pacientes en los que la terapia convencional no consigue normalizar la glicemia [14]. Generalmente, la dosis de exenatida para el tratamiento de la diabetes varía entre 5-10µg/12h por vía subcutánea [15]. Su biodisponibilidad subcutánea oscila entre 65-75% [16]. La Ex4 es el péptido natural, originalmente extraído de las secreciones salivares del lagarto *Heloderma suspectum*, comúnmente llamado monstruo de Gila (Figura 6).

Figura 6: *Heloderma suspectum*

La Ex4 es una cadena peptídica de 39 amino ácidos (4186.6 Da) [17] posee 53% de homología estructural con la hormona humana endógena GLP-1 (glucagon like peptide-1) (Figura 7), por tanto, actúa como agonista de los receptores pancreáticos del GLP-1, mimetizando sus acciones insulinotrópicas.

GLP-1: **H** A **E G T F T S D** V **S** S Y L **E** G Q **A** A K E **F I** A **W L** V K **G** R

EX-4: **H** G **E G T F T S D** L **S** K Q M **E** E E **A** V R L **F I** E **W L** K N **G** G P S S G A P P P S

Figura 7: Representación de las secuencias de aminoácidos del GLP-1 y exendina-4. Los residuos comunes aparecen resaltados en rojo.

Entre sus diversas acciones, la Ex4 estimula la secreción de insulina e inhibe la secreción de glucagón (principal hormona hiperglucemiante), ambos efectos son dependientes de los niveles de glucosa. Retrasa el vaciamiento gástrico que paradójicamente está incrementado en pacientes diabéticos y reduce la ingesta. Además, suprime la apoptosis y promueve la proliferación y neogénesis de las células β pancreáticas en modelos *in vitro* e *in vivo* [18]. Recientemente, un estudio en ratones *ob/ob* se ha demostrado que Ex4 previene la hipertensión arterial [19]. A diferencia de la insulina, presenta un riesgo de hipoglucemia muy reducido ya que sus acciones principales dependen de los niveles circulantes de glucosa. El riesgo de hipoglucemia se asocia con frecuencia a la co-administración con sulfonilureas [20].

No existen suficientes estudios sobre la biodisponibilidad oral de la Ex4 (B = 0.01%) [21], por ello se cree que el efecto de primer paso es el mismo que el GLP-1, sufriendo una reducción del 25% en el paso hepático [20]. Del mismo modo que el GLP-1, la eliminación se

produce por vía renal hacia el filtrado glomerular [16]. Sin embargo, la semivida plasmática de la Ex4 es notablemente mayor que la semivida del GLP-1 (t ½ < 2 min), gracias a la diferencia estructural que le aporta mayor resistencia frente a la hidrólisis enzimática por Di-Peptidil-Peptidasa-IV (DPP-IV), prolongando su semivida entre 1 y 3 horas.

2.2.1. Detección de exendina-4

Hasta el momento la cuantificación i*n vitro* mediante cromatografía (HPLC) presentaba un inconveniente debido a su escasa sensibilidad (concentración mínima ~100µg) [17]. Para mejorar la sensibilidad del método se empleó un detector de fluorescencia (FLD, Agilent Technologies, Alemania), basándose en la presencia del aminoácido triptófano en la cadena de Ex4. Con ellos se alcanzaron valores en la detección de ~5µg.

La determinación *in vivo* puede ser directa**,** cuantificando los niveles de Ex4 en sangre por RIA, o bien indirecta, a través de la variación en los niveles de insulina, péptido C o glucosa. Preferiblemente se emplea la determinación de glucosa por ser la más rápida y económica. Sin embargo, se debe tener en cuenta que la Ex4 en función de los niveles de glucosa del organismo produce 2 efectos opuestos sobre la glicemia:

- **Modelo diabético** (niveles de glucosa basal elevados): como consecuencia de la activación del receptor GLP-1 pancreático, la Ex4 normaliza los niveles de glucosa pre- y postprandial en humanos y animales diabéticos a largo plazo. Reduce la hemoglobina glicosilada (HbA1) en 0.8-1.0 y el peso corporal 1-3kg [18].

- **Modelo no diabético** (niveles de glucosa basal normales): incremento a corto plazo de la glucemia debido a la activación de un receptor diferente al receptor del GLP-1, que estimula el sistema nervioso simpático y la glándula adrenal. Este aumento se desarrolla tras 20 minutos y se prolonga durante 2 h tras administración intravenosa o intraperitoneal. Hasta el momento, este efecto se ha comprobado únicamente en ratas y puede ser utilizado como parámetro de respuesta de la acción sistémica de la Ex4 [22].

3. LIMITACIONES DE LA TERAPIA ACTUAL CON INSULINA Y EXENATIDA

La administración de **insulina** exógena es el tratamiento clave para diabetes tipo 1 y, para algunos casos de diabetes tipo 2. Sin embargo, el estrecho margen terapéutico y su corta semivida no permiten reproducir fielmente la secreción endógena mediante administración intravenosa y/o subcutánea, provocando riesgo de hipoglucemia, hiperinsulinemia periférica y ganancia de peso.

Otro inconveniente de las insulinas subcutáneas de efecto retardado es que presentan variabilidad en el perfil de acción entre diferentes personas, o incluso, dentro de la misma persona en distintos momentos [23]. A estas desventajas se suma la disconformidad del paciente por el uso de múltiples inyecciones molestas y dolorosas [24], riesgos de infección, inhabilidad del manejo de insulinas y deposiciones locales de insulina que provocan deposición de grasa e hipertrofia local que reduce la sensibilidad [25]

A diferencia de la insulina, con la administración subcutánea **exenatida** (exclusivamente para tratar la diabetes tipo 2) se reduce el número de efectos secundarios, gracias a que el mecanismo de acción de la exenatida resulta dependiente de los niveles de glucosa. Sin embargo, mantiene todos los inconvenientes asociados al uso de inyecciones repetidas.

4. NUEVAS DIANAS FARMACOLÓGICAS PARA EL TRATAMIENTO LA DIABETES MELLITUS.

Pese al avance científico en la búsqueda de nuevas terapias, no existe todavía en el mercado un tratamiento que consiga reproducir los niveles fisiológicos de insulina en paciente diabéticos. La investigación progresa en esta dirección gracias a la evaluación de los estudios en animales de experimentación, en los cuales se emplean modelos sanos, o bien, diabéticos. En general, los nuevos tratamientos con insulina emplean animales diabéticos para evaluar la eficacia. Al igual que en humanos, los animales pueden desarrollar la DM tipo 1 o tipo 2. Principalmente, nos centraremos en el desarrollo de modelos de DM tipo 1 caracterizada por la ausencia de insulina y, por tanto, los descensos en los niveles de glucosa son atribuidos a la administración de insulina exógena. Dentro de los biomodelos diabéticos tipo 1 se pueden clasificar según su mecanismo de desarrollo de la enfermedad en prototipos espontáneos o inducidos experimentalmente.

Los *biomodelos espontáneos* son animales que desarrollan la enfermedad por factores genéticos e inmunológicos. Sin embargo, estos modelos no son lo suficientemente comparables para el estudio de la diabetes tipo 1 en seres humanos.

Los *biomodelos inducidos* se consiguen a través de diversos métodos, entre los cuales está la manipulación genética (genes knock-out), el empleo de agentes infecciosos (virus), la extracción quirúrgica (animales pancreatectomizados) o la administración de sustancias químicas. La técnica más empleada es la administración agentes químicos como la **streptozotocina (STZ).** La STZ es un antibiótico de amplio espectro citotóxico (aprobado para el cáncer de páncreas) que administrado en dosis altas simples (50-70 mg/kg) por la vía intravenosa o intraperitoneal, causa la muerte de las células β en un plazo de 24h. También puede administrarse mediante dosis bajas subdiabetogénicas (5mg/kg) repetidas. Se administra a ratas, ratones, perros, hamsters, ovejas y monos. Además de la STZ, se emplean otras sustancias β destructoras como alloxan, clorotozin, vacor y la ciproheptadina, pero con un efecto menor [26].

4.1. Administración de insulina por vía transmucosal

Los investigadores, desde el descubrimiento de la insulina en 1921, han dedicado numerosos esfuerzos a buscar una forma de administración fácil, indolora y efectiva. En 2006, la comercialización de Exubera® (insulina humana inhalada) abrió una puerta en la mejora del tratamiento de los enfermos diabéticos insulino-dependientes, aunque debía ser co-administrada con insulinas subcutáneas u otros antidiabéticos orales. Sin embargo, 2 años más tarde, su posterior retirada del mercado puso de manifiesto los múltiples inconvenientes que se deben superar para conseguir un tratamiento eficaz de insulina por vías no invasivas. Actualmente, varias compañías están realizando estudios clínicos para lanzar al mercado nuevas propuestas para la administración de insulina. Entre ellas destacan, comprimidos de insulina conjugada con oligómeros anfifílicos para la absorción entérica (H1M2, Nobex Corporation),

insulina líquida en spray para nebulización bucal Oral-lyn® (Generex Corporation) y un parche adhesivo que libera insulina basal (Altea Development Corporation) [27].

El éxito de una formulación alternativa a la insulina subcutánea requiere, entre otros atributos, la plena optimización de un sistema de liberación, el cual debe poseer buena eficacia de encapsulación, prevenir de la degradación enzimática, conseguir permeabilidad epitelial, controlar la liberación del péptido y conservar la bioactividad durante la formulación y la liberación de la insulina en el lugar diana.

Como alternativa a la administración de insulina por vías invasivas, se han desarrollado sistemas que capaces transportar la insulina a través de la mucosa pulmonar [28, 29], bucal [30], ocular [31] o transdermal [32] demostrando ser eficaces para disminuir los niveles de glucosa. Asimismo, se han testado otros sistemas por vías menos convencionales; colónica [33, 34], rectal [35, 36], vaginal [37, 38] y uterina [39]. Sin embargo, la vía nasal y oral son las que gozan de mayor aceptación por el paciente, asegurando un mayor cumplimiento de la terapia. Entre las posibles estrategias para obtener una formulación con las características anteriores, las nanopartículas son potenciales candidatos proporcionando un medio estable y biocompatible a la insulina que garantiza su actividad biológica. En comparación con otros transportadores, como las micro- y nano- emulsiones, liposomas o micropartículas, las nanopartículas poseen mayor estabilidad en fluidos biológicos.

4.1.1. Nasal

En la actualidad, prácticamente todas las vías de administración han sido exploradas. Sin embargo, la vía oral y nasal son las que han

demostrado poseer gran potencial para el tratamiento de la diabetes. Múltiples estudios publicados por vía oral consiguieron disminuir los niveles de glucosa, incluso durante largos periodos de tiempo, como veremos en detalle más adelante. En cambio, el activo aclaramiento mucociliar de la vía nasal, evita el contacto prolongado del fármaco con el epitelio de absorción, impidiendo el mantenimiento de la concentración plasmática de insulina durante períodos prolongados. En general, los estudios con insulina por vía nasal presentan un perfil similar al obtenido tras la administración de insulina por vía intravenosa (acción rápida). Se caracterizan por una marcada reducción en los niveles de glucosa tras la administración que se recupera de forma rápida en las horas siguientes. Particularmente, en los estudios desarrollados con micro- y nanopartículas para liberación intranasal de insulina el descenso máximo de glucosa se produce entre 0.5-2 h y en ningún caso se mantiene después de 4-5 h de la administración (ver tabla 2). Consecuentemente, las insulinas diseñadas para administración nasal solo podrían sustituir a las insulinas de acción rápida, siendo su tratamiento complementado con otra insulina de acción más prolongada por vía subcutánea. Por esta razón y otras que se discuten más adelante, nos centraremos en la vía oral como objetivo para desarrollar un sistema de liberación de insulina capaz de simular la secreción endógena.

Tabla 2: Principales características de las propuestas de las formulaciones de nanopartículas para la administración por vía nasal de insulina testadas en animales[a].

Sistema[b]	Modelo animal	n	Ins IU/kg	T_{min} (h)	C_{min} (% glucosa basal)	Efecto-tiempo	Ref.
CS/TPP/Alg NP	Conejo sano	6	5	1	70	≤ 80%-0.5-2h	[40]
CS-NAC/TPP NP	Rata sana anestesiad	3-4	10	0.5	60	≤ 80%-0.25-2h	[41]
CS/CD/TPP NP	Conejo sano	6	5	1	65	≤ 80%-0.7-1.5	[42]
PEG-g-CS/TPP NP	Conejo sano	6	5	~1 3	PEG3gCS64:~40 PEG7gCS64;~40	≤ 75%-0.5-4h ≤ 75%-0.5-4h	[43]
EE-NP	Rata diabética	5	10	1	30	≤ 60%-0.5-2h	[44]
CS-TBA MP	Rata Sana	3	~40	0.5	30	≤ 60%-0.5-2h	[10]
CS-Gl/TPP NP	Rata (anestesiada)Oveja	5 6	~2 ~1.6	~2 0.5	~50 ~70	-	[45]
CS/TPP NP	Conejo sano	6	5	1	50	≤ 80%-0.5-2h	[11]

[a]Datos actualizados hasta Abril 2010 (orden cronológico)
[b]Abreviaturas: **CS**: Quitosano; **TPP**: Tripolifosfato; **Alg**: alginato; **NP**: nanopartículas, **CD**: ciclodextrina; **NAC**: N-acetil.l-Cisteina; **PEG**: polietilenglicol; **EE**: Epiclorohidrin emulsión; **CS-TBA**: quitosano tiolado; **MP**: Micropartículas; **CS-Gl**: glutamato de quitosano

4.1.2. Oral

Convencionalmente, la vía oral es la mejor aceptada por el paciente asegurando una mejora en el cumplimiento de la terapia, y consecuentemente, una mayor eficacia del tratamiento. Entre las ventajas que supondría la administración oral de insulina, la mayor está relacionada con su acción, ya que sería la única vía que conseguiría mimetizar la liberación de insulina tras la ingesta de alimentos de forma fisiológica y mejorar la homeostasis. La administración de insulina por vía oral teóricamente podría

reproducir la respuesta pancreática más fielmente que la administración parenteral. Tras la absorción intestinal, la insulina accedería directamente por la vena porta hepática suprimiendo la producción de glucosa hepática, y consecuentemente, reduciría los niveles de glucosa. Por tanto, supondría alcanzar directamente mayores concentraciones de insulina en el hígado reduciendo la insulinemia periférica. Con la terapia actual por vía subcutánea, sólo el 20% de la insulina administrada alcanza el hígado, demandando mayores cantidades de insulina para generar un efecto biológico que podrían provocar hiperinsulinemia periférica y el riesgo de hipoglucemia [7]. Otra ventaja estaría relacionado con el tiempo vida medio del péptido, ya que la degradación de la insulina parece reducirse tras la administración oral [3]

Sin embargo, el tracto gastrointestinal posee múltiples barreras fisiológicas y morfológicas para la vehiculización eficaz de insulina. Cuando la insulina atraviesa el estómago debe superar el medio ácido con la acción enzimática de la pepsina, posteriormente en el lumen del duodeno están las enzimas pancreáticas: tripsina, quimotripsina y carbopeptidasas que unidas con las endopeptidasas del borde del cepillo constituyen una barrera eficaz frente proteínas y péptidos. A esta barrera enzimática hay que sumarle, las bacterias de la flora intestinal, la capa de mucus y las células del epitelio intestinal. Aunque la insulina consiguiera alcanzar el lado apical y/o lateral de los enterocitos y se absorbiera a través de la mucosa, alcanzaría el medio intracelular de los enterocitos donde debe resistir la degradación de los lisosomas y la enzima degradadora de insulina (IDE) [7]. Otro factor limitante es la naturaleza hidrofílica de la insulina que limita el transporte transcelular por difusión (Fig. 8) [46]. Todas estas barreras contribuyen a que la biodisponibilidad de

la insulina por vía oral sea muy limitada (1-2 %) [47]. Por ello, con el fin de mejorar la biodisponibilidad se han propuesto numerosas estrategias capaces de proteger el péptido de la degradación

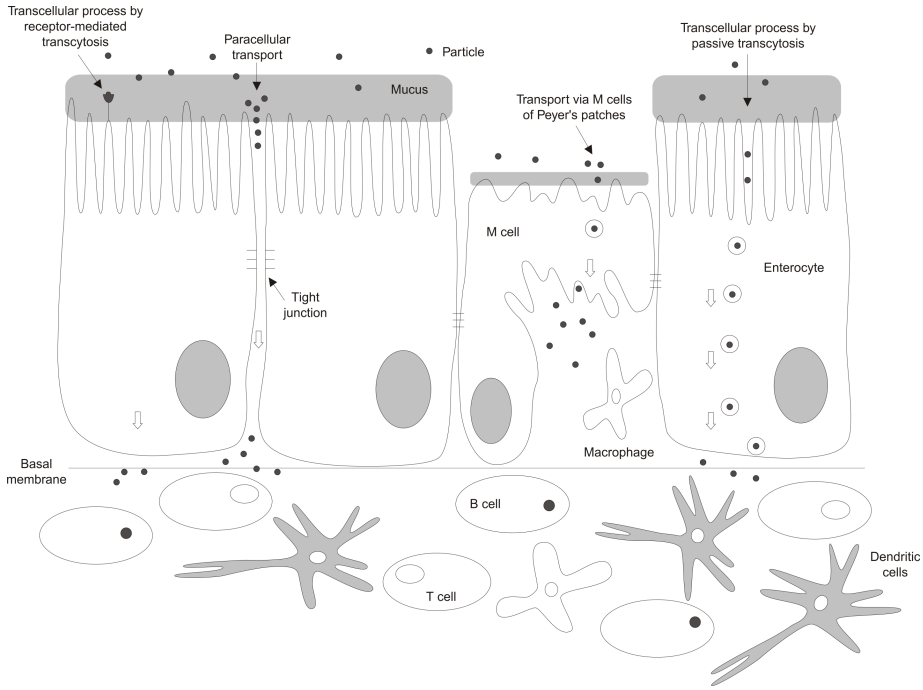


Figura 8: Mecanismos de transporte de sustancias a través del epitelio intestinal[46].

i.-Promotores de la absorción incrementan el transporte paracelular [48] y transcelular a través de diferentes mecanismos, incluyendo cambios en la fluidez de la membrana, disminuyen la viscosidad del mucus y abriendo las uniones íntimas. Los ejemplos comunes de promotores no específicos son las sales biliares [49, 50], n-lauryl-β-D-maltopyranoside [51], EDTA [51] or EPA and DHA [52].

ii.-Inhibidores enzimáticos disminuyen la velocidad de degradación de la insulina por trypsina, α-quimotrypsina, y elastasa, y en menor grado por enzimas de la membrana. Los inhibidores de polímeros conjugados [53, 54] y los derivados de ovomucoides [55]

iii.- Sistemas poliméricos mucoadhesivos incrementan el tiempo de residencia en el lugar de absorción, evitando la dilución o la degradación en el fluido del lumen. Con polímeros hidrofílicos, como poliacrilatos [56] derivados de celulosa [57] y quitosano [10, 58] se han desarrollado sistemas de liberación para aumentar la absorción del fármaco. Los polímeros tiolados (tiómeros) [59] catiónicos [60] y aniónicos [61] mediante enlaces intra- e intermoleculares prolongan el tiempo de descomposición controlando la liberación del péptido.

iv.- Sistemas de transporte nanoparticulados

Se han desarrollado numerosos sistemas coloidales para mejorar la liberación intestinal de péptidos: nano- y micropartículas poliméricas, nanocápsulas, microemulsiones, liposomas y micelas. La absorción por vía oral de péptidos asociadas a sistemas particulados puede ser mediante transporte paracelular, transcelular (endocitosis), o bien, a través de las células M, tejido linfoide asociado al intestino (GALT) (< 10 μm) (Fig. 8) [48].

Para este trabajo nos centraremos en sistemas nanométricos testados *in vivo* en distintos modelos animales para liberación de insulina por vía oral. Los estudios han sido divididos en varios grupos; polímeros naturales, polímeros sintéticos y liposomas. La Tabla 3 muestra los estudios que utilizan un polímero naturale, quitosano (CS). El CS se utilizó por su efecto mucoadhesivo como estrategia para incrementar el tiempo de residencia del sistema en el epitelio intestinal,

intensificando el contacto con el mucus y por tanto, reduciendo la degradación entre la liberación del fármaco y la absorción a través del epitelio. Por otra parte, el CS favorece la separación temporal de las uniones íntimas entre células (tight juntions) del epitelio intestinal, promueviendo el paso de sustancias a través de la membrana. Por estas razones principalmente, el CS fue seleccionado como base para el desarrollo de numerosas formulaciones de administración oral. En general, los nanosistemas de CS muestran perfiles de liberación de insulina que mantienen la reducción de los niveles plasmáticos de glucosa (≤ 50%) durante periodos superiores a 5 h (tiempo máximo alcanzado por vía nasal). Varios de los sistemas testados alcanzaron reducciones en la glucemia durante 24h. Entre ellos destaca, los nanocomplejos de liposoma-CS que decrecen los niveles de glucosa un 35% respecto del valor basal durante 2-24h postadministración en la rata [62] y las nanopartículas de CS/TPP (tripolifosfato) con 0.5-10% poloxamer 188 [70c] reducen los niveles durante 30 h en ratas diabéticas. Mientras que las nanopartículas que combinan dos polímeros naturales, alginato o dextrano con el quitosano [63, 64], necesitaron mayor dosis de insulina para reducir la glucemia durante ≤ 24 h. Por otra parte, los estudios que ensayaron nanopartículas con CS modificados [65, 66], mostraron reducciones de la glucemia de duración inferior a 12 h. Al igual que los complejos insulina-CS [67] o las nanopartículas de CS combinadas con ácido glutámico [68] o con metacrilatos [69].

Tabla 3: Sistemas nanométricos a base de un polímero natural, quitosano (CS), para la liberación de insulina por vía oral[a]

Sistema[b]	Modelo animal	n	Ins UI/kg	T_{min} (h)	C_{min}[c]	Efecto-duración	Ref.
L/CS complexes	Rata	7	10	24	55	≤ 65%-2-24h	[62]
TMC-Cys NP TMC NP	Rata	4	50	3 4	65 78	≤ 85% de 2-8h ≤ 85% de 2-5h	[66]
LSC/TPP NP	Rata diabética	4	60	6	65	≤ 80% de 3-7h	[65]
Ins/CS complex	Rata diabética	10	50	5	60	≤ 80% de 4-12h	[67]
Alginate/CS NP	Rata diabética	6	50 100	14	>60	≤ 80% de 2-18h	[64]
DS/CS NP	Rata diabética	6	100	14	65	< 80% de 4-24h	[63]
CS/γ-PGA NP	Rata diabética	6	30	5-10	50	≤ 50% de 5-10h	[68]
CS reduced gold NP	Rata diabética	6	50	2	70	≤ 80% de 1-3h	[70a]
CS/TMAEMC NP	Rata	6	100	8	65%	≤ 80% de 6-12h	[69]
CS/TPP NP 0.1% Pol 188	Rata diabética	8	14 21	10	60 40	≤50% de 10-24 ≤ 70% de 10-24	[70b]
CS/TPP NP 0.5-10% Pol 188	Rata diabética	8	10	15	40	≤50% de 15-32	[70c]

[a]Datos actualizados hasta Abril 2010 (orden cronológico)
[b]Abreviaturas: **Ins**:Insulina; **NP**:nanopartículas; **NC**:nanocápsulas; **CS:**quitosano; **L/CS**:nanocomplejos liposoma-quitosano; **TMC**:Trimetilquitosano; **Cys** Cisteina; **LSC**: Lauril Succinil Quitosano; **DS**:Dextrano Sulfato; **γ-PGA**:Poli(γ-glutamic acid); **TMAEMC:***N*-trimetilaminoetil metacrilato cloridrato; **TPP**: Tripolifosfato; **Pol 188**:poloxámero 188
[c]Concentración mínima de glucosa respecto del valor basal

Sin embargo, las nanopartículas de dextrano (DS) cubiertas con vitamina B_{12} (Tabla 4), utilizan el transportador de vitaminas para la absorción a través del tracto gastrointestinal prolongando la reducción de la glucemia durante 54 h [71, 72].

Tabla 4: Sistemas nanométricos a base de un polímero natural, dextrano (DS), para la liberación de insulina por vía oral[a]

Sistema[b]	**Modelo animal**	**n**	**Ins** UI/kg	**T_{min}** (h)	**C_{min}** (%)[c]	**Efecto-duración**	**Ref.**
VB_{12}/DS-NP conjugates	Rata diabética	6	20	M31: 5 M41:3-4 M42: 12	>30 30 >30	<70% de 24-54h <60% de 10-54h <60% de 24-54h (2ª fase)	[72]
VB12/DS NP conjugates	Rata diabética	4	20	5	>30	60% de 24-54h (2ªfase)	[71]

[a] Datos actualizados hasta Abril 2010
[b] Abreviaturas:**VB_{12}:** vitamina B_{12};
[c] Concentración mínima de glucosa respecto del valor basal

En la tabla 5 se resumen las principales características de los nanosistemas formados con polímeros sintéticos para liberación de insulina por vía oral. En general, los sistemas presentan tiempos de hipoglucemia superiores a 5 h, excepto un sistema con PEG (polietilenglicol) [73] y las nanopartículas de PLGA vehiculizadas con baja dosis de insulina [74b] revelaron descensos de glucemia durante tiempos inferiores a 5 h. Mientras que las partículas de PLGA con mayor carga de insulina [75] o combinadas con lípidos [75b] incrementaron el tiempo hasta las 24 h. Por otra parte, los nanosistemas formados con acrilatos mostraron perfiles superiores a 12 h [76, 77], alcanzando en algunos casos las 36 h [78] y días [77, 81]. De entre todos los polímeros sintéticos estudiados destaca el sistema formado con plurónico y ácido poliláctico que reduce los niveles de glucosa entre 5-24 h postadministración hasta un 75% respecto al valor basal [79].

Tabla 5: Sistemas nanométricos a base de polímeros sintéticos para la liberación de insulina por vía oral[a].

Sistema[b]	**Modelo animal**	**n**	**Ins** IU/kg	**T_{min}** (h)	**C_{min}** (% glucosa basal)	**Efecto-tiempo**	**Ref.**
PECA NP	Rata diabética	6	100	12-36	60	≤80% de 10-36	[78]
Tris/PC/PEG NP	Ratón diabética	10	29	3	55	≤75% de 3-4h	[73]
			72		45	≤75% de 2-5h	
PLGA NP PLGA/Hp55 NP	Rata diabética	6	20	2	20	≤80% de 2-8h	[73 b]
PLA-pluronic-PLA vesicles	Ratón diabético	5	50	6	25	≤25% de 5-23h	[79]
PLGA NP	Rata	5	50	10	60	≤75% de 4-24h	[75]
Ins-SPC complex /PLGA NP	Rata diabética	6	20	12	50	≤80% de 2-24	[75 b]
PBA NP	Rata diabética	5	10	<0.5	30	≤80% 1h	[50]
P(AA-g-PEG) NP	Rata diabética	4	50	8	80	-	[74]
PBCA NC	Rata diabética	5	50	7	70	≤80% de 6-16h	[76]
Ins-transferrin conjugates	Rata diabética	3-4	80	11	25	≤70% de 7-11h	[80]
PLGA NP	Rata	5	20	1	60	≤70%de 1-4h	[74 b]
PACA NC	Rata diabética	8	100	2	50	≤80% de 2-13d	[77]
PACA NC	Rata diabética	8	50	2	>50	≤50% de 2-13d	[81]

[a]Datos actualizados a diciembre 2009 (orden cronológico).
[b]Abreviaturas: **Ins**: Insulina; **NP**: nanopartículas; **NC**: nanocápsulas **PLGA**: poly(lactide-co-glycolide); **PECA:** Polietilcianoacrilato; **Tris/PC/PEG:** Tristearina/ Fosfatydilcolina/ Polietilenglicol; **SO**: Sodio Oleato; **M3,M4**:Amino alquil derivados de la vitamina B12; **Hp-55**: hypromelosa Ftalato, HPMCP-55; **SPC**: Fosfatidilcolina de soja **PBA**: poli-isobutil-acrilato; **P(AA-g-PEG):** Poli (acril acid-g-polietilenglicol); **PBCA:** poli (*iso*-butil cianoacrilato); **PBCA**: Poli(isobutilcianoacrilato); **PACA:** Polialquilcianoacrilato

En general, la mayoría de los sistemas de liberación de insulina por vía oral propuestos hasta el momento presentan niveles de reducción de la glucemia durante periodos prolongados (≥ 12 h) [62- 64, 67, 69,

70b-72, 75-78, 81], incluso en casos alcanzando varios días [71, 72, 77, 81]. Sin embargo, el mayor descenso de glucosa producido por estos sistemas se observa entre 5-14 horas tras la administración, que induciría un alto riesgo de hipoglucemia si este descenso no coincide con el horario de la ingesta de alimento.

La Tabla 6 muestra los ensayos de liposomas por vía oral en diferentes modelos animales. En general, los porcentajes de reducción de la glucemia son bastante limitados y no superan las 6h como sucede en los estudios por vía nasal.

Tabla 6: Sistemas liposomiales y micelares nanométricos para la liberación de insulina por vía oral[a] .

Sistema	**Modelo animal**	**n**	**Ins** IU/kg	**T_{min}** (h)	**C_{min}** (%)[b]	**Efecto-tiempo**	**Ref.**
ARM[c]	Rata diabética	5	25	4	70	≤80% de 4-24h	[83]
liposomas[d]	Rata diabética	7	12	1.7-5	~50	50% de 1.7-5h	[84]
Mucin- & PEG-liposomas	Rata	4	100	3	90 95	2-4h	[85]
liposomas[e]	Conejo diabético	6	100	2-4	40	<60% de 1-6h	[86]
liposomas[f]	Rata diabética	-	100	2	50	4h↑	[87]
liposomas[g]	Rata diabética	5	30 70	3	60 40	-	[88]

[a] Datos actualizados hasta Abril 2010
[b] Concentración mínima de glucosa respecto del valor basal
[c] Anhydrous reverse Michelle (micela reversa anhidra)
[d]fosfatiletanolamina
[e]lecitina-colesterol
[f]fosfatidilcolina-colesterol
[g] fosfatidilcolina-colesterol-dicetil fosfato

4.2.- Administración de Exenatida/Exendina-4.

A diferencia de otros fármacos, la biodisponibilidad por diferentes vías de administración de la exenatida es muy baja. Por vía subcutánea está aproximadamente entre 65-75 %, mientras que por vía intranasal e intraduodenal 1.68 % y ~0.005 %, respectivamente [6]. Aunque hasta el momento pocos estudios se han llevado a cabo para mejorar su administración. *Hargrove et al* sustituyeron un aminoácido de la cadena peptídica de la exenatida para incrementar su estabilidad plasmática *in vitro* [89], sin embargo *Son et al* consiguieron desarrollar un derivado de la exendina-4 de larga duración sin pérdida de actividad, ensamblando ácidos biliares en la cadena [90]. Únicamente, *Jin et al* desarrollaron un prototipo para vía oral. Ensamblaron vitamina H (biotina) en la secuencia aminoacídica del péptido, aumentando su estabilidad gastrointestinal, su biodisponibilidad (~4 %) y su efecto hipoglucemiante en ratones diabéticos [21]. Hasta el momento, no se ha desarrollado ningún sistema de liberación efectivo que permita la administración de Ex4 por vías no invasivas (p.e. oral o nasal).

A diferencia de la insulina, la Ex4 no requiere niveles constantes en sangre para mantener su acción. Por tanto, la administración de Ex4 por vía nasal se presenta como una alternativa adecuada por sus numerosos beneficios. El epitelio nasal presenta una gran superficie de absorción al estar cubierto de microvellosidades, está altamente vascularizado y los fármacos administrados pasan directamente a sangre evitando el efecto de primer paso por el filtro hepático. Además, permite la administración de bajas dosis de fármaco, el comienzo de la acción es muy rápido y es una vía fácilmente accesible.

A pesar del potencial de la vía nasal para la administración de fármacos, son numerosos los factores que limitan la absorción nasal. El aclaramiento mucociliar, la actividad enzimática y la barrera que constituyen el epitelio y la capa de mucus frente a moléculas de alto peso molecular e hidrofílicas. Por tanto, los grandes péptidos y proteínas consiguen atravesar el epitelio mediante endocitosis en bajas cantidades. Para solventar estas limitaciones de la vía nasal e incrementar la biodisponibilidad de péptidos como la insulina se han empleado múltiples estrategias: promotores de la absorción [91, 92], fosfolípidos [93], inhibidores enzimáticos [94], mucoadhesivos [45] y sistemas de liberación en polvo seco [95].

5. INTERÉS DE LA LECITINA Y EL QUITOSANO PARA LA FORMACIÓN DE NANOSISTEMAS.

Desde hace décadas, la lecitina y el quitosano han mostrado un mayor número de aplicaciones en biotecnología. El gran interés que suscitan ambos compuestos como vehículos para el transporte de moléculas activas proviene de sus propiedades. Ambas son de origen natural, biocompatibles y biodegradables y carecen de toxicidad. De aquí, que se hayan propuesto varios sistemas de transporte que combinan ambas moléculas usando diferentes métodos de preparación: micropartículas [96], nanopartículas [96b], nanocápsulas [97], complejos [67] y liposomas recubiertos de quitosano [98].

La **LECITINA (LEC)**, según la United States Pharmacopeia (USP), es un agente tensoactivo que está formado por una mezcla compleja de fosfolípidos insolubles en acetona, formada principalmente por fosfatidilcolina, fosfatidiletanolamina,

fosfatidilserina y fosfatidilinusitol (Fig. 9), combinadas en varias cantidades con otras sustancias, como los triglicéridos, ácidos grasos y en algunos casos con carbohidratos. Su composición varía dependiendo de la fuente y del grado de purificación, consecuentemente alterando también sus propiedades físicas. La lecitina es soluble en ácidos minerales y en hidrocarburos alifáticos y aromáticos. Sin embargo, es parcialmente soluble en alcoholes alifáticos y prácticamente insoluble en acetona y agua. Aunque la LEC es una sustancia lipófila, debido a la presencia de ácidos grasos esterificados de longitud variable, a su vez contiene un grupo fosfatidil de carácter polar. De ello, resulta su carácter anfifílico y su utilización extendida como emulsificante [99].

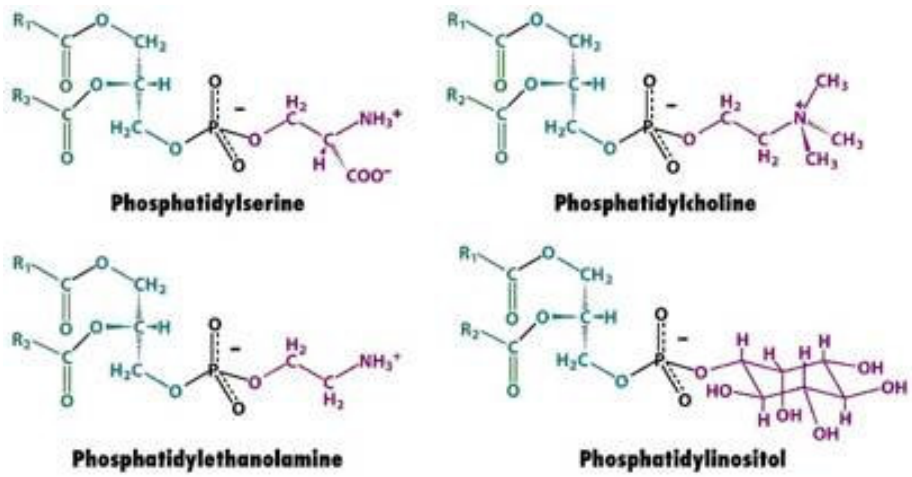


Figura 9: Estructura química de los principales componentes de la lecitina, donde R_1 y R_2 son cadenas de ácidos grasos.

Forma parte de todas las membranas celulares del organismo. Además, este compuesto tiene una gran variedad de aplicaciones

farmacéuticas (bases de supositorios; emulsificador, dispersante, y estabilizante en inyectables y en la nutrición parenteral; protector hepático en cirrosis alcohólicas), también se ha utilizado en cosmética y en productos alimentarios. Además, estudios recientes demuestran que la lisofosfatidilcolina de la LEC incrementa la captura de carotenoides en cultivo de células humanas Caco-2 y mejora la absorción oral de luteína en estudios in vivo [100, 101].

El **QUITOSANO (CS),** es un polisacárido pseudo-natural sintetizado mediante un proceso de desacetilación en medio básico a partir de la quitina, la cual está presente en el exoesqueleto de los crustáceos, insectos y algunos hongos. El CS es un co-polímero formado por una cadena lineal de unidades monoméricas de N-acetil-glucosamina y N-glucosamina distribuidas aleatoriamente. El grado de polimerización (G.P.) depende del número de residuos monoméricos que formen la cadena. El número de subunidades para clasificar el G.P en alto, medio o bajo es relativo. Por otra parte, el grado de acetilación (G.A) está definido por el porcentaje relativo de unidades de N-acetil-glucosamina presentes en el polímero. El G.A del CS puede oscilar entre ~1-~70 % (Figura 10). Sus condiciones de estabilidad dependen del G.A y son especialmente importantes para la producción de soluciones estables de quitosano, nanopartículas, macro- y microgeles y su administración.

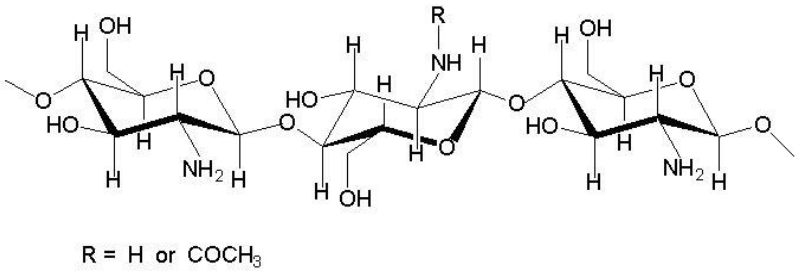


Figura 10: Estructura química del quitosano (CS)

A diferencia de la quitina, el quitosano forma sales con ácidos orgánicos (p.e. ácido glutámico) e inorgánicos, por tanto en disolución, los grupos amino están protonados y el polímero soluble está cargado positivamente. Las propiedades del CS, como su pK_0 y solubilidad, se pueden modificar variando su grado de acetilación y factores como el pH y la fuerza iónica [102].

El CS es un biomaterial especialmente interesante gracias a la combinación de sus propiedades mucoadhesiva y promotora de la absorción. Su adherencia, viene definida por el carácter catiónico de los residuos de glucosamina que le permiten interaccionar con los residuos negativos de ácido siálico presentes en el mucus. Además, a diferencia de los promotores de la absorción clásicos, permite la apertura de las uniones íntimas intercelulares ("tight juntions") de forma temporal, facilitando la absorción de numerosas moléculas a través de transporte paracelular y evitando lesiones en el epitelio [103, 104].

Habida cuenta de sus propiedades, el CS es un biopolímero muy útil para el desarrollo de materiales con diversas aplicaciones biomédicas. Se han propuesto múltiples sistemas para la liberación de fármacos compuestos por CS, entre ellos destacan las nanopartículas, los complejos preparados por diferentes técnicas, o

bien los sistemas coloidales recubiertos con CS (nanocápsulas o liposomas)

El CS puede ser degradado por numerosas enzimas quitosanasas, quitinasas, lisozima, o peptidasas [105-108], descomponiéndose en unidades de menor peso molecular como CS con menor G.P. y/u oligosacáridos de CS (COS, < 10kDa). Los oligosacáridos han sido ampliamente utilizados en alimentación y cosmética [21]. Recientes estudios demuestran que son capaces de reducir los niveles de glucosa tras administraciones prolongadas y en determinadas condiciones [109-112].

En este trabajo se desarrollarán sistemas nanométricos para la administración transmucosal de insulina y exendina-4 a base de lecitina y quitosano. El uso combinado de ambos materiales ha permitido aprovechar las ventajas que ofrecen desde el punto de vista físico-químico y biofarmacéutico.

PARTE I

Desarrollo de nanosistemas a base de lecitina para la administración de insulina por vía oral para el tratamiento de la diabetes mellitus tipo 1.

PARTE I

Antecedentes, hipótesis y objetivos

Artículo 1

Discusión

"Desarrollo de nanosistemas a base de lecitina para la administración de insulina por vía oral para el tratamiento de la diabetes tipo 1".

Antecedentes

1. El aporte exógeno de insulina por vía subcutánea a través de inyecciones combinadas, que constituye la base de la terapia actual para el tratamiento de la diabetes tipo 1, no consigue reproducir fielmente el perfil fisiológico requerido, provocando numerosos efectos secundarios. Actualmente, los nanosistemas propuestos para la liberación oral de insulina no presentan un perfil adecuado de liberación que permitan sustituir la administración subcutánea de las insulinas de acción corta.

2. Las interacciones electrostáticas (iónicas) entre ciertos componentes con cargas opuestas dan lugar a la formación de nanosistemas [113]. La insulina posee capacidad de la interacciona con fosfolípidos presentes en la lecitina [96, 114].

3. Sistemas coloidales desarrollados a partir lecitina [62, 75b, 85, 88] o quitosano [63, 64, 66] han permitido mejorar la absorción sistémica de insulina a través de la mucosa oral. Ambas moléculas unidas por interacción iónica forman, entre otros, nanopartículas que han demostrado encapsular eficazmente diferentes tipos de fármacos, entre ellos proteínas, Albúmina Sérica Bovina (BSA) [115] e incrementar la absorción de melatonina [116] y tamoxifeno [116b] en cultivos celulares de Caco-2.

Hipótesis

1. La administración oral podría reproducir la liberación de insulina producida por el páncreas más fielmente que la administración subcutánea. La insulina accederá al hígado directamente a través de la vena porta, suprimiendo la producción de glucosa hepática, reduciendo los riesgos de hiperinsulinemia periférica e hipoglucemia.

2. La interacción iónica entre cargas opuestas de la insulina y la lecitina producirá una estructura nanoscópica que potenciará la absorción de la insulina por vía oral.

3. La adicción de quitosano al sistema conferirá a la superficie de las nanopartículas la capacidad de interaccionar con la mucosa intestinal, a través de los residuos de ácido siálico presentes en la mucina. Esta interacción específica con las células epiteliales, promoverá la apertura de las uniones íntimas mejorando la absorción por vía oral

Objetivos

Teniendo en cuenta lo anterior, el objetivo general de la primera parte de este trabajo ha sido el desarrollo de nanosistemas capaces de incrementar la absorción de insulina tras administración por vía oral. El trabajo experimental llevado a cabo para alcanzar el objetivo global, se ha recogido en los apartados que se detallan a continuación:

1. Desarrollo y evaluación del comportamiento *in vitro* de las nanocomplejos de lecitina-insulina para la administración de insulina por vía oral. En este objetivo se desarrollará la optimización del proceso de preparación de los nanocomplejos, evaluando diferentes variables de formulación tecnológicas sobre las características finales de los sistemas.

2. Desarrollo y evaluación del comportamiento *in vitro* de las nanopartículas de lecitina y quitosano para la administración de insulina por vía oral. Se evaluará específicamente la incorporación del péptido al sistema, utilizando la posibilidad que ofrecen las proteínas de variar su carga según el pH del medio de disolución.

3. Estudio de los efectos *in vivo* de los nanocomplejos y las nanopartículas cargadas con insulina mediante administración oral en diferentes modelos experimentales diabéticos. Se pretende evaluar si los nanosistemas de lecitina pueden favorecer la absorción sistémica de insulina administrada por vía oral. Se analizarán la influencia de la presencia o ausencia de quitosano en el sistema sobre la respuesta in vivo en dos modelos de roedores diabéticos.

PARTE I

Antecedentes, hipótesis y objetivos

Artículo 1

Discusión

ARTÍCULO 1

Lecithin-based nanocarriers for oral delivery of insulin in diabetic rodents

Valle-Gallego A[1,2]., Goycoolea F.M[1]., Mallo F[2]. and Alonso M.J[1*]

[1]*Departement of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Santiago de Compostela, Spain*
[2]*Laboratory of Endocrinology, Faculty of Biology, University of Vigo. Spain*

*Corresponding author: Prof. Alonso MJ.
Tel: + 34 981 594627
Fax: + 34 981 547148
E-mail: mariaj.alonso@usc.es

Manuscript submitted to publication

Abstract

This work has addressed the development of two different types of nanostructures intended as nanocarriers for the effective oral delivery of insulin (INS). Namely, nanocomplexes of lecithin (LEC) and INS and nanoparticles comprising LEC and chitosan (CS) loaded with INS. Different formulations of LEC–INS nanocomplexes at varying mass ratios of both components were developed with adequate physical characteristics (Z-average size ~209 nm; ζ~-44mV); high production yield (~60%) and excellent INS association efficiency (A.E. ~97-100%). These systems also exhibited high *in vitro* stability in simulated gastrointestinal fluids with little release of INS in intestinal conditions (pH 6.8, <~20%). In turn, INS-loaded LEC–CS nanoparticles were also formulated at different LEC/CS mass ratios and optimal prototypes (average sizes of 212±9 nm, ζ~+32±1 mV) with high INS association efficiency (> 80%) were achieved. These systems exhibited adequate colloidal stability in simulated biological fluids. Both systems after i*n vivo* assays in STZ-induced diabetic rats revealed the capacity to improve the systemic absorption of INS peroral. The decrease of glycemic levels (~54%) was achieved 5 h after oral gavage of LEC–INS nanocomplexes. In turn, the oral administering of LEC/CS 5/1 nanoparticles to STZ-induced diabetic mice led to a reduction in hyperglycaemia by 80% 2 h post-administering, a response that was prolonged for 12 h. Essentially similar results were confirmed in STZ-induced diabetic rats, where a rapid onset of action (~0.5 h) and a longer duration were observed, in contrast with the slow onset (~3 h) and short duration obtained with

LEC/INS nanocomplexes in the same animal model. The results of this study are regarded as promising towards achieving adequate prototypes for oral administering of insulin in diabetes therapy.

Key words: insulin, nanoparticles, nanocomplexes, lecithin, oral delivery

1. Introduction

The rational development of insulin (INS) delivery systems is beset of complex requirements so as to meet the needed pharmacokinetics/ pharmacodynamics associated with an adequate diabetes mellitus therapy [1] in order to normalize INS plasma profile to regulate systemic glucose levels. Currently, the conventional diabetes treatment combines the administering of INS by subcutaneous route with different duration (rapid-, short-, intermediate- and long-acting INS) [2]. Over the last decades, attempts to overcome the limitations and drawbacks associated with conventional subcutaneous INS therapy have been made. However, despite the known disadvantages of the subcutaneous route, thus far, only limited success has been achieved with alternative treatments able to reproduce as closely as possible the physiological profile resulting from endogenous insulin secretion [3]. Among the alternative routes of insulin delivery, the oral (enteric gastrointestinal) route is undoubtedly the most preferred one. In fact, oral insulin administering seems to be the most convenient and advantageous route from a physiological standpoint. This is due to the fact that insulin absorbed by intestinal epithelium reaches the liver through the portal vein and can directly inhibit hepatic glucose output. This effect on the liver is essential in the maintenance of glucose homeostasis while avoiding peripheral hyperinsulinemia [4]. However, the bioavailability of insulin peroral is known to be very small (<2 %) [5]. This is the consequence of a number of factors, namely, the low absorption of INS by the intestinal epithelia, its susceptibility to acidic and proteolytic degradation during the transit through the gastrointestinal tract and the rapid clearance from the site of absorption. Consequently,

an effective delivery system for INS, among other challenges, must be able to overcome these physical and metabolic barriers so as to facilitate its transport to the blood stream across the intestinal epithelia.

To this end, various approaches have been adopted, including utilizing INS derivative conjugates and prodrugs with high stability against degradation by gastrointestinal enzymes [6], INS associated antiproteases and hydrogels or combined with absorption enhancers (cyclodextrins, bile salts or surfactants) [4]. However, nanocarrier systems have emerged as highly promising systems to increase the intestinal uptake of peptides [7]. The nanoscopic dimensions provide these systems with an extremely large surface-to-volume ratio and surface functionality [8]. Liposomes [9-11], nanocapsules [12, 13] and nanoparticulate systems [14-18] have been studied as potential delivery systems for oral administering of insulin. This type of carriers has been made out of biocompatible materials including polymers and lipidic compounds of natural and synthetic origin [13, 19-22]. However, the pharmacokinetic/pharmacodynamic parameters are strongly influenced by the composition of the system.

A great deal of commendable research efforts have aimed to develop an adequate carrier for oral delivery of insulin that can effectively replace subcutaneous insulin. A recent study has addressed the development of hybrid biodegradable nanoparticles of copolymers of polylactic and polyglycolic acid (PLGA) and an insulin-phospholipid complex for INS's oral administering achieving a relative bioavailability of 7.7% in STZ-induced diabetic rats [20, 21]. Most of the effective developed systems

present a long-term response (hypoglycaemic effect ≥ 12 h) with a maximum decrease 5 h post-administering. These have included both natural (CS or dextran sulfate [14, 15, 19, 22-29] or synthetic [13, 20, 30, 31] polymers. However, the insulin therapy requires an oral formulation with a rapid onset of action and a prolonged effect that matches the maximum hypoglycaemic effect during the increase of glucose after meals. Hence, any improvement in this direction would represent a significant step towards a successful system. Motivated by this overall goal, in the present work, we have addressed the pharmacological performance of lecithin-insulin (LEC–INS) nanocomplexes and of INS-loaded lecithin-chitosan (LEC–CS) nanoparticles, both produced under a very simple and mild technique, on the enhancement of absorption of INS by oral route in two different rodent models.

LEC is a lipid mixture of phospholipids that has been frequently used for liposome and micelle formation and is largely employed in pharmaceutical or nutraceutical formulations [32, 33]. In this regards, lysophosphatydylcholine of LEC has been credited to markedly enhance the uptake of carotenoids solubilized in mixed micelles by Caco-2 human intestinal cells and *in vivo* studies of rats [34, 35]. In turn, the rationale behind the use of CS in the second type of systems addressed in this work was due to the firmly established pharmacological effects that CS-based nanocarriers have shown to improve the absorption and pharmacological performance of insulin administered peroral [7, 8, 17, 36, 37] and to enhance its systemic absorption after nasal administering [38-40]. Indeed, CS has been found to facilitate the interaction of

nanocarriers with mucosae and, hence, to improving the permeability and absorption of peptide drugs [41]. Interestingly, it has been proved that CS is able to interact readily with LEC by means of ionic and hydrophobic interactions [42-45] thus forming a new self-organized system [46, 47]. In several studies, LEC–CS nanoparticles have been found to be effective to entrap drugs of different type, such as tamoxifen [48], progesterone [47] or BSA [46]. Nanoparticles of this type have also been found to enhance the permeability of melatonin through Caco-2 cell monolayers, a common in vitro model of the intestinal epithelium [49], but to the best of our knowledge, their efficacy to deliver INS by oral route has not yet been tested in vivo.

2. Material and methods

2.1.-Materials

CS in the form of hydrocloride salt (Protasan UP-Cl 113, Mw = 125 kDa, deacetylation degree: 86%) was purchased from Pronova Byopolimer, A.S. (Norway). Lecithin (LEC) (Epikuron 145V) was donated by Cargill Texturizing Solutions, S.A (Barcelona, Spain). Details of composition (according with the supplier) of LEC are: phospholipid complex min 97% (phosphatidylcholine min 45%; phosphatidylethanolamine min 10%; phosphatidylinositol 3%; phosphatidic acid 3%; lyso-phosphatidylcholin 4%; phosphorus 2.7-3.4%). INS from bovine pancreas [Ref. Nº-I5500, *M*w 5.7 kDa, p*I* 5.3, Zn traces ~0.5%] was supplied from Sigma-Aldrich Chemie (Steinheim, Germany). Milli-Q quality water (18.2 MΩ.cm) was utilized throughout.

2.2. -Preparation of LEC–INS nanocomplexes

These nanocomplexes were obtained by interaction of negatively charged LEC's lipid components and positively charged INS's aminoacids. To find the optimum composition a series of blends of both components were explored. To this end, LEC aliquots of varying volumes of a LEC ethanolic stock solution (25 mg/ml) were injected to INS aliquot solutions (12.5 mg/ml) with a micropipette under magnetic stirring during 10 min at room temperature. The volumes of both stocks were adjusted so as to achieve nanocomplexes of lecithin/insulin (LEC/INS) mass ratios in the range 2/1 to 60/1. For the formulations of these systems, INS was dissolved in acetic acid solution of varying concentration in the range 0.1 to 2.0% (v/v).

2.3. - Preparation of INS-loaded LEC–CS nanoparticles

The general experimental protocol to prepare unloaded LEC–CS auto-assembled nanoparticles was that previously described by Sonvico et al [47]. In brief, CS aqueous solutions (0.027-0.56 mg/ml) were prepared by fully dissolving the polymer in water. To prepare INS-loaded nanoparticles two strategies were tested. Accordingly, one strategy involved that INS was dissolved in NaOH (pH~11.2) (i.e. bearing net negative charge) and subsequently added to a LEC ethanolic solution (25 mg/ml), this anionic solution was further on incorporated into a CS aqueous solution under gentle magnetic stirring for ~10 min at room temperature. Under the second strategy, INS was dissolved in acetic acid 1% (pH~3.3) (i.e. bearing net positive charge). The procedure was identical to the previous one in all respects except that INS was blended

in the CS solution. In both cases, the LEC–CS ratios assayed were in the range 5 to 80 (w/w).

2.4. – Isolation and yield production

The INS-loaded LEC–CS nanoparticles were isolated by ultracentrifugation (138800 × g, 2 hours, 15 °C) in vials on a bed of glycerol (~50 μL) carefully deposited at the bottom of the vial. The supernatant was removed cautiously with a pipette.

The processing yield of LEC–INS nanocomplexes and INS-loaded LEC–CS nanoparticles was determined by centrifuging (138800 × g, 2 h, 15 °C) accurately weighed aliquots of the nanoparticles in vials without added glycerol. The supernatants were carefully separated and the centrifuged pellets were freeze-dried for 2 days and subsequently weighed (n = 3).

The yield production was calculated as follows:

$$\text{Yield} \quad (\%) = \frac{\text{Nanocarriers weight}}{\text{Total solids weight}} \quad \text{x } 100$$

2.5. - Physical characterization

The Z-average size distribution of the colloidal systems was analyzed by photon correlation spectroscopy (PCS). To this end, aliquots of the various systems were diluted to the appropriate concentration with filtered ultrapure water so as to achieve the adequate counts per second. Measurements were performed at 25 ºC with an angle detection of 90º. The zeta potential (ζ) was calculated from the mean electrophoretic

mobility values, which were determined by laser Doppler anemometry (LDA). The suspensions were diluted with KCl 1 mM and placed in the electrophoretic cell where a potential of ±150 mV was established. The PCS size measurements were performed using either a Zetasizer® 3000 HS or a NanoZS ZEN 3600 fitted with a red laser light beam (λ=632.8 nm) (Malvern Instruments, Malvern, UK). The overall differences between measurements made on each type of instruments were within 21±6 nm. LDA measurements were performed in the 3000 HS instrument.

The morphology of nanocomplexes was examined by Transmission electron microscopy on a Philips CM12 TEM instrument (Eindhoven, The Netherlands). To this end, 10 μL of a 2% solution of phosphotungstic acid was mixed with an equal volume of a 1:100 dilution of nanocomplexes in water. Immediately afterward, an aliquot of 5 μL was immobilized on a copper grid coated with a Formvar® membrane and allowed to dry.

2.6. - INS association efficiency

The efficacy of association of INS was calculated indirectly, by quantifying of free INS in the supernatant after isolation as described above. The INS concentration was analyzed by HPLC (Agilent Technologies, Germany) according with the method described by Krauland [50].
The association efficiency (A.E.) was calculated as follows:

$$\text{A.E. (\%)} = \frac{\text{Total INS amount} - \text{Free INS amount}}{\text{Total INS amount}} \times 100$$

2.7. - Stability in simulated gastric and intestinal fluids

The colloidal stability of both INS-loaded systems was evaluated during incubation at 37°C under horizontal shaking (~100 rpm) in simulated gastric (SIG, pH=1.2) and intestinal (SIF, pH=6.8) fluids (both without enzymes) (*USP 31 - NF 29*. 1st ed. Vol. 1. 2008).

2.8. - In vitro release studies

INS release studies were performed by incubating aliquots of nanocomplexes or isolated nanoparticles in 1.5 ml of simulated fluid for each sample at 37 °C under horizontal shaking (~100 rpm). The INS concentration incubated in the release media was increased to 15μg/ml regarding sink conditions in order to detect the minimum released concentration of INS by HPLC analysis. At appropriate time intervals (30, 60, 120, 240 360 min), the samples were taken out to the ultracentrifuge at 4 ºC to separate the released INS from the system. The amount of INS released was determined also by HPLC [50].

2.9. -In vivo studies

In this study, we addressed the effects on the efficacy to induce a pharmacological response after oral administering INS-loaded systems in two diabetic models: (i) the effect of LEC–INS nanocomplexes in

diabetic rats (ii) after incorporation of CS, the effect of INS-loaded LEC–CS in diabetic mice (iii) and diabetic rats.

Healthy adult male Wild-type mice and Sprague–Dawley rats were housed in cages (five animals each) at 21±1°C in a 12:12 h light:dark cycle (all experiments were performed in the light cycle) and were fed and watered *ad libitum*. After 3 days, diabetes mellitus was induced by single injection of streptozotocin (STZ, 70 mg/kg i.p) in 0.1M citrate buffer (pH 5) and confirmed after 24 h by detecting glucose in urine. The diabetic animals were maintained with 4.5-6IU/kg/12 h of subcutaneous INS prior to the oral experiment. To monitor of the diabetes progression, glucose in urine and the body weight were quantified daily during one week. Following the procedure, three days before oral administering, the animals were placed in a metabolic cage (4 mice/cage; 1 rat/cage) in order to check the food, drink and urine. The animals were fasted for 12 h previous to oral assay, but allowed water *ad libitum*. Animals were kept conscious during the experiments. Hence, blood samples were collected from the tail vein prior to oral administering to establish the baseline glucose levels, and at different times after dosing. Urine samples in mice were collected from each cage at time points 0 and 8 h. The plasma was separated by centrifugation at ~2500 ×g for 5 min. Changes in plasma glucose levels were determined by Glucose RTU™ kit (BioMerieux® SA, France).

2.10. Statistical analysis

Data are expressed as means-S.E.M. *In vivo* data were analysed by Kruskall-Wallis test followed by U-Mann Whitney's post hoc test was

used for comparisons between the two independent groups. Chi-squared (χ^2) test was used selectively for comparing data on the percentages of decrease glycaemia (≥40%) between the dependent variable (glucose level) and the independent variable (treatment of INS-loaded LEC/CS nanoparticles) in STZ-induced diabetic rats.

3. Results and discussion

One of the most elusive aims in modern pharmaceutical technology has been to achieve an efficient delivery system of insulin (INS) by routes alternative to subcutaneous injection [3]. In this context, the rationale behind the present study was to develop nanocarrier systems that would take advantage of the expected properties of nanocomplexes formed between INS and LEC and of INS-loaded LEC–CS nanoparticles to preserve the bioactivity of the peptide during both the process of formulation and its subsequent transit trough the gastrointestinal tract as well as to ferry it through the intestinal mucosa, thus enhancing its absorption and bioavailability. As a first step toward this end, the preparation conditions of LEC–INS nanocomplex were optimized in terms of mass ratio of both constituents. Subsequently, CS was incorporated in prototype formulations of LEC–INS nanocarriers with a view to modify their surface properties, thus effectively, improving the mucoadhesion and intestinal absorption of INS. Both LEC–INS nanocomplexes and INS-loaded LEC–CS nanoparticles were

characterized and their potential as oral delivery vehicles was assayed in two diabetic rodent models.

3.1. Production and Characterization of LEC–INS nanocomplexes

The ability of INS to interact with the major constituents of LEC, such as phosphatidylcholine, can be conceived as the result of the formation of a polyion-surfactant complex between oppositely charged ionic groups on both molecules at the appropriate pH [51]. Systems of this type have been exploited in early studies for the development of insulin-loaded liposomes for oral delivery [11] and their general physicochemical properties have been addressed elsewhere [52, 53].

In the present work, preliminary experiments were conducted to optimize the size dimensions of nanocomplexes of LEC–INS. A series of blends of LEC and INS at varying LEC/INS mass ratios were explored. To this end, INS was dissolved in aqueous acetic acid solutions of varying concentration in the range 0.01–2 % (v/v), thus inducing a varying degree of protonation of INS (*pI* ~ 5.3). The obtained systems were inspected visually and three types of systems were identified according with their appearance: clear solutions, white opalescent colloidal suspensions and aggregates (visible precipitates). The experimental size data were used to construct a surface response map in terms of the LEC/INS mass ratio and acetic acid concentration (Figure 1). Aggregates were not considered for the construction of this plot. The obtained structures were arbitrarily assigned to three categories according with their dimensions, namely, structures ≤ ~90 nm, ~90 – 180 nm and ≥ ~180 nm. The incorporation

of increasing positive charge of INS with respect to LEC led to an increase the compensated electroneutral regions, entailing an increase on the hydrophobic character of electrostatic complexes and thus, led to a slight increase in their size. To account for this behaviour, the nanocomplexes are formed as INS becomes progressively more charged and also when the LEC/INS mass ratio <~15/1. Meanwhile, the overall increase in LEC/INS mass ratio up to a value of ~20/1 is clearly accompanied by a pronounced reduction in particle size, with no further decrease beyond this ratio. Interestingly, in the narrow range of composition at LEC/INS mass ratios ~20/1 – 30/1 at low concentrations of acetic acid appear very small species (≤ ~90 nm), suspected to be comprised by liposomes. Hence, the region defined by the size range ~90 – 180 nm can be expected to comprise a mixture of nanocomplexes and liposomes.

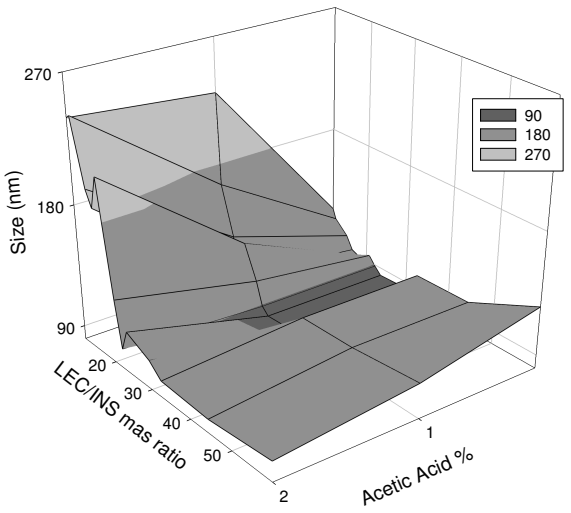


Figure 1. Response surface map showing the effect of lecithin/insulin mass ratio and acetic acid concentration on the particle size of lecithin-insulin nanocomplexes.

Size distribution curves obtained by DLS representative of LEC/INS mass ratios ~60/1, 16/1 and 10/1 along with liposomes comprising solely lecithin are shown in Figure 2. It reveals that the curve corresponding to the system of LEC/INS mass ratios ~60/1 exhibits a bimodal size distribution with predominance of a population of ~120 nm and the presence of a small fraction of particles of size ~20 nm. This bimodal distribution is also observed for the systems at LEC/INS mass ratio 16/1 and for lecithin liposomes, though the predominant peak is shifted to ~60 nm while the small one to ~10 nm. Whereas the system with proportionally less LEC contents (LEC/INS mass ratio ~10/1) shows a single population with monomodal size distribution corresponding with the nanocomplexes formation.

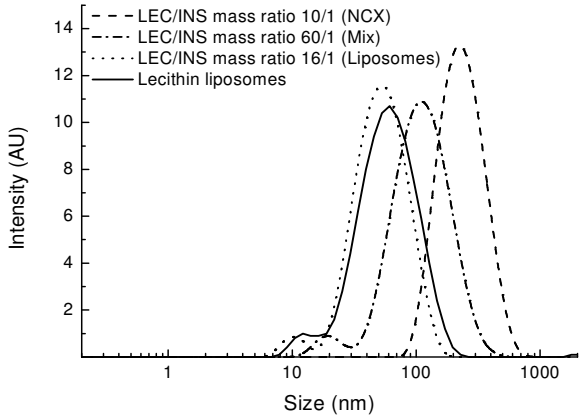


Figure 2. Size distribution of the scattered light intensity as determined by photocorrelation spectroscopoy in water at 25ºC of lecithin-insulin systems with mass ratios (w/w): 10/1 nanocomplexes (dash line); 60/1 mixture of nanocomplexes and liposomes (dash-dot line); liposomes 16/1 (short dot). Liposomes formed with lecithin (continuos line).

For subsequent experiments a prototype formulation was prepared with a LEC/INS mass ratio 10 and by dissolving INS in 1% (v/v) acetic acid. The composition for this prototype was selected on the grounds of its size (209±18 nm) and more so, on its high association efficiency for insulin (A.E. ~97–100%) as shown in Table 1. Indeed, INS A.E. for this prototype formulation was almost total representing a clear functional advantage over the conventional liposomes with notably lower affinity for drug binding (1- 53%) [9, 11, 54, 55]. Such high INS A.E. is explained as the consequence of positively charged INS at pH below its isoelectric point (INS *pI*~5.3), that favours its electrostatic interaction with negatively charged phospholipidic macroions in LEC [45]. LEC–INS complexes in general showed negative surface charge (ζ~-42±4 mV) with high yields of production (60±6%). From the biopharmaceutical standpoint, it is worth pointing out that the high negative zeta potential could be expected to favour the bioadhesivity and absorption by both M-cells and enterocytes during oral delivery, as suggested in previous studies [8]. These nanocomplexes also exhibited high stability (> 90 min) in simulated intestinal fluid, yet another *sine qua non* pre-requisite for their use as oral drug delivery vehicles.

The morphology and features of LEC–INS systems of mass ratios 60 and 10 were probed by TEM. As evidenced in the representative micrographs of the LEC – INS of mass ratio 60 (Fig. 3a), it can be appreciated that spherical assemblies with a core–shell structure were formed. A closer inspection reveals that these assemblies are constitued by a core sorrounded by a shell of variable thickness. In turn, the TEM images of nanocomplexes, corresponding with LEC/INS mass ratio 10 (Figure 3b),

reveal the presence of species of irregular shape and size and it is not possible to discern the presence of a bilayer.

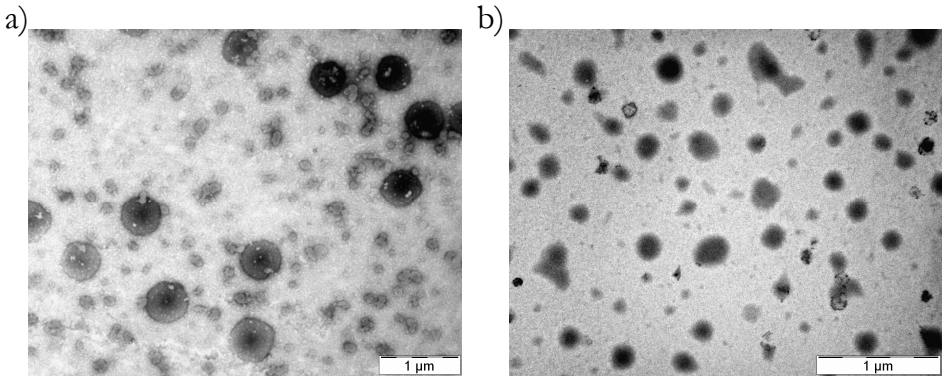


Figure 3. Transmission electron micrographs of lecithin – insulin mass ratio 60/1(a) and 10/1 (b)

3.2. Production and characterization of INS loaded LEC– CS nanoparticles

LEC–CS nanoparticles were the second class of systems addressed in the present study. The rationale behind the incorporation of CS in the formulation was to achieve mucoadhesivity and improve the absorption of INS. Preliminary trials were carried out to explore the optimal composition of unloaded (blank) nanoparticles of this kind [47]. The unloaded nanoparticles produced at LEC/CS ratios varying from 5/1 to 20/1 (w/w) invariably displayed positive ζ values, while those comprising a low amount of CS (LEC/CS 60/1 to 80/1) experienced a charge inversion with negative surface charge [47]. Hence, a series of systems of varying composition were formulated by holding fixed the amount of LEC while varying that of CS incorporated, so as to afford blends of

LEC/CS mass ratios from 5/1 to 80/1. The ionic character of INS was also modified by dissolving the peptide either in acetic acid 1% (v/v) or in NaOH 0.1 N, thus bearing either a positive or negative net charge, respectively.

Interestingly, when INS was incorporated bearing a net positive charge, nanoparticles were formed only at LEC/CS mass ratios of 5/1 to 10/1 (i.e. at high amounts of CS), while formulations incorporating proportionally less CS (LEC/CS 60/1 to 80/1) invariably produced aggregates. The stabilization brought in by CS in systems of lower LEC/CS mass ratio can be accounted for as the formation of a hydrophobic complex between LEC–INS nanocomplexes and neutralized LEC–CS species, presumably involving lipidic bilayers. In turn, aggregation due to an excess of LEC could be explained as consequence of surface charge neutralization attained by the oppositely charged groups of INS and CS, leading to electrostatic repulsion among particles, and thus, a loss of stabilization [45, 46]. A schematic representation of the proposed interactions occurring in the system is shown in Figure 4a. Notice in Table 1 that regardless of the LEC/CS mass ratio, these nanoparticles exhibited very similar average particle size (230 – 241 nm) and positive surface charge (ζ~+28±1 mV) values. The results are consistent with the idea that the excess of positive charged amino groups of CS at the surface is required for its colloidal stabilization. This is in agreement with results for unloaded LEC–CS nanoparticles [43]. Despite the fact that INS was bearing similar charge as CS, a high INS association efficiency (~99-100%) was attained. Small

angle X-ray scattering (SAXS) studies conducted in unloaded LEC–CS nanoparticles systems have postulate that multilamellar structures are formed in these systems. The outer layer is thought out to be formed by a single bilayer [43, 44]. The formation of these multilamellar phospholipidic structures entrapping INS molecules can account the high INS A.E.

Table 1/ Physic-chemical characteristics of insulin-loaded nanosystems (mean ± S.D., n = 3).

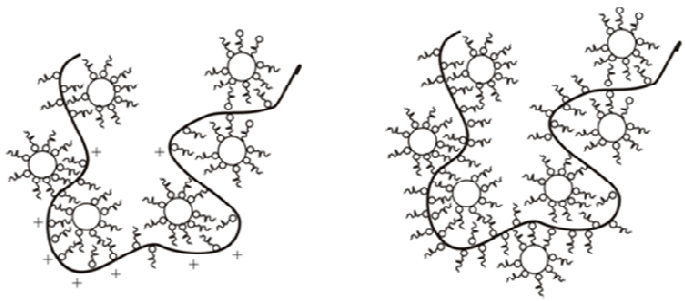
Insulin [a]	LEC/CS mass ratio (w/w)	Size (nm)	P.D.I	ζ (mV)	Insulin A.E. (%)	Stab.[b] (pH 6.8)
+	LEC-INS[c]	209±18	0.18-0.21	-42±4	97-100	+++
+	5/1	230±5	0.24-0.29	+28±1	99-100	_
+	10/1	241±4	0.31-0.36	+28±1	99-100	_
-	5/1	212±9	0.26-0.40	+32±1	83±2	+
-	60/1	248±8	0.15-0.19	- 25±6	22±6	+++
-	80/1	285±14	0.20-0.41	- 20±2	10±5	+++

[a]Insulin dissolved in acetic acid 1% (positive charge) or NaOH 0.1N (negative charge).
[b] Stability in simulated intestinal fluid was classified as: (+++) stable ≥ 90 min, (+) stable < 60 min and (-) unstable (immediate aggregation).
[c]Lecithin-insulin nanocomplexes (LEC/INS mass ratio 10/1 w/w).

By contrast, particles harnessed when INS was incorporated in the formulation bearing negative net charge, were formed regardless of the amount of CS (i.e. LEC/CS 60/1 to 80/1). The particle diameter was very similar than for the rest of the nanosystems (203–299 nm). In turn,

the zeta potential of systems comprising high CS contents (i.e. LEC/CS 5/1) attained highly positive values (+32±1 mV). Meanwhile, for formulations comprising a low content of CS (LEC/CS 60/1 to 80/1) a charge inversion was observed (-20 – -25 mV) as the complexation between oppositely charged groups goes beyond charge neutralization, in line with previous studies [43]. This phenomenon is also accompanied by a moderate overall increment in particle size (i.e. ~30%) with a drastic reduction in the INS A.E. from 83±2% for LEC/CS 5/1 to 10±5% for LEC/CS 80/1, presumably as the expected consequence of the displacement of the peptide by LEC competing by CS's positive charged sites. In these systems one can envisage that INS is directly bound to CS and hence, LEC competes for the available positive charges in the polysaccharide. The negative ζ values of these systems can be due to the formation of LEC lipid bilayers placed at the nanoparticle surface, thus orienting the negative phospholipidic polar heads of LEC towards the surface, as schematically represented in Figure 4b.

a)



b)

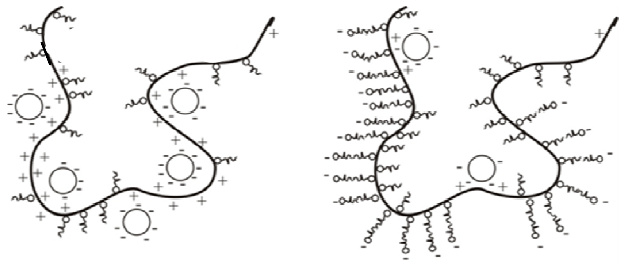


Figure 4. Schematic representation of the interactions proposed to occur in insulin–loaded lecithin–chitosan nanoparticle systems when insulin bears either (a) positive or (b) negative net charge. Chitosan polymer (line), insulin (big circles) and lecithin ( )

Based on these results, the nanoparticle formulation LEC/CS 5/1 was selected for further experiments due to the adequate optimal physicochemical properties and stability in gastrointestinal fluids.

3.3 Stability in gastric and intestinal simulated media

Before proceeding with the *in vivo* studies, it was necessary to confirm that the developed prototype nanosystems were stable in conditions that

simulate those found in the biological environment of the oral mucosae. To this end, the evolution of Z-average particle size of nanosystems was assayed in SGF (pH 1.2) and SIF (pH 6.8) without the presence of enzymes. Accordingly, the evolution of Z-average diameter of LEC–INS nanocomplexes and INS-loaded LEC–CS nanoparticles was monitored during 90 min at 37ºC under horizontal shaking as already shown in Table 1.

In general, the LEC/CS systems presented excellent stability in SGF for up 90 min as illustrated in the Z-average size evolution traces in Figure 5. Notice that the LEC–INS system without CS showed excellent stability in SIF and SGF during the whole experiment. This represents a clear advantage over LEC-based liposomes that are not stable in SGF [56]. On the other hand, all LEC–CS formulations prepared with INS dissolved in acetic acid (i.e bearing positive net charge) were not stable in SIF (pH 6.8). This fact was expected, as a consequence of the neutralization of $-NH_3^+$ groups of CS (i.s. CS's intrinsic pK_o=6.0±0.1 [57], thus leading to the aggregation of the nanoparticles. Only LEC–CS systems incorporating the negatively charged form of INS showed stability after incubation in SIF, particularly those with negative ζ values (results not shown). However, the LEC/INS 5/1 formulation did also show adequate stability albeit bearing a positive surface charge (ζ =+32±1 mV), with only a moderate increasing trend in size during incubation in SIF (Figure 5). In this case, the presence of INS electrostaticaly bound to CS chain could explain this stability.

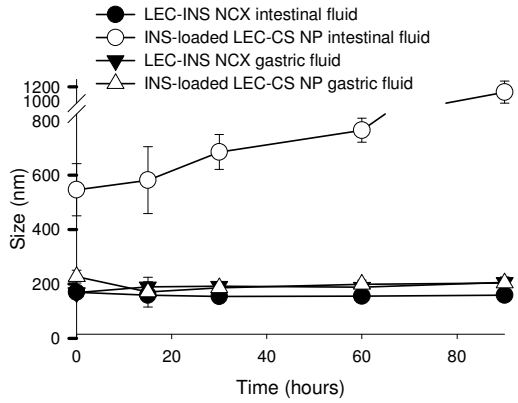


Figure 5. Evolution of particle size during incubation in a simulated gastric (pH 1.2) (triangles) and intestinal fluid (pH 6.8) (circles) without enzymes at 37 ◦C of: lecithin–insulin nanocomplexes (filled symbols) and insulin–loaded lecithin–chitosan nanoparticles (empty symbols) (mean ±S.D., n = 3).

3.4. In vitro release studies

INS release profiles from LEC–INS and LEC–CS nanocarriers were investigated in simulated intestinal medium without enzymes, pH 6.8 (Fig. 6). Both systems displayed a release profile characterized by a low initial burst (< 20%) and no further peptide was released for up to 4 hours. The initial release in both cases was attributed to the dissociation of the peptide associated at the surface, whereas a second slower process corresponded to the release of the well associated peptide molecules, showing a high affinity for the components of the formulation.

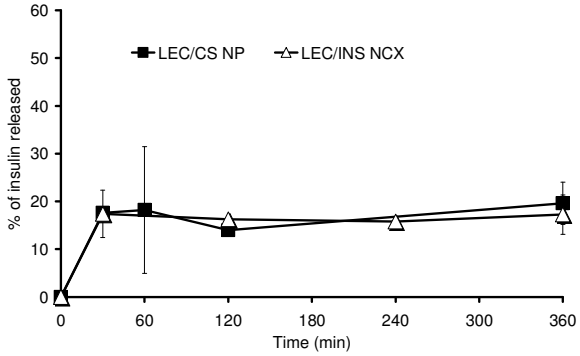


Figure 6. *In vitro* release profiles of insulin from lecithin/chitosan 5/1 nanoparticles (black square) and lecithin/insulin 10/1 nanocomplexes (white triangle) in simulated intestinal fluid without enzymes pH 6.8 at 37 ◦C. (mean ±S.D., $n = 3$).

The comparison of our results with those obtained by INS-loaded liposome systems and LEC–CS nanoparticles loaded with other drugs, evidenced interesting differences. In liposome systems, the percentage of released INS increased notably (from 30% to 97%) depending on composition [9, 58]. Otherwise for LEC–CS nanoparticles no release of tamoxifen was evidenced in simulated gastrointestinal fluid without enzymes [48], whereas melatonin was released ~60% at pH 5.6 [49]. In our case, the strong electrostatic interaction of the peptide with the compounds of nanoparticles would justify the low released amounts of INS under the experimental conditions of the study. The high affinity of INS for LEC or CS was confirmed in gastric medium, showing < 10% released during 45 min (data not shown).

3.5. Efficacy of LEC–INS nanocomplexes after oral administering in STZ-induced diabetic rats

LEC–INS nanocarriers (dose of INS 35 IU/kg) were administered intragastrically using a catheter (stainless steel curved feeding needles from Harvard apparatus) to STZ- induced diabetic rats. As control, we used the unloaded liposomes and saline solution. To this end, all animals (234±12g) received 1.2 ml of each treatment.

In Figure 7, the results indicate that the formulation led to a decrease in glucose plasma levels during ~3-7 h after oral administering, reaching this maximum reduction (~30 %) at 5 h. However, unexpectedly, the control groups showed a notable hyperglycaemic effect that lasted up to 10 h, possible due to stress caused to the animals during the experiment. Hence, the hypoglycaemic effect produced by LEC–INS nanocomplexes was statistically significantly greater than for the controls groups during 11 h post-administering. Moreover, the observed effect in the present work of nanocomplexes was significantly greater than that induced by liposomes coated with mucin or PEG [9]. According with these results, it can be argued that LEC–INS nanocomplexes were able to protect INS against gastrointestinal degradation and overcome the intestinal barrier.

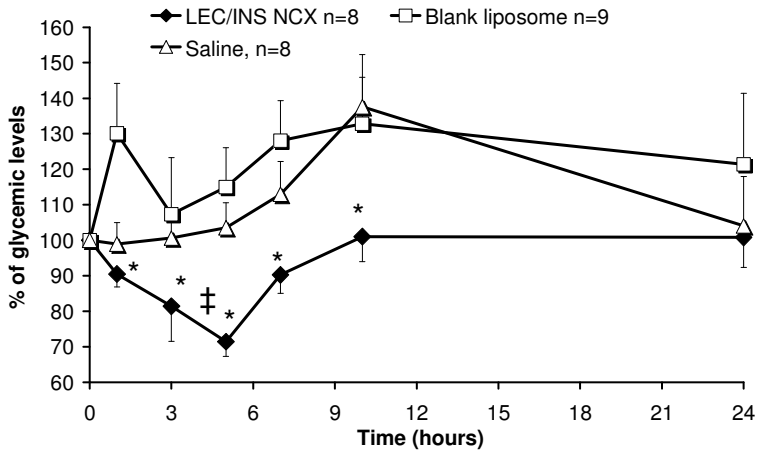


Figure7. Relative plasma glucose levels achieved in streptozotocin-induced diabetic rats after oral administering (mean± S.E.M, n=8) of lecithin-insulin nanocomplexes (filled diamonds), liposomes (empty squares) and saline (empty triangle). *Statistical significant differences ($p \leq 0.05$) with respect to liposomes and saline control groups, except ‡ ($p \leq 0.065$) respect to the saline group. The dose of insulin administered was ~35IU/kg of body weight

3.6. Efficacy after oral administering of INS-loaded LEC–CS nanoparticles in STZ-induced diabetic mice

Given the promising results obtained with LEC–INS nanocarrier systems in diabetic rat model, we further investigated the role of introducing CS to a different nanoparticle harnessed formulation. The rationale behind this was to benefit of the well-established properties of nanocarriers bearing CS. Namely CS's mucoadhesive properties and hence a prolonged time of response and also of an expected greater INS's absorption as a result of a permeabilization effect induced by CS across the intestinal epithelia. In this regards, in previous studies it was reported that CS improves the oral absorption and the pharmacological effect of

INS administered orally [1, 14, 15, 27]. In this study, we have evaluated the pharmacological performance behaviour of INS-loaded LEC–CS nanoparticles in STZ-induced diabetic mice (19±1g) as a model of diabetes [59]

For this purpose, we selected the LEC/CS 5/1 formulation containing ~166 IU/kg of INS, and we also assayed unloaded or blank nanoparticles and water as controls. The treatments were administered in a final volume of 0.250 ml. Figure 8 shows the profiles of the glucose levels achieved following oral administering. Most important response was obtained for INS loaded LEC–CS nanoparticles showing a notable decrease down to 80% of the basal glucose levels, achieving normoglycemic levels (T_0=555.2±57.6 mg/dl, T_{2h}=91.16±6.4 mg/dl). Indeed, the reduction of glycaemia was maintained below 60% for at least 12 h. It was found that a maximum hypoglycaemic effect was produced after 2 h. In this regards, INS-loaded LEC–CS nanoparticles represent an advance over the conventional longer-term INS nanosystems applied by oral route that produce the maximum decrease of glucose after 5 h of administering. This decrease in glycemic plasma profiles was also confirmed by the analysis of glucose concentration in urine. This assay showed that the mice treated with INS nanoparticles presented a strong reduction respect the basal values and control water group (Fig. 9).

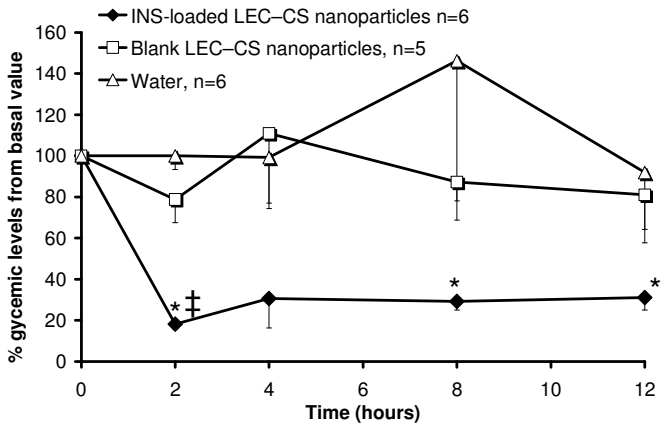


Figure 8. Relative plasma glucose levels achieved in streptozotocin-induced mice after oral administering; (mean± S.E.M, *n*=3-6) of insulin-loaded lecithin-chitosan 5/1 nanoparticles (filled diamonds), blank 5/1 lecithin-chitosan nanoparticles (empty square) and water (empty triangle). *Statistical significant differences ($p \leq 0.05$) with respect to blank nanoparticles control group. ‡ Statistical significant differences ($p \leq 0.05$) with respect to the water group. The dose of insulin administered was ~166IU/kg of body weight

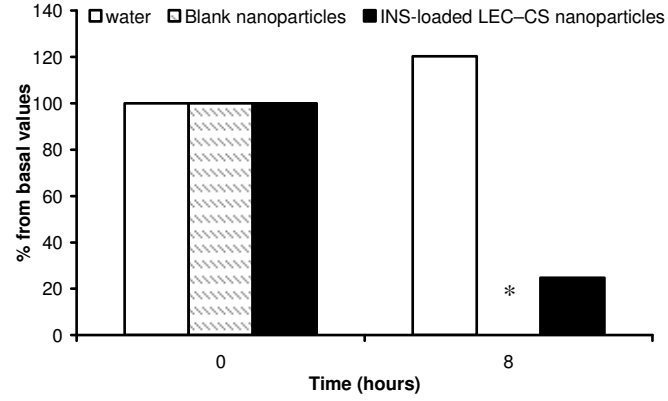


Figure 9. Relative glucose levels in urine achieved in streptozotocin-induced diabetic mice after oral administering of insulin-loaded lecithin-chitosan nanoparticles (black bars), unloaded lecithin-chitosan nanoparticles (grey bar) and water (white bars). The dose of insulin administered was ~166IU/kg of body weight. * Absence of urine at 8 h.

3.7. Efficacy after oral administering of INS-loaded LEC–CS nanoparticles in STZ-induced rats

As shown above LEC–CS nanoparticles per oral gavage are able to improve the pharmacological effect of INS administered orally in diabetic mice. To corroborate this effect an *in vivo* assay was conducted in conscious STZ-diabetic rats using unloaded nanoparticles and saline as controls. As illustrated in Figure 10, the average of glucose levels after dosing did not show significant differences between the assayed groups. A closer analysis, however, revealed that 30% of the animals in the group treated with INS-loaded LEC–CS nanoparticles experienced a reduction in glucose levels of ≥40 %. Hence, a contingency table was constructed (Table 2) to assess the statistical significance resulting of the comparison of the number of animals that gave a positive response in the various groups using the χ^2 test. It was found that the number of animals that experienced a positive response in the group of INS-loaded LEC–CS nanoparticles achieved high statistical significance ($p \leq 0.005$). For the sake of clarity, in Figure 11 are depicted the glycaemia profile obtained only for the animals that gave a positive response (n=4). In those animals, the average of glucose levels revealed that INS-loaded LEC–CS nanoparticles reduced the glycemia by ~54 % and the hypoglycaemic effect was prolonged for ~24 h, with respect to the control groups. It can be appreciated that the maximum percentage of reduction of glucose was of greater magnitude than the effect observed with LEC–INS nanocomplexes in diabetic rats (Figure 7), even when they treated with different INS dose. This may be related with the diferente administering

dose. Regarding the incorporation of CS to the nanosystem, it was found that it does represent an improvement due to the onset of action that was produced 0.5 h after oral administering in contrast with that observed for LEC/INS nanocomplexes (onset of effect ~3 h). Indeed, the pattern of response of INS-loaded LEC–CS nanoparticles is very similar to that achieved by subcutaneous injection of insulin NPH 2.5 UI/kg INS in the same animal model [1], although the oral nanosystem prolonged the effect during ~24 h whereas that the glucose reduction with INS injection persisted for ~8 h.

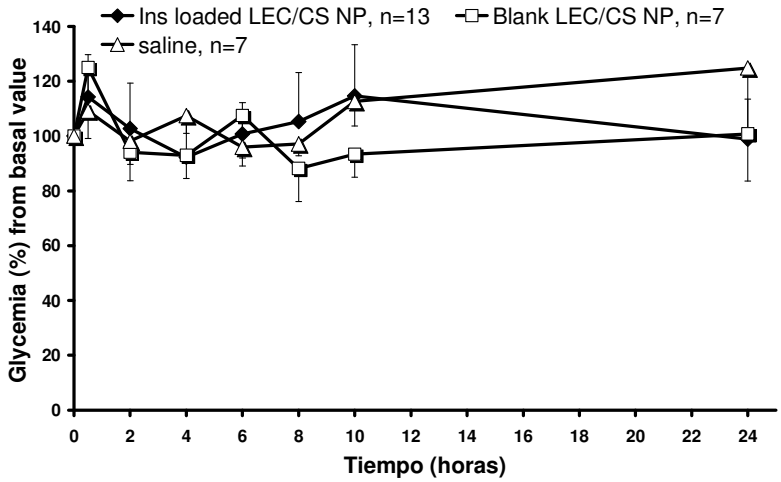


Figure 10. Relative plasma glucose levels achieved in streptozotocin-induced diabetic rats after oral administering (mean ± S.E.M, n=7-13) of insulin-loaded lecithin-chitosan nanoparticles (filled diamonds), blank lecithin-chitosan nanoparticles (empty square) and water (triangle triangle). The dose of insulin administered was ~190UI/kg of body weight

Table 2: Contingency table for the glucose effects of insulin-loaded nanoparticles, unloaded nanoparticles or saline in plasma levels after oral administering in STZ-induced diabetic rats.

	≥ 40% decrease of glycemia respect to the basal values		
Treatment	**positive**	**negative**	**total**
Insulin-loaded NP	$4^{a,*}$	9	13
Unloaded NP	0	7	7
Saline	0	7	7

$^{a}C_{min}$ (%)= 46,5 ± 15.7 (mean ± S.E.M), T_{mim} 4 h ; T(C ≤ 80 %)= 0.5- 24 h
Statistically different respect to the control groups ($p \leq 0.005$*)

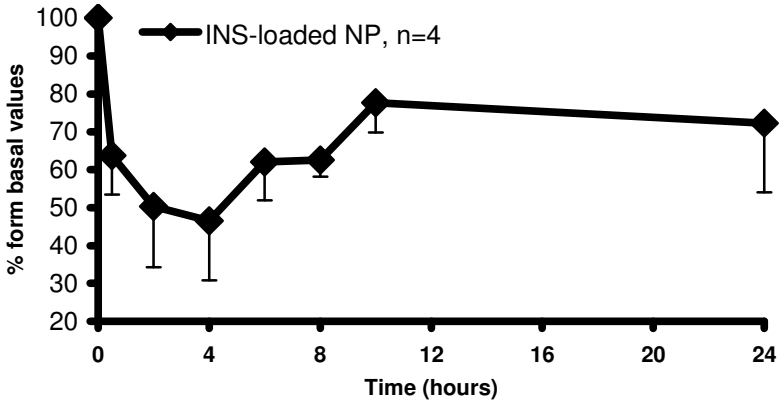


Figure 11. Relative plasma glucose levels achieved in STZ-induced diabetic rats that gave a positive response (≥ 40% decrease of glycemia respect to the basal values) after oral administering; of insulin-loaded lecithin-chitosan nanoparticles (mean ± S.E.M, n=4).

In agreement with previous studies [14, 15, 60], the results of this study confirm that CS nanoparticles enhance the systemic absorption of bioactive peptides such as INS after oral administration. Moreover, the

INS-loaded LEC–CS nanoparticles evidenced the potential advantage to achieve a prolonged hypoglycemic response after oral administering.

4. Conclusion

Two novel INS-loaded formulations comprising lecithin as a major component were developed and proved effective for oral administering of INS, according with proof of concept in two STZ-induced diabetic rodent models. Both can be prepared by a very simple and mild process, thus preserving the bioactivity of INS. LEC–INS nanocarriers optimized in terms of size were adequate to protect INS against the gastrointestinal degradation and to enhance its absorption. The incorporation of CS in INS–loaded LEC–based nanoparticles reduces the onset of action and prolongs the absorption of INS leading to a prolonged hypoglycaemic response during ~24 h.

Acknowledgements

Financial support of the European Union from the Nanobiosaccharides project. (Ref No. 013882 of call FP6–2003–NMP–TI–3–Main) is gratefully acknowledged. We are grateful to Lucas G., Mayte A. and Manuel G-L. for his help during *in vivo* studies.

References

1. Lin, C., et al., *Recent strategies and methods for improving insulin delivery.* Drug Development Research, 2004. 63(4): p. 151-160.
2. Farmaceúticos, C.O.d., *Catálogo de Medicamentos. Consejo Plus.* 1ª ed, ed. C.G.d.C.O.d. Farmaceúticos. 2009.
3. Takei, I. and T. Kasatani, *Future therapy of diabetes mellitus.* Biomedicine and Pharmacotherapy, 2004. 58(10): p. 578-581.
4. Damgé, C., C.P. Reis, and P. Maincent, *Nanoparticle strategies for the oral delivery of insulin.* Expert Opinion on Drug Delivery, 2008. 5(1): p. 45-68.
5. Crane, C.W. and G.R. Luntz, *Absorption of insulin from the human small intestine.* Diabetes, 1968. 17(10): p. 625-627.
6. Kipnes, M., et al., *Control of postprandial plasma glucose by an oral insulin product (HIM2) in patients with type 2 diabetes.* Diabetes Care, 2003. 26(2): p. 421-426.
7. Florence, A.T., et al., *Nanoparticles as carriers for oral peptide absorption: Studies on particle uptake and fate.* Journal of Controlled Release, 1995. 36(1-2): p. 39-46.
8. Jung, T., et al., *Biodegradable nanoparticles for oral delivery of peptides: Is there a role for polymers to affect mucosal uptake?* European Journal of Pharmaceutics and Biopharmaceutics, 2000. 50(1): p. 147-160.
9. Iwanaga, K., et al., *Oral delivery of insulin by using surface coating liposomes. Improvement of stability of insulin in GI tract.* International Journal of Pharmaceutics, 1997. 157(1): p. 73-80.
10. Kisel, M.A., et al., *Liposomes with phosphatidylethanol as a carrier for oral delivery of insulin: Studies in the rat.* International Journal of Pharmaceutics, 2001. 216(1-2): p. 105-114.
11. Patel, H.M. and B.E. Ryman, *Oral administration of insulin by encapsulation within liposomes.* FEBS Letters, 1976. 62(1): p. 60-63.
12. Damgé, C., et al., *Poly(alkyl cyanoacrylate) nanospheres for oral administration of insulin.* Journal of Pharmaceutical Sciences, 1997. 86(12): p. 1403-1409.
13. Watnasirichaikul, S., et al., *In-vitro release and oral bioactivity of insulin in diabetic rats using nanocapsules dispersed in biocompatible microemulsion.* Journal of Pharmacy and Pharmacology, 2002. 54(4): p. 473-480.
14. Sarmento, B., et al., *Alginate/chitosan nanoparticles are effective for oral insulin delivery.* Pharmaceutical Research, 2007. 24(12): p. 2198-2206.

15. Sarmento, B., et al., *Oral bioavailability of insulin contained in polysaccharide nanoparticles*. Biomacromolecules, 2007. 8(10): p. 3054-3060.
16. Yin, L., et al., *Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery*. Biomaterials, 2009. 30(29): p. 5691-5700.
17. Sonaje, K., et al., *In vivo evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery*. Biomaterials, 2009. 30(12): p. 2329-2339.
18. Calvo, P., et al., *Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers*. Journal of Applied Polymer Science, 1997. 63(1): p. 125-132.
19. Chalasani, K.B., et al., *Effective oral delivery of insulin in animal models using vitamin B12-coated dextran nanoparticles*. Journal of Controlled Release, 2007. 122(2): p. 141-150.
20. Cui, F., et al., *Biodegradable nanoparticles loaded with insulin-phospholipid complex for oral delivery: Preparation, in vitro characterization and in vivo evaluation*. Journal of Controlled Release, 2006. 114(2): p. 242-250.
21. Cui, F.D., et al., *Preparation of insulin loaded PLGA-Hp55 nanoparticles for oral delivery*. Journal of Pharmaceutical Sciences, 2007. 96(2): p. 421-427.
22. Elsayed, A., et al., *Formulation and characterization of an oily-based system for oral delivery of insulin*. European Journal of Pharmaceutics and Biopharmaceutics, 2009. 73(2): p. 269-279.
23. Carvalho, E.L.S., et al., *Mucosal Delivery of Liposome–Chitosan Nanoparticle Complexes*. In Nejat Düzgüneş ed, ed. M.i. enzymology. Vol. 465. 2009 Burlington: Elsevier Inc. 289-312.
24. Chalasani, K.B., et al., *A novel vitamin B12-nanosphere conjugate carrier system for peroral delivery of insulin*. Journal of Controlled Release, 2007. 117(3): p. 421-429.
25. Liin, Y.H., et al., *Preparation and characterization of nanoparticles shelled with chitosan for oral insulin delivery*. Biomacromolecules, 2007. 8(1): p. 146-152.
26. Pan, Y., et al., *Bioadhesive polysaccharide in protein delivery system: Chitosan nanoparticles improve the intestinal absorption of insulin in vivo*. International Journal of Pharmaceutics, 2002. 249(1-2): p. 139-147.

27. Pan, Y., et al., *Relationship between drug effects and particle size of insulin-loaded bioadhesive microspheres.* Acta Pharmacologica Sinica, 2002. 23(11): p. 1051-1056.
28. Qian, F., et al., *Chitosan graft copolymer nanoparticles for oral protein drug delivery: Preparation and characterization.* Biomacromolecules, 2006. 7(10): p. 2722-2727.
29. Zhenqing, H., et al., *Use of natural plant exudates (Sanguis Draxonis) for sustained oral insulin delivery with dramatic reduction of glycemic effects in diabetic rats.* Journal of Controlled Release, 2004. 97(3): p. 467-475.
30. Graf, A., et al., *Oral insulin delivery using nanoparticles based on microemulsions with different structure-types: Optimisation and in vivo evaluation.* European Journal of Pharmaceutical Sciences, 2009. 37(1): p. 53-61.
31. Xiong, X.Y., et al., *Vesicles from Pluronic/poly(lactic acid) block copolymers as new carriers for oral insulin delivery.* Journal of Controlled Release, 2007. 120(1-2): p. 11-17.
32. Batzri, S. and E.D. Korn, *Single bilayer liposomes prepared without sonication.* BBA - Biomembranes, 1973. 298(4): p. 1015-1019.
33. Betageri, G.V.J., S. A.; Parsons, D. L., Liposome Drug Delivery Systems; Technomic Publishing: Lancaster, PA., 1993.
34. Sugawara T, K.M., et al., *Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human intestinal cells.* J Nutr 2001. 131: p. 2921-7.
35. Lakshminarayana R, et al., *Enhanced lutein bioavailability by lyso-phosphatidylcholine in rats.* Mol Cell Biochem, 2006. 281:: p. 103-10.
36. Alonso-Sande, M., *Nanopartículas poliméricas modificadas con manosa: Nuevos sistemas destinados a la administracion oral de macromoléculas terapéuticas*, in *Departamento de Farmacia y Tecnología Farmaceútica*. 2006, Santiago de Compostela: Santiago de Compostela. p. 255.
37. Lee, D., et al., *Thiolated chitosan/DNA nanocomplexes exhibit enhanced and sustained gene delivery.* Pharmaceutical Research, 2007. 24(1): p. 157-167.
38. Fernandez-Urrusuno, R., et al., *Enhancement of nasal absorption of insulin using chitosan nanoparticles.* Pharmaceutical Research, 1999. 16(10): p. 1576-1581.
39. Goycoolea, F.M., et al., *Effect of molecular weight and degree of acetylation on the physicochemical characteristics of chitosan nanoparticles.* Avances in chitin of sciences, 2007. X.

40. Teijeiro-Osorio, D., et al., *New generation of hybrid poly/oligosaccharide nanoparticles as carriers for the nasal delivery of macromolecules.* Biomacromolecules, 2009. 10(2): p. 243-9.
41. Takeuchi, H., et al., *Mucoadhesive nanoparticulate systems for peptide drug delivery.* Advanced Drug Delivery Reviews, 2001. 47(1): p. 39-54.
42. Fang, N., et al., *Interactions of phospholipid bilayer with chitosan: Effect of molecular weight and pH.* Biomacromolecules, 2001. 2(4): p. 1161-1168.
43. Gerelli, Y., et al., *Structure of self-organized multilayer nanoparticles for drug delivery.* Langmuir, 2008. 24(20): p. 11378-11384.
44. Gerelli, Y., et al., *Structure and organization of phospholipid/polysaccharide nanoparticles.* Journal of Physics Condensed Matter, 2008. 20(10).
45. Magdassi, S., et al., *Formation of positively charged microcapsules based on chitosan-lecithin interactions.* Journal of Microencapsulation, 1997. 14(2): p. 189-195.
46. Chuah, A.M., et al., *Formation of biocompatible nanoparticles via the self-assembly of chitosan and modified lecithin.* Journal of Food Science, 2009. 74(1).
47. Sonvico, F., et al., *Formation of self-organized nanoparticles by lecithin/chitosan ionic interaction.* International Journal of Pharmaceutics, 2006. 324(1): p. 67-73.
48. Barbieri, S., et al., *Chitosan/Lecithin Autoassembled nanoparticles: a new carrier for oral delivery.* 9th International Conference of the European Chitin Society. EUCHIS. , 2009.
49. Hafner, A., et al., *Melatonin-loaded lecithin/chitosan nanoparticles: Physicochemical characterisation and permeability through Caco-2 cell monolayers.* International Journal of Pharmaceutics, 2009. 381(2): p. 205-213.
50. Krauland, A.H. and M.J. Alonso, *Chitosan/cyclodextrin nanoparticles as macromolecular drug delivery system.* International Journal of Pharmaceutics, 2007. 340(1-2): p. 134-142.
51. Perry, M.C., et al., *The interaction of insulin with phospholipids.* Biochemical Journal, 1971. 125(1): p. 179-187.
52. Dubin, P.L. and J.M. Murrell, *Size distribution of complexes formed between poly(dimethyldiallylammonium chloride) and bovine serum albumin.* Macromolecules, 1988. 21(7): p. 2291-2293.

53. Leung, P.S., et al., *A study of polycation-anionic-surfactant systems.* Colloids and Surfaces, 1985. 13(C): p. 47-62.
54. Al-Achi, A. and R. Greenwood, *Intraduodenal administration of biocarrier-insulin systems.* Drug Development and Industrial Pharmacy, 1993. 19(11): p. 1303-1315.
55. Pons, M., et al., *Liposomes obtained by the ethanol injection method.* International Journal of Pharmaceutics, 1993. 95(1-3): p. 51-56.
56. Filipović-Grčiać, J., et al., *Mucoadhesive chitosan-coated liposomes: Characteristics and stability.* Journal of Microencapsulation, 2001. 18(1): p. 3-12.
57. Rinaudo, M., et al., *Solubilization of Chitosan in Strong Acid Medium.* International Journal of Polymer Analysis and Characterization, 1999. 5(3): p. 267-276.
58. Rowland, R.N. and J.F. Woodley, *The stability of liposomes in vitro to pH, bile salts and pancreatic lipase.* Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism, 1980. 620(3): p. 400-409.
59. Sullivan K.A, et al., *Mouse model in diabetic neurophaty.* Neurobiology of Disease, 2007. 28(3): p. 276-85.
60. Garcia-Fuentes, M., et al., *A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery.* European Journal of Pharmaceutical Sciences, 2005. 25(1): p. 133-143.

PARTE I

Antecedentes, hipótesis y objetivos

Artículo 1

Discusión

DISCUSION PARTE I

Los materiales y métodos empleados para la realización de este trabajo están descritos en el artículo correspondiente.

Como se ha mencionado previamente, el objetivo general de la primera parte de esta tesis ha sido desarrollar un sistema nanoparticular destinado a la administración de insulina por vía oral. Para ello, y teniendo en cuenta las propiedades descritas en la introducción, se seleccionaron lecitina (LEC) y quitosano (CS) para diseñar dos tipos de sistemas. Por una parte, un sistema simple (nanocomplejos) preparado por la interacción de LEC con el principio activo, la insulina (INS). Y un sistema mixto (nanopartículas) que combina LEC y CS. La técnica utilizada para elaborar estos sistemas es sencilla, versátil y no requiere la utilización de compuestos orgánicos, temperatura ni energía [96b]. En ambos sistemas, la principal variable estudiada durante el proceso de formación fue la carga neta de la insulina. En los nanocomplejos de LEC-INS, el péptido cargado negativamente presenta diferente grado de ionización. En tanto que en las nanopartículas de LEC-CS, la insulina se incorporó al sistema cargada positiva o negativamente, al mismo tiempo que se varió la cantidad de quitosano añadida. De este modo, se estudiaron las propiedades fisicoquímicas de los sistemas nanoparticulares formados, su capacidad de asociar y liberar insulina y su estabilidad en diferentes fluidos fisiológicos simulados representativos de los jugos gastro-intestinales. De esta forma, se identificó la mejor formulación de insulina destinada a la administración por vía oral. Además, se estudiaron los niveles de glucosa tras la administración oral *in vivo* en diferentes modelos animales experimentales de diabetes tipo I (ratón y rata tratados con STZ: streptozotocina)

Preparación y caracterización de nanocomplejos de LEC-INS y de las nanopartículas de LEC-CS

Para la elaboración de los **nanocomplejos de LEC-INS** se combinaron dos técnicas sencillas empleadas en la preparación de nanosistemas, liposomas por inyección etanólica de lecitina [117] y otra técnica desarrollada en nuestro grupo de investigación, la gelificación iónica [113]. En la primera parte del trabajo experimental, se procedió a la optimización del proceso de obtención de los nanocomplejos de lecitina, evaluándose distintos factores: la relación de masa de LEC e INS, y el grado de ionización de la INS, el cual se modifica por el pH del medio de disolución de la insulina (*p.I*~5.4). Para este trabajo se utilizó acido acético en una rango entre 0.1-2% (v/v).

Para la caracterización de los nanocomplejos de LEC-INS, en una primera etapa se evaluó visualmente el aspecto, realizando una clasificación en función de la composición de la formulación. A continuación, se midió el tamaño de partícula, y sé visualizó la morfología de los sistemas mediante microscopía de transmisión electrónica.

De acuerdo a su aspecto las formulaciones se clasificaron en 3 tipos: agregados (presencia de precipitados), opalescente y transparente. El posterior análisis de tamaño realizado mediante espectroscopía de correlación fotónica (PCS), mostró que los nanosistemas se encuentran en un rango que oscila entre 84 y 240 nm, ya que las formulaciones con precipitados fueron descartadas. Los datos experimentales del tamaño fueron utilizados para crear un mapa de

superficie-respuesta (Fig. 1) entre la relación de masa LEC/INS y la concentración de acido acético empleada para disolver la INS. Los resultados fueron clasificados en varias categorías según su tamaño; liposomas, aquellos con un tamaño ≤ ~90 nm [117] y que aparecían como suspensiones transparentes, mientras que los nanocomplejos presentan un tamaño > ~180 nm con suspensión opalescente. Sin embargo, en la región intermedia coexisten liposomas y nanocomplejos donde el tamaño de partícula medido varía entre 90-180 nm y se ve reflejado en el gráfico de las curvas de distribución de tamaño obtenidas por PCS (Fig. 2).

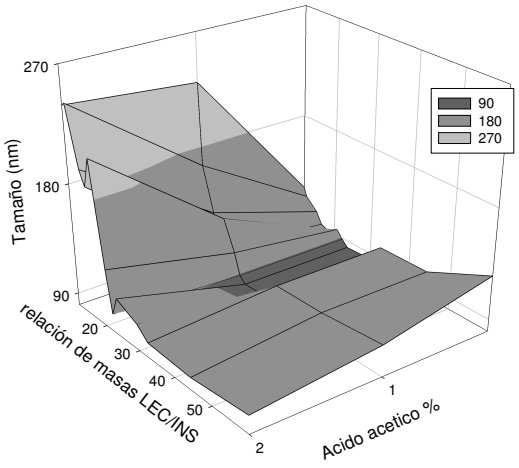


Figura 1: Mapa de superficie del efecto de la relación de masas entre la lecitina/insulina y la concentración de ácido acético sobre el tamaño de partícula.

En la figura 2 se representan las curvas bimodales correspondientes a la relación LEC/INS 60/1, 16/1, 10/1 y liposomas formados únicamente con lecitina. La curva perteneciente a la relación 60/1 y 16/1 revela la presencia de dos poblaciones, al igual que la curva control de liposomas. Estas 3 curvas bimodales revelan la presencia

de dos poblaciones, cuyo pico menor corresponde a una población de tamaño inferior, 10-60 nm. Por el contrario, la curva de distribución de la relación LEC/INS 10/1 es monomodal, con una única población de nanopartículas con tamaño medio de 210±2 nm, correspondiente con los nanocomplejos. Este comportamiento revela que el exceso de lecitina es determinante para la formación de nanocomplejos, así como el incremento en la cantidad relativa de insulina y por tanto, de su protonación. Ello indica que el tamaño aumenta a medida que se neutralizan las cargas de la interacción.

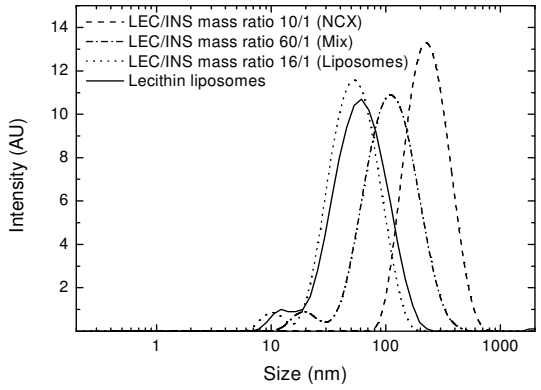


Figura 2: Curvas de distribución de tamaño de los liposomas (línea continua) y sistemas de lecitina-insulina de relación de masas (p/p) 16/1 (línea punteada), 60/1 (líneas y puntos), 10/1 (línea discontinua) en agua a 25°C

En general, los nanocomplejos de LEC-INS muestran superficie cargada negativamente (ζ~-42±4mV) con elevado rendimiento de producción (60±6%). Desde el punto de vista biofarmacéutico, vale la pena indicar que el alto potencial negativo zeta podría favorecer el bioadhesividad y la absorción por células de M y los enterocitos durante la administración oral, como se ha sugerido en estudios anteriores [48].

Una vez evaluados los distintos factores que afectan a la preparación de los nanocomplejos de LEC-INS, se seleccionó la formulación 10/1 LEC/INS preparada con ácido acético al 1%. Para finalizar la caracterización del sistema, se realizó un estudio morfológico con previa tinción con ácido fosfotungístico que se comparó frente a la relación 60/1 LEC/INS (Fig. 3). La visualización mediante microscopia de transmisión electrónica (TEM) reflejó que la relación 60/1 está formada por un núcleo recubierto por una capa de espesor variable (figura 3a). Sin embargo, la imagen de TEM de los nanocomplejos (LEC/INS 10/1) revelan la presencia de especies de tamaño y forma irregular en las que no es posible distinguir una bicapa (Fig. 3b)

a) b)

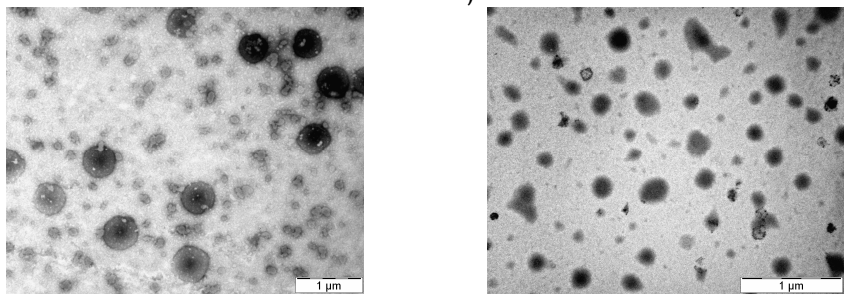


Figura 3. Fotografía de microscopía de transmisión electrónica de lecitina-insulina 60/1 (a) y 10/1 (b)

La elaboración de la **nanopartículas de LEC-CS** fue desarrollada por Sonvico-Cagnani *et al* en colaboración con el grupo de investigación Nanobiofar [30]. La nanopartículas sin fármaco fueron preparadas para diferentes relaciones de masa entre LEC/CS. Para los cocientes 5/1-20/1 LEC/CS, las nanopartículas presentan carga superficial positiva. Sin embargo, al disminuir la cantidad de quitosano hasta alcanzar relaciones de masa 60/1-80/1, la carga superficial se invierte, presentando ζ negativos. Para las relaciones intermedias

LEC/CS 30/1-50/1 se formaban agregados [96b]. En nuestro estudio, para la incorporación la insulina en la nanopartículas se estudiaron dos estrategias modificando la carga de la insulina (positiva o negativa). Variando el pH del medio de disolución del péptido (ácido o básico), se altera la carga neta, y por tanto, condiciona ligeramente el método de preparación. Para llevar a cabo la primera estrategia, la INS se disolvió en ácido acético al 1%, presentando carga neta positiva. Posteriormente, la INS se incorpora a la solución de CS que también presenta carga positiva, minimizando la interacción entre ambos componentes. El método de incorporación de INS aplicado fue el mismo para todas las relaciones LEC/CS, pero se formaron únicamente nanopartículas cuando la cantidad de CS fue suficientemente alta (5/1-30/1), mostrando carga positiva (+28±1 mV) al igual que las nanopartículas sin fármaco. Sin embargo, cuando se redujo la cantidad de CS se formaron agregados y precipitados, debido a la falta de estabilización coloidal en el sistema cuando alcanza valores de carga cercanos a la electro-neutralidad [96, 96b, 115]. Por otra parte, para desarrollar la segunda estrategia, la INS se disolvió en NaOH 0.01N presentando carga negativa. Por tanto, para formar las nanopartículas se incorporó en la disolución de LEC. En este caso, las nanopartículas se formaron para las mismas relaciones de masas que las nanopartículas sin INS; apareciendo nanopartículas con carga superficial positiva y negativa (Tabla 1).con tamaño 202-299nm. Los valores de ζ negativos de estos sistemas pueden ser debido a la formación de bicapas lipídicas de la LEC colocados en la superficie de nanopartícula, por tanto orientando las cabezas negativas fosfolipídicas polares de la LEC hacia la superficie.

Asociación y liberación de la insulina *in vitro* a partir de los nanocomplejos y nanopartículas

La eficacia de asociación presentó porcentajes diferentes al variar el pH de la disolución de insulina. En general, cuando la insulina presentó carga positiva disuelta en ácido acético, la eficacia de asociación (E.A.) del péptido en el sistema es total (97-100%) tanto en los nanocomplejos como en los sistemas con quitosano. El incremento en la eficacia de asociación conseguido supuso una gran ventaja sobre la baja afinidad de la INS en los sistemas tradicionales, como los liposomas [85, 87, 88, 118]. La elevada E.A. en ambos nanosistemas puede explicarse como consecuencia de la carga positiva de la INS al estar disuelta a bajo pH ($p.I._{INS}$ ~ 5.3), que favorece la interacción electrostática con la carga negativa fosfolipídica de la lecitina [96]. Por otra parte, estudios previos realizados con Dispersión de rayo X de ángulo pequeño (SAXS) en nanopartículas de LEC/CS sin carga han permitido postular la formación de estructuras multilaminares [119, 120] capaces de atrapar insulina.

Sin embargo, los porcentajes de asociación variaron cuando la insulina está disuelta en un pH básico. La mejor asociación se presentó cuando el sistema incorpora la mayor cantidad de quitosano (Tabla 1). Este comportamiento se explicaría como consecuencia del desplazamiento de la insulina al competir con la lecitina por su unión al quitosano. En estos sistemas uno puede prever que la INS interacciona directamente con el CS y de ahí, LEC compite por las cargas disponibles positivas del polisacárido.

Para los estudios posteriores se seleccionó la formulación de nanopartículas que presentó mayor eficacia de asociación, características físico-químicas más favorables y mayor estabilidad en medios simulados. La formulación de nanopartículas LEC/CS 5/1 cargada con la insulina negativamente cumplía con estos requisitos.

Tabla 1: Características fisicoquímicas de los nanosistemas de lecitina.

Insulina[a]	**LEC/CS**[b]	**Tamaño** (nm)	**I.P**[c]	**ζ** (mV)	**E.A (%)**	**Estab**[d]
+	LEC-INS[e]	209±18	0. 18-0. 21	-42±4	97-100	+++
+	5/1	230±5	0.24-0.29	+28±1	99-100	_
+	10/1	241±4	0.31-0.36	+28±1	99-100	_
-	5/1	212±9	0.26-0.40	+32±1	83±2	+
-	60/1	248±8	0.15-0.19	- 25±6	22±6	+++
-	80/1	285±14	0.20-0.41	- 20±2	10±5	+++

[a] Insulina disuelta en ácido acético 1% (carga positiva) o NaOH 0.1N (carga negativa).
[b] Relación de masas (p/p)
[c] Índice de polidispersión
[d] Estabilidad en fluido intestinal simulado (pH 6.8) fue clasificada en: (+++) buena ≥ 90 min, (+) adecuada < 60 min e (-) inestable (agregación inmediata).
[e] Nanocomplejos de lecitina-insulina (10/1).

La liberación *in vitro* de la insulina se estudió a 37°C y con agitación horizontal para mimetizar las condiciones fisiológicas. Los perfiles de liberación obtenidos se representan en la Figura 4. Se observa un perfil bifásico, caracterizado por una rápida liberación inicial ("efecto de estallido") seguida de la ausencia de liberación durante 4 horas. La liberación inicial se atribuye a la disociación del péptido localizado en la superficie del sistema, mientras que la segunda etapa se debe a la

alta afinidad del péptido por los componentes del nanosistema que evitan su liberación al medio. La liberación de INS de los nanocomplejos y de las nanopartículas difiere del perfil de liberación de estudios previos con liposomas, oscilando entre 30- 97% dependiendo de la composición del sistema [85, 121]. Mientras que para las nanopartículas de LEC/CS no existen estudios de liberación con proteínas, aunque el estudio de liberación con tamoxifeno en fluidos gastrointestinales sin enzimas demostró la ausencia de liberación [116b]. Sin embargo, hasta un 60% de la melatonina asociada a las nanopartículas fue liberada durante 4h de incubación en pH 5.6 dependiente de la relación de masa entre el quitosano y la lecitina [116]. En nuestro estudio, la incorporación de quitosano a la formulación no mostró cambio alguno sobre la liberación.

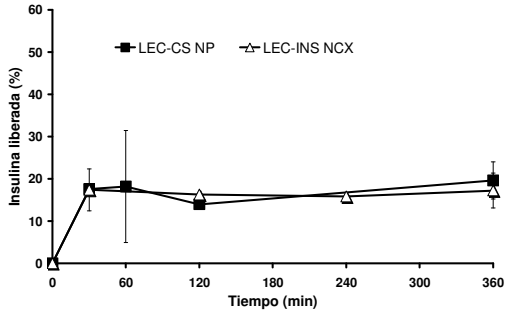


Figura 4. Perfil de liberación de la insulina *in vitro* a partir de nanopartículas lecitina/quitosano 5/1 (cuadrados negros) y nanocomplejos de lecitina/insulina 10/1 (triángulos blancos) en medio intestinal simulado pH 6.8 sin enzimas a 37 ◦C y agitación horizontal (media ± D.E., *n* = 3).

Estabilidad

La evaluación de la estabilidad de estos sistemas se realizó siguiendo la evolución del tamaño de partícula tras su incubación en fluidos gastro-intestinales simulados (USP XXIII) durante 90 min. Los

nanocomplejos y la formulación de nanopartículas LEC/INS 5/1 con la insulina cargada negativamente presentaron excelente estabilidad en fluido gástrico simulado (Fig. 5). Por otra parte, las nanopartículas de LEC-CS que incorporan la INS disuelta en ácido acético (p.e. con carga neta positiva) no fueron estables en fluido intestinal simulado (pH 6.8). Esto se atribuye a la agregación resultante de la neutralización de los grupos $-NH_3^+$ en el CS (pK_0 ~6.0±0.1) [122]. Sin embargo, las partículas formadas con INS cargada negativamente demostraron poseer muy buena estabilidad, aún para la formación de baja relación de masa LEC/CS que tuvieran carga superficial positiva. En este caso la estabilidad se puede atribuir a la interacción de la INS con la cadena de CS.

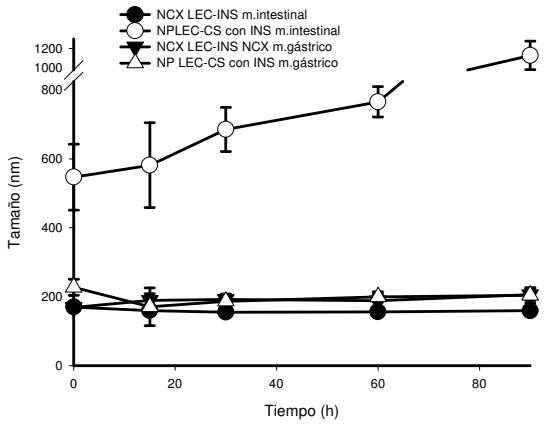


Figura 5. Evolución del tamaño de partícula durante la incubación en medio intestinal pH 6.8 (círculos) y gástrico pH 1.2 (triángulos) simulados sin enzimas de nanocomplejos (NCX) lecitina-insulina (LEC-INS) (símbolos negros) y nanopartículas (NP) de lecitina-quitosano (LEC/CS) cargadas con insulina (INS) (símbolos blancos) a 37°C (media ±D.S, n = 3).

Estudios in vivo.

Respuesta farmacológica tras la administración oral de nanocomplejos le lecitina-insulina en rata diabética

Inicialmente investigamos la eficacia de los nanocomplejos de LEC-INS administradas mediante vía oral en rata diabética, comparando la respuesta con dos grupos controles: los liposomas y el salino. Inesperadamente, los grupos control provocaron un incremento en la glucemia durante 10 h probablemente debido al estrés del animal en el experimento. Sin embargo, la formulación de nanocomplejos de LEC-INS 10/1 redujo los niveles de glucosa hasta un ~30%, alcanzando su máximo 5 horas después de la administración. El efecto observado sobre la glucemia con los nanocomplejos fue mayor que la reducción inducida por diferentes tipos de liposomas administrados por vía oral [84, 85]. De acuerdo a estos resultados, los nanocomplejos demostraron ser capaces de proteger la insulina de la degradación gastrointestinal y aumentar su absorción (Fig. 6).

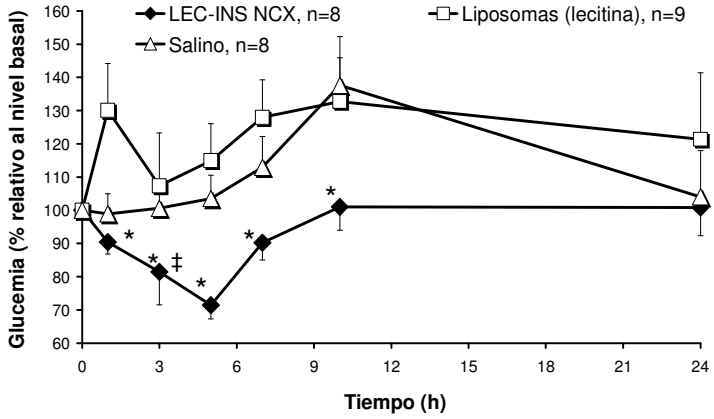


Figura 6. Niveles relativos de glucosa en plasma en ratas diabéticas conscientes tras administración oral (media ± E.E, n=8) de nanocomplejos LEC/INS 10/1 (diamantes negros), liposomas (cuadrados blancos) y el salino (triángulos blancos). La dosis administrada fue ~35UI/kg.*Diferencias estadísticamente significativas ($p \leq 0.05$) con respecto a ambos grupos control, excepto ‡ ($p \leq 0.065$) respecto al grupo salino.

Respuesta farmacológica tras la administración oral de nanopartículas de lecitina-quitosano en ratón diabético

Tras los resultados conseguidos con los nanocomplejos lecitina-quitosano, se refuerza la hipótesis de que la incorporación de CS en la formulación supondría un beneficio para la formulación. Entre las posibles mejoras se pretende prolongar el tiempo de residencia del sistema gracias a sus propiedades mucoadhesivas, e incrementar la absorción del fármaco al modificar la permeabilidad del epitelio intestinal. De acuerdo a la hipótesis planteada, la Figura 7 demuestra el descenso de los niveles de glucosa en el grupo tratado con las nanopartículas de LEC/CS (5/1) cargadas con insulina. En las 2 h posteriores a la administración, se alcanzaron valores normoglicémicos con una reducción del 80% de los valores basales, prolongándose el efecto durante las 12 h siguientes con reducciones mayores del 60% respecto a la situación basal. Estos resultados presentan una mejora frente a los efectos obtenidos con otros nanosistemas preparados con quitosano, [62, 63, 65-67, 69,70,70a] y con polímeros sintéticos [50, 73-76, 78, 80], en los que la C_{min} de glucosa ≥60% del valor basal y T_{min} se produce a partir de las 5 h tras la administración. Los resultados de glicemia encontrados en sangre fueron confirmados con los niveles de glucosa en orina (glucosuria) después de 8 h de administración (Figura 8).

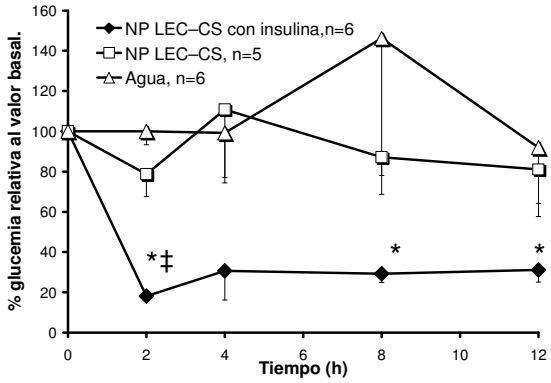


Figura 7. Niveles relativos de glucosa en plasma en ratón diabético conscientes tras administración oral (media ± E.E, n=3-7) de nanopartículas de lecitina-quitosano, relación LEC/CS 5/1 cargadas con insulina (diamantes negros), nanopartículas LEC/CS 5/1 blancas (cuadrados blancos) y agua (triángulos blancos). La dosis administrada fue ~166UI/kg. *Diferencias estadísticamente significativas ($p \leq 0.05$) con respecto al grupo tratado con NP blancas ‡Diferencias estadísticamente significativas ($p \leq 0.05$) con respecto al grupo tratado con agua.

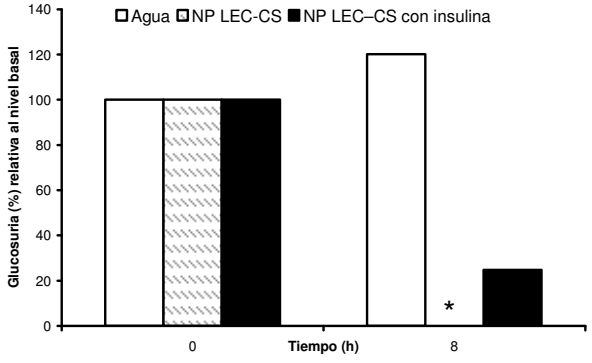


Figura 8. Niveles relativos de glucosa en orina en ratas diabéticas conscientes tras administración oral (n=1 jaula con 4 ratones)) de nanopartículas de lecitina-quitosano, relación LEC/CS 5/1 cargadas con insulina (barras negras), sin carga (barra gris) y agua (barras blancas).* ausencia de orina a las 8h para el grupo de las nanocápsulas blancas

Respuesta farmacológica tras la administración oral de nanopartículas de lecitina-quitosano en rata diabética anestesiada

Tras la administración de la formulación de insulina por vía oral, el parámetro más empleado para determinar la eficacia del tratamiento es la variación de los niveles de glucosa. Sin embargo, como hemos visto, la glucemia es un parámetro que varía por múltiples causas, entre ellas, el estrés es un factor que causa incrementos erráticos en los niveles de glucosa. Para evitar este inconveniente causado por la manipulación del animal durante la administración y recogida de sangre, se optó por la utilización de animales anestesiados.

La figura 9 representa variación de los niveles de glucosa en sangre tras la administración de nanopartículas de LEC/CS (5/1) cargadas con insulina por vía oral. En el mismo experimento se compararon las variaciones de insulina tras la administración en solución por vía intravenosa.

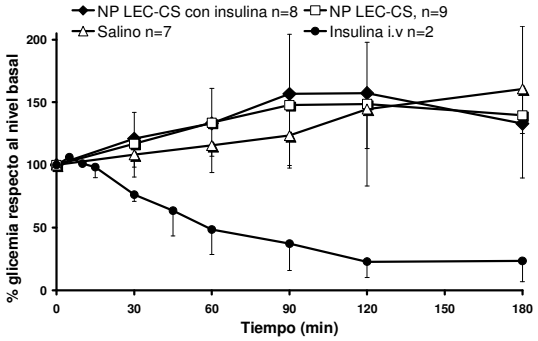


Figura 9: Evolución de los niveles de glucosa en plasma tras la administración oral de las nanopartículas cargadas con insulina (diamantes negros), nanopartículas sin carga (cuadrados blancos) y el salino (triangulos blancos) en ratas diabéticas anestesiadas. Solución de insulina se administró por vía intravenosa (círculos negros), (media ± E.E). La dosis administrada por vía oral 50UI/kg, mientras que por vía i.v 2UI (~7.3UI/

El gráfico muestra que, tanto la formulación cargada con insulina como los controles, producían un aumento en la glucemia. Este incremento inesperado se atribuye al efecto del anestésico que causa potentes efectos depresores cardiocirculatorios y respiratorios que modifican el metabolismo de la glucosa impidiendo reproducir la respuesta fisiológica de un animal consciente. Este efecto también podría deberse eventualmente a la respuesta contrareguladora causada por el muestreo de sangre sobre los glucocorticoides o catecolaminas que tienen efectos hiperglucemiantes. Además, el control de insulina administrada por vía i.p a las ratas anestesiadas presenta un marcado retraso en el tiempo de respuesta de descenso de glucosa.

Respuesta farmacológica tras la administración oral de nanopartículas de lecitina-quitosano en rata diabética.

Partiendo de los resultados obtenidos en ratón diabético, se abordó el estudio el efecto hipoglucemiante de las nanopartículas de LEC-CS en rata diabética por administración de STZ consciente.

En este caso, el efecto medio encontrado en el grupo tratado con nanopartículas de LEC/CS cargadas con INS no disminuyó la glucosa en el plasma (Figura 10), contrariamente a lo observado en el modelo ensayado, el ratón.

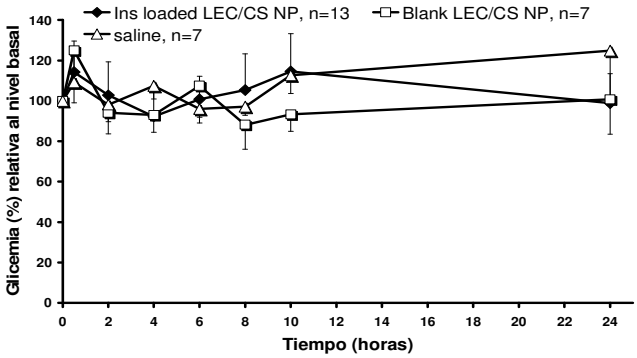


Figura 10. Niveles relativos de glucosa en plasma en ratas diabéticas conscientes tras administración oral (media ± E.E) de nanopartículas de lecitina-quitosano, relación LEC/CS 5/1 cargadas con insulina (diamantes negros), nanopartículas de lecitina-quitosano, relación LEC/CS 5/1 sin carga (cuadrados blancos) y agua (triangulos blancos).La dosis administrada fue ~190UI/kg.

Sin embargo, en el estudio pormenorizado de los resultados se encontró que hasta el 30% de las ratas tratadas con nanopartículas cargadas con INS por vía oral demostraron disminuir la glucosa. Los resultados estadísticos se analizaron mediante el test de χ^2 creando una tabla de contingencia (Tabla 2) cuya hipótesis de partida es la respuesta hipoglucemiante ≥40% en cualquier punto tras la administración. Los animales del grupo que recibieron las NP cargadas con INS presentaron respuestas con valores de glucosa significativamente menor respecto de las halladas en los grupos control (*p≤0.005)*

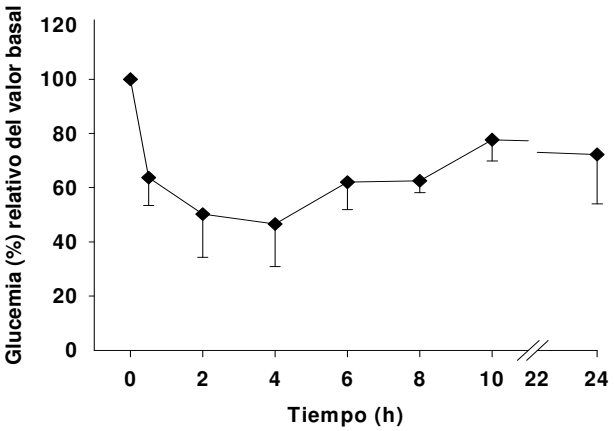
Tabla 2: Tabla de contingencia para los efectos de las nanopartículas de lecitina-quitosano cargadas con insulina (NP –INS), nanopartículas de lecitina-quitosano sin carga (NP –Bl) o salino tras administración oral sobre los niveles de glucosa en el plasma de ratas diabéticas.

H_0: ≥ 40% descenso de glicemia respecto al nivel basal			
Tratamiento	**positivo**	**negativo**	**total**
NP -INS	4[a,*]	9	13
NP -Bl	0	7	7
Salino	0	7	7

[a]C_{max} (%)= 46,5±15.7 (datos ± E.E); T_{max} 4 h; T= 0.5- 10 h (≥ 20 %)
*Diferencia estadísticamente significativa respecto de los grupos controles (*p≤0.005*)

El perfil hipoglucemiante del 30 % de las ratas resultó similar (figura 11A) a la curva de reducción de la glucemia demostrada tras la administración subcutánea de 2.5UI/kg de INS NHP en el mismo modelo diabético (Figura 11B) [68]. Las NP de LEC-CS consiguieron extender la respuesta más allá de las 8 h, prolongándose hasta 24 h después de la administración oral

a)



b)

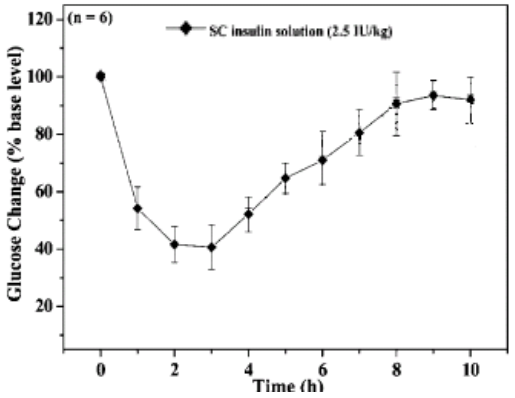


Figura 11: Efecto hipoglucémico en plasma en ratas diabéticas conscientes tras administración a) nanopartículas de lecitina-quitosano (LEC/CS 5/1) cargadas con insulina por vía oral, (190UI/kg) (media ± E.E, n=4). b) solución de insulina bovina por vía subcutánea (2.5 UI/kg) (n=6) [68].

Estos resultados ponen de manifiesto, una vez más, la capacidad del quitosano para interaccionar con el epitelio intestinal y favorecer la absorción de la insulina.

PARTE II

Aplicación de las nanocápsulas de lecitina-quitosano para la vehiculización transmucosal de exendina-4 para el tratamiento de la diabetes mellitus tipo 2

PARTE II

Antecedentes, hipótesis y objetivos

Artículo 2

Artículo 3

Discusión

Parte 2: "Aplicación de las nanocápsulas de lecitina-quitosano para la vehiculización transmucosal de exendina-4 para el tratamiento de la diabetes mellitus tipo 2"

Antecedentes

1. Exenatida, péptido sintético de la exendina-4, ha sido recientemente comercializada para el tratamiento de la diabetes mellitus tipo 2 por vía subcutánea. Su vía de administración limita las indicaciones del fármaco.

2. El quitosano es un polisacárido cuyas características de biodegradabilidad, mucoadhesión y efecto promotor de la absorción a través de diferentes epitelios, lo convierten en un candidato único para el desarrollo de nuevos sistemas de liberación de macromoléculas de utilidad terapéutica a nivel de mucosas. El grado de acetilación, indicativo de la proporción de grupos aminos con residuos acetilados presentes en la molécula de quitosano determina sus propiedades [102, 123] y su estabilidad en diferentes medios [105, 124].

3. El quitosano es la base de numerosos sistemas de administración oral de péptidos [58], entre ellos, las nanocápsulas. Las nanocápsulas de quitosano presentan gran versatilidad como vehículos para la administración de péptidos. El efecto se atribuye a la capacidad del núcleo oleoso formado con lecitina para proteger el péptido [81] mientras que la cubierta de quitosano aumenta la absorción por vía nasal [97] y oral [125-127].

Hipótesis

1. La formación de las nanocápsulas con distintas variedades de quitosano, inducirá una variación en las propiedades de superficie de las nanocápsulas que modula su estabilidad en los distintos medios.

2. La elección del tipo de quitosano para formar las nanocápsulas específicas para cada vía, presentará una mejora en la absorción de la exendina-4 través de las mucosas.

Objetivos

Teniendo en cuenta los antecedentes expuestos y la hipótesis de partida, el objetivo general de la segunda parte de esta tesis se ha centrado en el desarrollo específico de nanocápsulas de quitosano capaces de encapsular eficazmente exendina-4 y transportar el péptido a través de la mucosa nasal y/o oral. Desde el punto de vista práctico, el trabajo experimental llevado a cabo para alcanzar el objetivo general se ha desarrollado según los objetivos concretos que se detallan a continuación.

1. Preparación y caracterización de la nanocápsulas utilizando quitosanos con diferente grado de acetilación y peso molecular. Estudio del efecto del grado polimerización y acetilación sobre las características fisicoquímicas y estabilidad en diferentes medios fisiológicos (artículo 2).

2. Evaluación de la capacidad de las nanocápsulas para asociar y liberar exendina-4 en prototipos específicos desarrollados con quitosano de diferente grado de acetilación. Para ello, es necesario desarrollar un método analítico eficaz y eficiente para la determinación de bajas concentraciones de exendina-4 (artículo 3).

3. Estudio del potencial uso de las nanocápsulas de quitosano como sistemas para la administración nasal y oral de exendina-4. Estudios *in vivo* (artículo 3).

PARTE II

Antecedentes, hipótesis y objetivos

Artículo 2

Artículo 3

Discusión

ARTÍCULO 2

Chitosan–based nanocapsules: Physical characterization, capsaicin encapsulation efficiency and stability in biological media

Goycoolea F.M.[1*], Valle-Gallego, A.[1], Stefani R.[2], Mencicchi B.[1], Santander-Ortega M.J[3], Remuñán-López C.[1],Alonso MJ.[1]

[1]*Department of Pharmacy and Pharmaceutical Technology. Universidad de Santiago Compostela, Campus Sur s/n Santiago de Compostela, 15782 Spain;*

[2]*Dipartimento di Chimica e Tecnologie Farmaceutiche ed Alimentari, via Brigata Salerno 13, 16147 Genova, Italy*

[3]*Department of Applied Physics, University of Granada, Av. Fuentenueva S/N, 18071 Granada, Spain*

*Corresponding author: Francisco M. Goycoolea
E-mail: fm.goycoolea@usc.es

(Manuscript submitted to publication)

Abstract

Core-shell nanocapsules (NCs) intended for the encapsulation of capsaicin, a lipophilic drug currently used in pain therapy, were obtained by solvent displacement. To this end, we have investigated the influence of the degree of N–acetylation (DA ~1.4 – 56%) of chitosan (CS) of low and high molecular weight (M_w) here denoted as LDP CS (low degree of polymerization, DP ~63 – 82; M_w ~ 9.5 – 13.2 kDa) CS and HDP DP (high degree of polymerization DP ~766 – 1442; M_w ~ 122 – 266 kDa), respectively, on the NC's physical properties, capsaicin encapsulation efficiency and colloidal stability in biological media. The resulting NCs had an average particle size within the range of ~150-200 nm and a spherical morphology, irrespective of CS's DA and M_w. The zeta potential (ζ) was invariably highly positive (+26-47 mV) and exhibited a monotonic decreasing trend concomitant with the increase in CS's DA. The encapsulation efficiency of capsaicin in NCs comprising CS LDP-DA 51 was very high (~90%). In general, all systems showed high stability in Minimal essential medium Eagle (MEM) and in endothelial cell growth medium (ECGM), regardless of CS type. However, the stability in Roswell Park Memorial Institute medium (RIMI-1640) was restricted to specific formulations (HDP27 and HDP56 and all LDP CS). The results of this work reveal that varying CS's characteristics allows fine tuning of the physical properties and biological stability of CS–based nanocapsules that could be utilized for future studies addressing intracellular and/or mucosal delivery of lipophilic drugs such as capsaicin.

Keywords: nanocapsules, chitosan, capsaicin, biological stability,

1. Introduction

Colloidal nanocapsules (NCs) comprised by an oily core, lecithin and a hydrophilic coat of chitosan (CS), a natural aminopolysaccharide, have been one of the systems at the focus of our research due to their promising potential as an effective drug delivery platform for transmucosal administration of peptides, lipophilic drugs and vaccines (Calvo et al., 1997; Prego et al., 2006a, 2006b; Prego et al., 2006; Valle-Gallego et al., 2010; Lozano et al., 2008; Vicente et al., 2008). The driving physicochemical mechanism whereby these systems are formed, is by spontaneous emulsification or solvent displacement (López-Montilla et al., 2002), whereby an homogeneous liquid-liquid nucleation occurs as acetone and ethanol, initially admixed in the organic phase, migrate to the aqueous phase, yielding an o/w nanoemulsion stabilized by the surfactant (lecithin) adsorbed at the o/w interface. Either during its formation or in a subsequent incubation step, a water soluble polymer can be incorporated as coat by ionic interaction with the phospholipids of lecithin, thus effectively, yielding a core-shell colloidal stable nanocapsule structure.

Our rationale for the design of new nanocarrier systems, such as oily-core nanocapsules and matrix nanospheres, has been to exert control not only the size, but also the surface properties and composition of these systems, so that the stability in biological fluids and the interaction with mucosal surfaces can be adapted to achieve an adequate transmucosal drug delivery. In this regards, it has been recognized that CS significantly

improves the ability of NCs to transport drugs across the corneal epithelium (Calvo et al., 1997). Further evidence has shown that CS NCs are excellent prototypes for the nasal (Prego et al., 2006b) and oral (Prego et al. 2006a; Prego et al. 2006) delivery of biologically active peptides, such as salmon calcitonine (sCT). Other studies have revealed that these systems are effective for the intracellular delivery vehicle of cytotoxic lipophilic drugs, such as docetaxel (Lozano et al., 2008). In a parallel study, we have also addressed the utilization of nanocapsules coated with a CS of Mw ~ 266 kDa and degree of N-acetylation (DA) of 56% for the encapsulation of exendin–4 (Valle-Gallego et al., 2010), a recently antidiabetic peptide currently prescribed for the treatment of diabetes melitus type II. It was found that these systems are able to encapsulate >90% of the peptide drug at the oily core and they are effective for the intranasal delivery of the drug. Moreover, this nanocapsule system has shown to be an effective intracellular delivery vehicle for the intranasal administering of vaccines, i.e. Hepatitis B surface antigen (Vicente et al., 2008).

Due to the fact that CS is commercially available either as the neutral polymer or in various salt forms (typically as the hydrochloride or glutamate salts), with varying M_w and DA, previous studies have addressed the role of the salt form and M_w of CS utilized to coat the NCs on their physical properties (size and zeta potential) (Calvo et al., 1997), stability and drug encapsulation efficiency. The *in vitro* cytotoxicty and *in vivo* transport properties during oral administration to rats were also examined (Prego et al., 2006b). The results of these studies have

consistently shown that the size and, to less an extent, the zeta potential, are influenced by CS's M_w. In general, it has been observed that an increase in CS's M_w leads to larger NCs and to a slight elevation in ζ, while the type of CS salt form had no discernible effect on the physical properties. These effects were hypothesized to stem in differences in the thickness of the NC's coat (Prego et al., 2006a). In turn, *in vitro* studies in Caco-2 cell monolayers have revealed that neither CS's salt form nor the M_w (in the range 160-450 kDa) have a significant effects on the viability of the cells after an exposure to NCs during 2 h nor in the transepithelial electrical resistance, TEER (Prego et al., 2006a). Meanwhile, in the same study, it was found that the presence of the CS coat was essential for the capacity of NCs to achieve a significant pharmacological hypocalcemic response after oral administration of sCT-loaded NCs to rats, but no differences in this capacity were associated to CS's M_w.

In the present study, we have carried out a systematic investigation on the role of the far less studied effect of CS's degree of acetylation (DA) on the physical properties and stability on biological fluids of CS NCs. We have extended the lower range of M_w of CS of previous studies, to ~10 kDa and have worked with the neutral form of CS dissolved in stoichiometric amounts of acetic acid. From the influence of CS's DA and M_w on the zeta potential and on the relative thickness of the shell coating the nanocapsules at varying pH, here we advance a model to account for the way in which CS organizes at the NC's surface and account for differences observed in the stability of NCs in simulated biological conditions. For selected NCs, we have also studied the capacity

to associate capsaicin (8-methyl-N-vanillyl-6-nonenamide), a lipophilic drug, in the oily core of the nanocapsules. Capsaicin, the pungent vainilloid found in hot chilli peppers, is approved for the treatment of chronic pains (e.g. arthritis, migraine, diabetic neuropathy) by topical administration due to the analgesic activity associated to its capacity to depleting substance P in small fibre nociceptor neurons on which transient receptor potential vainilloid-1 (TRPV1) cation channel is predominantly located (Cortright and Szallasi., 2004; Bevan and Szolcsányi., 1990). As many proalgesic pathways converge on TRPV1 and this nocisensor is upregulated and sensitized by inflammation and injury, TRPV1 is thought to be a central transducer of hyperalgesia and a prime target for the pharmacological control of pain. Moreover, the activation of TRPV1 by capsaicin enables the influx of membrane-impermeate local anaesthetics such as lidocaine derivatives into the intracellular space, thus allowing to block conduction only in sensory neurones that express the TRPV1 receptor. This could be exploited in that local anaesthetics can be made selective for nociceptive afferent neurones, avoiding their unwanted action on non-nociceptive sensory, autonomic and motor neurons (Binshtok et al., 2007). In light of this, identifying new potential nanocarriers for the delivery of capsaicin intracellularly, transdermally and/or to the brain by intranasal administering, so as to regulate the activity of TRPV1 receptors, appeals as a potential emerging therapeutic strategy. To complement the study, we have also addressed the stability of NCs in commercial biological media commonly used to grow mammalian cells to identify the most promising systems for future studies in this direction.

2. Materials and methods

2.1. Materials

A series of CS samples were prepared and purified from a parent batch of CS obtained from chitin sourced from squid pen supplied by Dr. Dominique Gillet of Mahtani Chitosan Pvt Ltd (France). To this end, purified CS was depolymerised under nitrous acid generated from $NaNO_2$ (Allan and Peyron., 1995) in order to obtain two CS batches of high and low degree of polymerization (M_w ~122 kDa and 11 kDa, respectively, as determined by HPLC SEC-MALLS), here referred to as HDP CS and LDP CS, respectively. Portions of HDP CS and LDP CS samples were further N–acetylated under homogeneous conditions by adding the needed stoichiometric amount of acetic anhydride in 1,2–propanediol (Lamarque et al., 2005) so as to afford CS with DA values (determined by ^{1}H NMR) varying in the range 1.6 to 56% for HDP CS and 1.4 to 51% for LDP CS. A commercial CS sample of pharmaceutical grade, ChitopharmS® (M_w ~279 kDa and DA ~20% as certified by supplier), was a kind gift from Cognis GmbH (Düsseldorf) and was used without further purification; lecithin (Epikuron 145V) was from Degussa (Spain); Miglyol 812® (caprylic/capric triglycerides) was supplied by Lemmel (Spain). Capsaicin (purity ≥ 99.0%) was purchased from Sigma-Aldrich (Stenheim, Germany); all other reagents and solvents were of analytical or HPLC grade. Milli-Q quality water was used throughout.

2.2. Preparation of nanocápsulas

CS-based nanocapsules were prepared according with the protocol originally developed in our laboratory (Calvo et al., 1997) slightly modified by avoiding the use of poloxamer in the aqueous phase. Briefly, 20 mg of lecithin were dissolved into 250 □ L of ethanol, then 62.5 □ L of Miglyol 812® were added, followed by addition of 4.75 mL of acetone. Immediately afterwards this organic phase was poured into of 10 mL of CS solution (0.5 mg/mL) in 5% stoichiometric excess of acetic acid. Immediately upon addition of the organic phase into the aqueous CS solution it turned milky. Acetone, ethanol and a portion of the volume of water were evaporated in a rotavapor at 40°C for ~8 min to a final volume corresponding with one third of the original one. A nanoemuslion (NE) was prepared under an identical protocol as that used for CS NCs, but without including CS in the aqueous phase.

2.3. Physical characterization

2.3.1. Size and zeta potential

The size distribution of NCs was determined by photocorrelation spectroscopy using non-invasive backscattering (PCS-NIBS, measurement angle 173º) and the zeta potential (ζ) was measured by mixed laser Doppler velocimetry and phase analysis light scattering (M3–PALS), both using a Malvern Zetasizer NanoZS Zen 3600 (Malvern Instruments, U.K.) fitted with a red laser light (λ=632.8 nm).

2.3.2. Chitosan association

The net proportion of CS associated with the NCs was measured indirectly by quantification of the concentration of the polymer remaining in the aqueous medium after separating the NCs by ultracentrifugation at 18000 rpm for 1 h at 20°C on a Beckman centrifuge Coulter Optima L-90K (Fullerton, CA). To this end, the colorimetric test based in the reaction of CS with Cibacron brilliant red 3B-A (Muzzarelli 1998) was scaled to a microplate assay.

2.3.3. Transmission electron microscopy (TEM)

The ultrastructure of the various systems was probed on a Philips CM12 TEM instrument (Eindhoven, The Netherlands). To this end, 10 μL of a 2% solution of phosphotungstic acid was mixed with an equal volume of a 1:100 dilution of nanocapsules in water. Immediately afterward, an aliquot of 5 μL was immobilized on a copper grid coated with a Formvar® membrane and allowed to dry.

2.4. Drug encapsulation

To incorporate capsaicin into the NCs an identical preparation protocol to the one described above was adopted but incorporating an accurately weighed amount of the drug (5% load) dissolved it in the ethanolic lecithin solution. The CS samples tested for this purpose were LDP-DA 1.4, LDP-DA 51 and ChitopharmS®. The encapsulation efficiency (E.E.) of capsaicin was determined indirectly by calculating the difference

between the total amount of capsaicin incorporated in the formulation and that determined remaining in the aqueous medium after separating the NCs by ultracentrifugation at 18000 rpm for 1 h at 20°C on a Beckman centrifuge Coulter Optima LK-90K (USA). Capsaicin contents were determined by an HPLC-FLD method as described elsewhere (Wang et al., 2001).

2.5 Stability in biological media

The colloidal stability of the developed NCs was investigated in the following cell culture media: a) Minimal essential medium Eagle (MEM M4655, Sigma-Aldrich) (pH 7.45), supplemented with 10% (v/v) of fetal bovine serum (FBS, GIBCO-Invitrogen) and 1% (v/v) of L-glutamine (Sigma-Aldrich), and 1% (v/v) of penicillin/streptomycin solution (GPS, Sigma); b) Endothelial cell growth medium (ECGM, PromoCell GmbH, Heidelberg) (pH 7.41) supplemented with 5% (v/v) fetal calf serum (PromoCell GmbH, Heidelberg), 0.4% (v/v) endothelial cell growth supplement/heparin (PromoCell GmbH, Heidelberg), 10 ng/mL epidermal growth factor (PromoCell GmbH, Heidelberg) and 1 μg/mL hydrocortisone (PromoCell GmbH, Heidelberg); c) RPMI-1640 medium (PAA Laboratories GmbH) (pH 7.34) supplemented with 6% (v/v) fetal bovine serum (PAA Laboratories GmbH), 1% (v/v) L-glutamine and 0.2%(v/v) penicillin/streptomycin solution (GPS, Sigma-Aldrich). The stability was evaluated in terms of the evolution of the particle size distribution over time during incubation at 37°C for up to ~48h. Size

measurements at given time intervals were registered by PCS-NIBS as described above.

3. Results

3.1. Chitosan samples characteristics

The characteristics of the CS samples utilized in this study are shown in Table 1. These CS samples are currently being used in several parallel ongoing studies addressing their bioactivity and that of nanoparticles and nanocapsules against plant, fungi, bacterial and cultured mammalian cells.

Table 1. Physicochemical characteristics of chitosan samples

Chitosan Sample	D.A[a] (%)	M_w	M_n	I_p	DP[d]
HDP-DA1	1.6[a]	123900[c]	89000[c]	1.39[c]	766
HDP-DA11	11.0[a]	122100[c]	75330[c]	1.62[c]	737
HDP-DA27	27.5[a]	143000[c]	85710[c]	1.67[c]	818
HDP-DA56	56.0[a]	266100[c]	139700[c]	1.90[c]	1442
LDP-DA1	1.4[a]	13200[c]	11130[c]	1.19[c]	82
LDP-DA9	9.2[a]	9572[c]	5914[c]	1.62[c]	58
LDP-DA27	27.8[a]	11530[c]	8120[c]	1.42[c]	67
LDP-DA51	51.0[a]	11420[c]	6012[c]	1.90[c]	63
ChitopharmS®	20.0[b]	279200[b]	97100[b]	2.88[b]	1648

[a] Degree of acetylation as determined by ^{1}H NMR spectroscopy

[b] Data according with specifications given by supplier

[c] Parameters determined by GPC–HPLC with multidetection (MALLS–DRI): weight average molecular weight (M_w); number average molecular weight (M_n); polydispersity index ($Ip = M_w/M_n$);

[d] Degree of polymerization (DP = M_w /molar mass per residue)

3.2. Nanocapsules characterization

3.2.1 Z-average size

The nanoemulsions appear without CS-coating had a Z–average size of 151 ± 2 nm. In turn, nanocapsules were invariably afforded after coating with the CS samples used. Figure 1 shows the dependence of Z-average size of the NCs coated with HDP CS and LDP CS samples of varying DA. Notice in Figure 1a that for HDP CS NCs the size was found to lie in the range ~175–219 nm and polydispersity index, PI ~0.14 – 0.19, whereas LDP CS systems exhibited slightly lower average sizes that varied in the range ~127–158 nm (PI ~0.13 – 0.21) for CS with DA 1.2 to 27.8%; whereas for LDP-DA51 though, the size was notably greater (~189 ± 12 nm). In general, the PDI values, derived from the second cumulant of the fitted decay autocorrelation function, were small (<~0.21) and monomodal size distribution curves were invariably recorded for all systems. Notice that LDP-DA51–coated NCs exhibited a greater size than the rest of particles coated with LDP CS of DA 1.4 – 27.5%. The increment in size is not as notable for HDP-DA56–coated as for LDP-DA51 NCs.

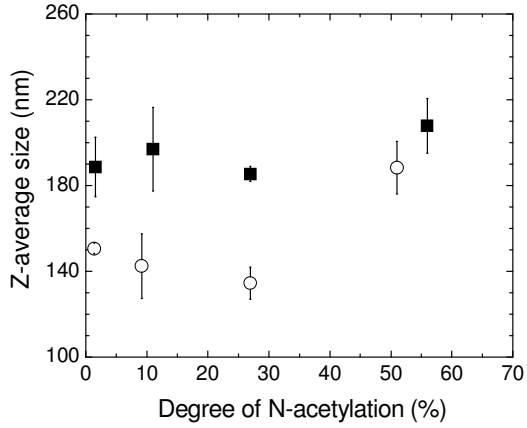


Figure 1. Effect of the variation of the degree of N-acetylation of chitosan on the Z-average diameter of core–shell nanocapsules comprising chitosan of high (squares) or low (circles) molecular weight (water at 25ºC; mean ± SD; *n*=3).

3.2.2 Zeta potential (ζ)

By contrast with the variations in size with DA, ζ was found to decrease under a non-linear monotonic trend with increasing DA for both LDP CS and for HDP CS NCs (Figure 2). For systems comprising LDP CS, □ decreased from +39 to + 26 mV (i.e. 33% reduction) while for HDP CS NCs, it did so from +47 to + 36 mV (i.e. 23% reduction). In both cases the reduction in ζ corresponded with the change in CS's DA between ~1.4 and ~56% (Figure 2). This is rationalized as the consequence of the reduction in CS's positive charge density due to the decrease in the molar fraction of D-glucosamine residues. In both cases, a linear negative dependence was observed for DA ≥ ~9%. Overall, LDP-based systems exhibited slightly less positive ζ values than did systems comprising HDP CS.

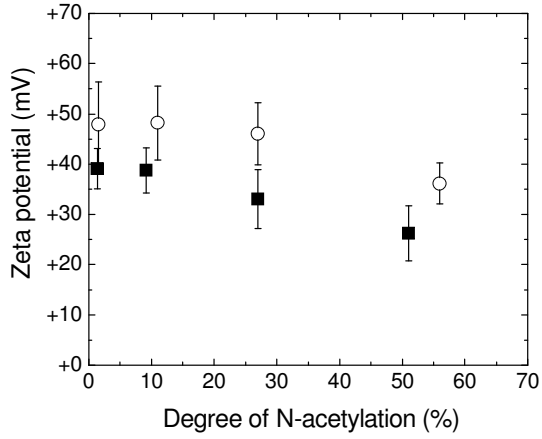


Figure 2. Effect of the variation of the degree of N-acetylation of chitosan on the zeta potential (ζ) of core–shell nanocapsules comprising chitosan of high (squares) or low (circles) molecular weight (NaCl 1mm at 25ºC; mean ± SD; *n*=3).

3.2.3 Chitosan association

In a separate experiment, the relative amount of CS adsorbed to the NCs was assessed from the depletion of CS's original concentration by determining the remaining amount in the supernatant after isolation of NCs by centrifugation. It was interesting that regardless of CS's M_w and DA, nearly all the polysaccharide amount (> 96.8 % of the original added amount) was incorporated into the NCs (Figure 3).

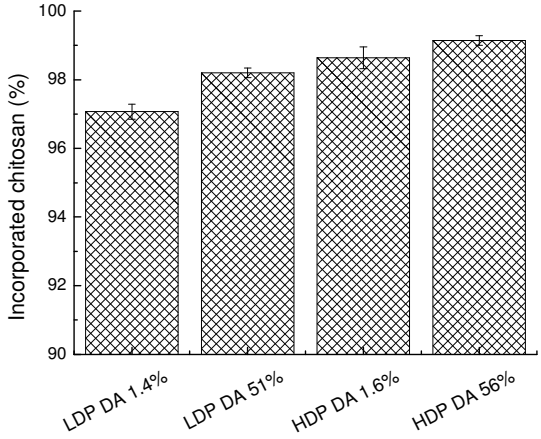


Figure 3. Estimated proportion (%) of chitosan adsorbed at the nanocapsules with respect to the originally added amount (0.5 mg/mL).

3.2.4 Transmission electron microscopy (TEM)

The nanocapsules exhibited a spherical morphology, as unequivocally visualized by TEM for representative systems (Figure 4). In general, the CS NCs (Figures 4a – 4e) exhibited a smoother surface than did the nanoemulsion (Figure 4f). The size of the various imaged NCs lied within the range as determined by PCS-NIBS. A close inspection of the TEM images reveals the presence of a core–shell structure on the various NCs. The coat–shell structure however, is also appreciated for the nanoemulsion (Figure 4f), hence it cannot be attributed solely to adsorbed CS. More detailed aspects of the surface topolgy either lie below the spatial resolution achieved by TEM (i.e. ~4–5 nm) or the contrast achieved after staining with phsophotunstic acid was not high enough.

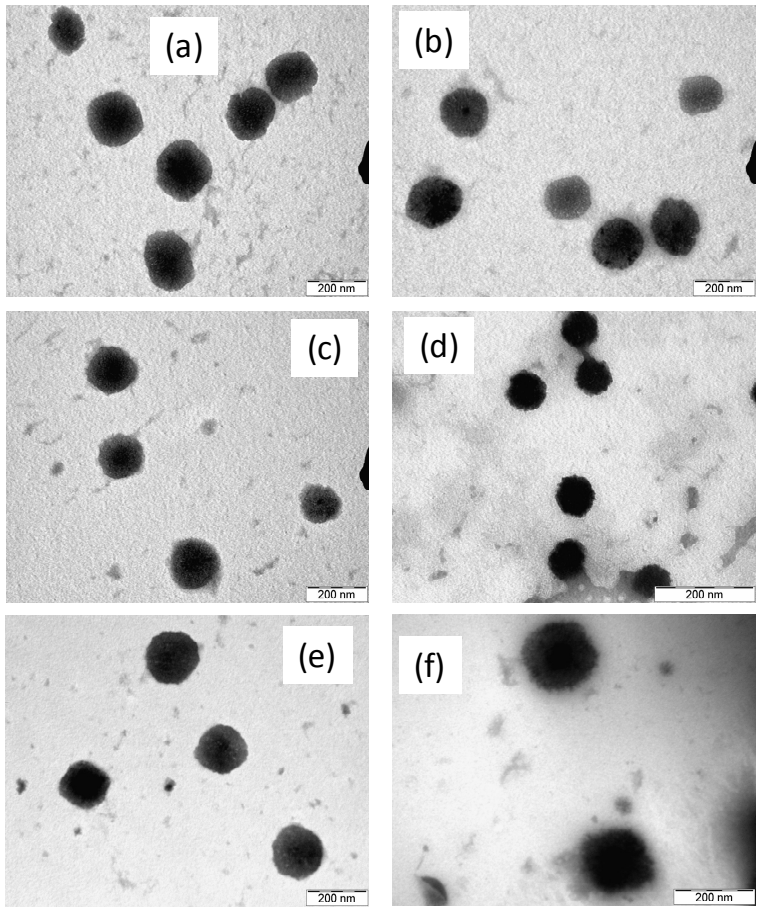


Figure 4. Transmission electron microscopy (TEM) images of nanocapsules made with CS: a) LDP-DA1; b)LDP-DA51; c) HDP-DA1.6; d) HDP-DA11; e) ChitopharmS® and e) nanoemulsion. Magnification as shown in bars.

3.3 Drug encapsulation

Capsaicin was loaded into NCs comprising LDP CS (DA 1.4 and 51%) and commercial CS (ChitopharmS® Mw ~280 kDa; DA ~20 %). Loading of capsaicin did not have any effect on the Z-average size nor size distribution of any of the formulations as shown in Table 2. In turn,

ζ values neither showed variation with the capsaicin load and the values remained very close to those found in blank nanoparticles (Figure 2). The encapsulation efficiency (E.E.) of capsaicin in the tested formulations was high and varied in the range 72-96 % (Table 2). Interestingly, the highest E.E. was measured in systems comprising LDP-DA 51 CS whilst slightly lowest in systems formulated with ChitopharmS®. This can be accounted for on the basis of the differences in M_w and in the physical characteristics of the CS coat structured at the NC's surface, thus affecting the efficacy of entrapment of capsaicin. Nevertheless, the capsaicin encapsulation efficiency found for CS-based NCs of the present work compared closely with previous studies that have nanoencapsulated capsaicin in a gelatin-gum arabic-tannin nanocapsule prepared by complex coacervation with average E.E. of 81% (Xing et al. 2005). The high encapsulation efficiency coincides well with that achieved for other liposoluble peptide drugs, such as exendin–4, in which > 80% of the peptide is encapsulated (Valle-Gallego et al., 2010). Other water soluble peptide drugs, such as sCT have been encapsulated under much lower efficiency (~44.1 ± 3.3 %) (Prego et al., 2006a) due to the charge displacement of sCT by CS.

3.4 Stability in biological media

The stability of NCs was studied during incubation in MEM, ECGM and RPMI-1640, three commonly used commercial media where human cells are cultivated. To this end, the evolution of the particle size distribution was probed by dynamic light scattering (PCS-NIBS) at varying time

intervals up to 48 h at 37ºC. MEM and RPMI are normally used for the cultivation of human cell lines adhered as monolayers or suspended such as normal and neoplastic leukocytes, including fresh lymphocytes and dendritic cells, while ECGM is used to cultivate endothelial cells (e.g. HUVEC). Figure 5 illustrates the time evolution of the particle size distribution curves for representative curves of LDP CS and HDP CS during incubation in supplemented MEM (pH ~7.4) of low (Figures 5a and 5c) or high (Figures 5b and 5d) degree of acetylation. The rest of the tested NCs in MEM had very similar curves to those shown in Figures 5b-5d. The inspection of the plots reveals that, in general, essentially similar Gaussian monomodal size distributions were recorded for all NC systems regardless of the type of CS coat during up to 48 h. The only noticeable difference was registered for HDP-DA1 NCs (Figure 5c) whose plots appeared shifted towards greater values than the rest of the NCs, and also that slightly smaller size values were recorded after 24 and up to 48 h, though the nanoparticles remained stable. The stability of NCs was also studied under an identical protocol during incubation in ECGM and RPMI-1640 cell culture media. Figure 6 shows the kinetics of the evolution of the Z-average size during incubation in these two media. It can be appreciated that the time evolution of the size of both LDP and HDP CS–coated NCs in ECGM (Figures 6a and 6c) depended on CS's DA. The NCs comprising CS of low DA (both LDP and HDP) did show a monotonic increase in size at early incubation times. The elevation in size, however, was moderate and not accompanied by a discernible aggregation process. In turn, systems comprising CS of greater DA, even when they had a lower ζ, were noticeably stable, with virtually no

variation in particle size during the duration of the experiment. By contrast with the results observed in MEM and ECGM, in RPMI-1640 the NCs were much less stable, particularly those comprising HDP CS of low DA. In fact, for NCs containing HDP CS of DA 1.6% aggregated immediately upon addition into the medium as visually observed. Notice in Figure 6d that NCs comprising HDP CS of DA 11% attained particle sizes larger than 1 □m after 200 min and continued increasing beyond this time. For LDP CS, the increase in particle size was also evident for CS of low DA (DA~1.4 and 9%), though these systems did not experience visible aggregation.

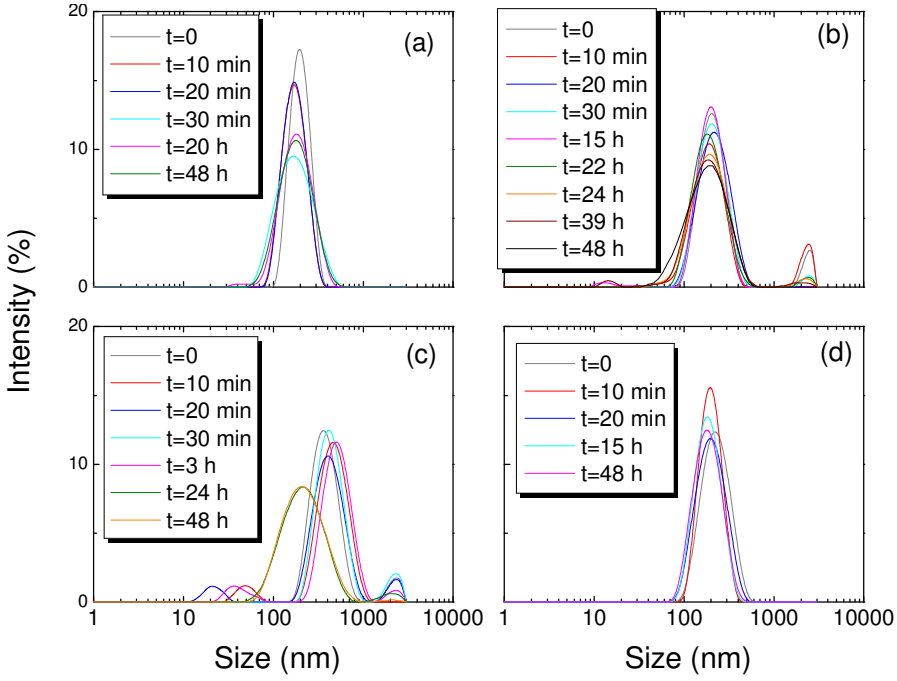


Figure 5. Time evolution of the size distribution curves of nanocapsules incubated (37ºC) in MEM culture medium for nanocapsules comprising the following chitosan types: a) LDP-DA1; b) LDP-DA51; c) HDP-DA1 and d) HDP-DA56.

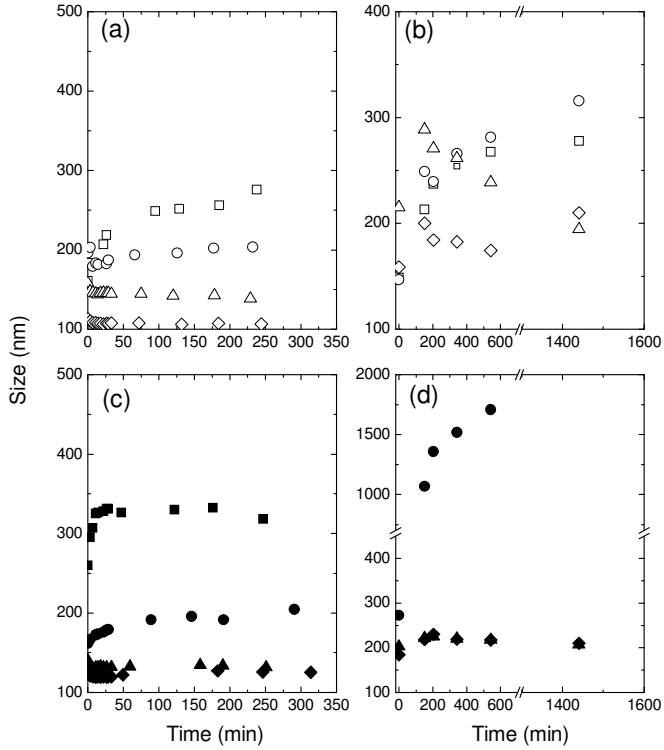


Figure 6. Variation of the Z-average particle size during incubation (37ºC) in ECGM (frames a and c) or RPMI (frames b and d) of chitosan nanocapsules comprising LDP (frames a and b) or HDP (frames c and d) chitosan of degrees of N-acetylation (%) as follows: 1.4 (empty squares), 1.6 (filled squares), 9.0 (empty circles), 11.0 (filled circles), 27 (filled and empty triangles), 51.0 (empty diamonds) or 56.0 (filled diamonds).

4. Discussion

Differences in the overall thickness of the shell that coats the NCs' surface can explain the greater overall particle size found for HDP CS over LDP CS-containing systems, as suggested in previous studies (Prego et al., 2006). It was rather unexpected, though, to notice that LDP 51

NCs exhibited a greater size than the rest of particles with LDP CS of DA 1.4 – 27.5%. As a possible explanation to this phenomenon, we can speculate that CS's M_w does not only affect the overall thickness of the coating shell itself but it also entails differences in the way CS organizes itself at the NC's surface. Shorter chains of LDP CS are likely to pack themselves more densely as a consequence of a flatter arrangement of greater cooperativity than that attained by longer chains of HDP CS, presumably due to a entropic disadvantage associated to the local confinement of larger polymer stretches the NC's surface. In turn, the more hydrophobic and more electroneutral character of CS of high DA (Lamarque et al., 2005) can account for the stacking of an overall greater amount of sample LDP-DA 51, leading to an increase in the shell thickness. Indeed, in CS with DA > ~20% the polyelectrolyte behaviour in solution has been suggested to be replaced by that of isolated charges in a hydrophobic environment with increasing DA (Lamarque et al., 2005; Sorlier et al., 2002). The thickness of the shell layer depends on the nature of the surface, the absolute adsorbed amount of CS and on non-electrostatic surface-CS interactions (e.g. hydrophobic). In previous studies addressing it has been demonstrated that the adsorption of CS to lipid surfaces is mediated predominately by electrostatic interactions of CS and the strength of the interaction is strongly dependent on pH. The suggested mechanism for the interaction is by a flat adsorption of CS at negatively charged solid surfaces (Fäldt et al., 1993). Independent recent studies that have also addressed the interaction of CS of M_w varying in the range ~50 to 500 kDa with lipid-based unilamellar vesicles, also argue that, regardless of pH, CS adsorbs flat on the membrane surface of these

systems (Quemeneur et al., 2007; Quemeneur et al., 2008). One can envisage that the longer polymer chains favour the adsorption of HDP CS under low cooperativity leading to formation of loops and 'trains' type of structures. While LDP CS chains, the greater cooperative binding of CS leads to flatter structures.

Consistent with this interpretation, it was observed that the surface zeta potential, ζ, values of all the tested NCs was invariably highly positive, in agreement with previous studies conducted on CS nanocapsules (Calvo et al., 1997) and in CS "decorated" unilamellar lipid vesicles (Quemeneur et al., 2007; Quemeneur et al., 2008). This is also diagnostic evidence that CS forms a polycationic coat around the core-shell nanoemulsion, hence forming a nanocapsule structure. Indeed, nanoemulsions exhibit a highly negative zeta potential (ζ ~ -54 ± 1 mV) that upon coating with CS they experience a large inversion in ζ from highly negative to highly positive values, consistent with the occurrence of CS at the nanocapsules surface (Calvo et al., 1997).

The observed different behavior in ζ among the various NCs with LDP CS and HDP CS suggest that there are differences in the organization of CS chains around the nanocapsule's shell that are associated with CS's DA and M_w. In the case of LDP CS of low DA, short highly charged stiff polymer chains can be envisaged to pack themselves more closely at the lecithin oil-water interface, thus effectively, leading to an overall greater charge neutralization of phospholipid negative charges. A more regular distribution of LDP CS around the NC's surface than that achieved by

HDP CS can also account for the overall lower values found in LDP CS. This interpretation is also consistent with a flat, hence compact, arrangement of LDP CS at the NC's surface. This suggestion is consistent with the studies of adsorption of CS on LUVs (Quemeneur et al., 2007; Quemeneur et al., 2008), in which two distinct regimes of behaviour were clearly observed for the dependence of ζ on the proportion of CS coverage for CSs of 50 or 500 kDa. At low degrees of coverage, no dependence on M_w was observed, as a consequence of a flat adsorption of CS chains regardless of their size, whereas at high degrees of coverage, LUVs coated with high M_w CS showed larger ζ values. This behaviour was interpreted as a consequence to the formation of loops by the high M_w polymer, in agreement with our own interpretations to the differences in size found between NCs with LDP CS or HDP CS NCs given above. Interesting to notice was that for both LDP CS and HDP CS the decrease in ζ values is not proportional to the increase in the molar proportion of neutral N-acetyl-glucosamine residues and they only decrease significantly at high DA (>27%), a phenomenon that can be explained due to the formation of hydrophobic domains that penetrate into the phospholipids interface, an interpretation that is also in line with previous studies addressing the interaction of chitosan with phospholipid bilyarers (Fang et al., 2001). A schematic model of the envisaged type of local interactions between CS and the lecithin phsopholipid membrane at the NC's surface is presented in Figure 7.

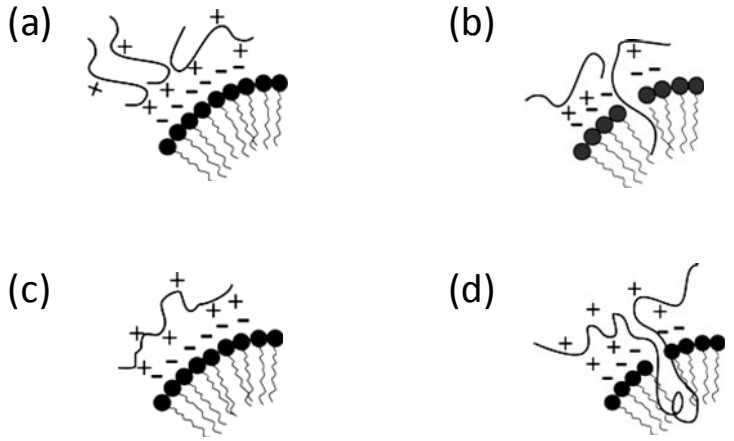


Figure 7. Schematic models for the interaction of chitosan and lecithin at the nanocapsule's surface. a) Chitosan of low M_w (LDP) and low degree of N-acetylation; b) Chitosan of low M_w (LDP) and high degree of N-acetylation; c) Chitosan of high M_w (HDP) and low degree of N-acetylation and d) Chitosan of high M_w (HDP) and high degree of N-acetylation

Close inspection of TEM images revealed that although subtle variations in contrast are appreciated among the general aspect of the various systems between the core and the shell, it is difficult to account for these in terms of the suspected presence of flat or coiled patterns of adsorption of CS at the NC's surface CS associated with CS's varying DA or M_w. Moreover, normal TEM images, in contrast with those obtained by cryo-TEM techniques, may not reflect detailed features the hydrated state of the NC's surface due to drying and shrinkage during the fixation procedure (van Helden et al., 1981). Ongoing collaborative synchrotron SAXS studies conducted by our Group are presently addressing more detailed aspects of the local topology of these NC's at the mesoscale.

The fact that CSs with nearly half as much charged residues (DA~51-56%) adsorbed equally efficiently than did the nearly fully charged polymers (DA~1.4-1.6%), is in full keeping with the previous suggestion that the mechanism of CS adsorption is not mediated solely by electrostatic interactions between oppositely charged $-NH_3^+$ groups in CS and phospholipidic polar heads of lecithin at the NC's surface but must also involve other type of interactions, such as hydrophobic forces.

In previous works, lipophilic drugs, such as diazepam (Calvo et al., 1997) or docetaxel (Lozano et al., 2008) have been effectively associated to CS nanocapsules as they can readily be loaded at the oily core. In the present study, we have chosen capsaicin, due to its current and potential future pharmacological use for a number of therapies. Besides, its inclusion in a nanovehicle may offer a strategy to control its delivery during topical, transmucosal or intracellular delivery. The capacity of nanocapsules to entrap capsaicin allows to anticipate the potential utilization of these systems from the inherent bioactive properties of CS (e.g. mucoadhesitvity) along with the possibility to achieve a prolonged release of the drug during topical or nasal administering.

Finally, the results for the studies of stability in biological cell culture media revealed that in general the NCs were very stable as compared with CS-based nanoparticle systems prepared by ionotropic gelation. These series of experiments allowed to conclude that CS NCs were extremely stable during incubation in MEM for up to 48 h and in ECGM for up to 4 h. The practical relevance of these results is that these systems can be

utilized for a wide range of studies in mammalian cell lines, including endothelial cells. On the other hand, it is striking to note that despite the fact that the pH of fresh MEM and ECGM (pH ~7.45) culture media is well above CS's pK_a (~6.0 – 7.0) and hence its amino groups are likely to be almost fully neutralized, the integrity of the NCs persists during prolonged times (e.g. as much as 48 h in MEM). Hence, the stability displayed by these systems at high salt concentrations cannot be explained neither by DLVO theory nor steric hindrance (Santander-Ortega et al., 2010a; Santander-Ortega., 2006). In this case, the hydrophilic character of CS originates another stabilization mechanism based on repulsive hydration forces, which become significant when a large amount of hydrated ions are accumulated in the proximity of the hydrophilic surface of the nanocapsules (López-León et al., 2008; Santander-Ortega et al., 2010b). Moreover, M_w and DA of CS affect its conformation onto the oily core, creating more hydrophilic nanocapsules at low degrees of polymerization and high degrees of acetylation (Santander-Ortega 2009). This explanation agrees with the lower stability displayed by the HDP systems with low DA; which, although they bear higher ζ, are more hydrophobic and hence they are less stable at high salt concentrations. An explanation of the markedly distinctive stability exhibited by the NCs in MEM, ECGM and RPMI-1640, is that although the three media share roughly similar composition in terms of salts, aminoacids and vitamins, there are subtle differences in the concentration and type of specific components of their formulation. Indeed, RPMI-1640 contains 5-fold greater concentration of Na_2HPO_4 than that in MEM. Phosphates easily tend to form ion pairs with chitosan (Santander-

Ortega et al., 2010a) and, consequently they can induce coagulation not only by charge cancelation, but also by bridging mechanism. In a parallel study, these effects were studied in detail for the individual contributions of NaCl, $CaCl_2$ and $MgCl_2$ (Santander Ortega et al., 2009) to the aggregation and re-stabilization of the same series of CS NCs along with the instability regions for each of these cations towards the NCs have been identified. In addition, lyotropic effects arising from the type and concentration of the occurring ions, particularly of negatively charged species, may also modify the hydration shell of the NC's surface, as suggested since the early studies conducted on surfactant micelles in the presence of various salts (Anackearn and Ghose., 1963).

5. Conclusions

In summary, we have developed core–shell nanocapsules bearing an oily nucleus, a surfactant membrane and a hydrophilic polymer coat were obtained by spontaneous emulsification and stabilization by lecithin and concomitant interaction with CS of varying M_w and degree of N-acetylation. Electrostatic and most likely also hydrophobic attractive forces are involved in the interaction between CS and lecithin as evidenced by the fact that regardless of CS's DA, the particles retain most of the polysaccharide at their surface and their Z–average size does not vary much within the range ~140–200 nm. Their surface charge (ζ), however, decreases as the DA increases beyond ~27%. All the nanocapsule systems exhibited remarkable stability in MEM and ECGM

cell culture media and those bearing CSs of DA > 27% were stable also in RPMI–1640, an attribute of high practical relevance for future *in vitro* studies with a variety of cell lines. Capsaicin, a currently established drug for pain therapy was effectively encapsulated (72–96 %). The results of this work allow anticipating the promising potential use of CS–based NCs for the intracellular and mucosal delivery of capsaicin.

Acknowledgements

Support from the European Union for projects Nanobiosaccharides (Ref No. 013882 FP6), BioNanoSmart-DDS (Ref. No. 908111 FP7-PEOPLE Programme Marie Curie) and Consolider Nanobiomed (CSD 2006-00012, Spain) and from the "Consejería de Innovación, Ciencia y Tecnología de la Junta de Andalucía" projects P07-FQM-2496 and P07-FQM-03099 are gratefully acknowledged.

References

Allan, G.G. & Peyron, M. 1995, "Molecular weight manipulation of chitosan I: Kinetics of depolymerization by nitrous acid", *Carbohydrate Research,* vol. 277, pp. 257-272.

Anackearn, E.W. & Ghose, D.H.M. 1963, "Counterions and micelle size. I. Light scattering by solutions of dodecyltrimethylammonium salts", *Journal of the American Chemical Society,* vol. 67, pp. 1713-1715.

Bevan, S. & Szolcsányi, J. 1990, "Sensory neuron-specific actions of capsaicin: mechanisms and applications", *Trends Pharmacol. Sci.,* vol. 11, pp. 330-333.

Binshtok, A.M., Bean, B.P. & Woolf, C.J. 2007, "Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers", *Nature,* vol. 449, pp. 607-610.

Calvo, P., Remunan-Lopez, C., Vila–Jato, J.L. & Alonoso, M.J. 1997, "Development of positively charged colloidal carriers: chitosan-coated polyester nanocapsules and submicron emulsions", *Colloid Polymer Science,* vol. 275, pp. 46-53.

Cortright, D.N. & Szallasi, A. 2004, "Biochemical pharmacology of the vanilloid receptor TRPV1. An update", *Eur. J. Biochem.,* vol. 271, pp. 814-819.

Fäldt, P., Bergenstahl, B. & Claesson, P.M. 1993, "Stabilization by chitosan of soybean oil emulsions coated with phospholipid and

glycocholic acid", *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* vol. 71, pp. 187-195.

Fang, N., Chan, V., Mao, H.Q. & Leong, K.W. 2001, "Interactions of phospholipid bilayer with chitosan: effect of molecular weight and pH", *Biomacromolecules,* vol. 2, pp. 1161-1168.

Lamarque, G., Lucas, J.L., Viton, C. & Domard, A. 2005, "Physicochemical behavior of homogeneous series of acetylated chitosans in aqueous solution: Role of various structural parameters.", *Biomacromolecules,* vol. 6, pp. 131-146.

López-León, T., Santander□ Ortega, M.J., Ortega-Vinuesa, J.L. & Bastos-González, D. 2008, *J. Phys. Chem. C,* vol. 112, pp. 16060.

López-Montilla, J.C., Herrera-Morales, P.E., Pandey, S. & Shah, D.O. 2002, "Spontaneous Emulsification: Mechanisms, Physicochemical Aspects, Modeling, and Applications", *Journal of Dispersion Science and Tehnology,* vol. 23, pp. 219-268.

Lozano, M.V., Torrecilla, D., Torres, D., Vidal, A., Domínguez, F. & Alonso, M.J. 2008, "Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers", *Biomacromolecules,* vol. 9, pp. 2186-2193.

Muzzarelli, R.A.A. 1998, "Colorimetric determination of chitosan", *Analytical Biochemistry,* vol. 260, pp. 255-257.

Prego, C., Torres, D. & Alonso, M.J. 2006a, "Chitosan nanocapsules as carriers for oral peptide delivery: Effect of chitosan molecular weight and type of salt on the in vitro behaviour and in vivo effectiveness", *Journal of Nanoscience and Nanotechnology,* vol. 6, no. 9-10, pp. 2921-2928.

Prego, C., Torres, D. & Alonso, M.J. 2006b, "Chitosan nanocapsules: a new carrier for nasal peptide delivery", *Journal of Drug Delivery Science and Technology,* vol. 16, no. 5, pp. 331-337.

Prego, C., Torres, D., Fernandez-Megia, E., Novoa-Carballal, R., Quinoa, E. & Alonso, M.J. 2006, "Chitosan-PEG nanocapsules as new carriers for oral peptide delivery - Effect of chitosan pegylation degree", *Journal of Controlled Release,* vol. 111, no. 3, pp. 299-308.

Quemeneur, F., Rammal, A., Rinaudo, M. & Pepin-Donat, B. 2007, "Large and Giant Vesicles "Decorated" with Chitosan: Effects of pH, Salt or Glucose Stress, and Surface Adhesion", *Biomacromolecules,* vol. 8, pp. 2512-2519.

Quemeneur, F., Rinaudo, M. & Pepin-Donat, B. 2008, "Influence of Molecular Weight and pH on Adsorption of Chitosan at the Surface of Large and Giant Vesicles", *Biomacromolecules,* vol. 9, pp. 396-402.

Santander-Ortega, M. J., Lozano-Lopez, M. V., Bastos-Gonzalez, D., Peula-Garcia, J.M. & Ortega-Vinuesa, J.L. 2009, "Novel core-shell lipid-chitosan and lipid-poloxamer nanocapsules: stability by hydration forces", Colloids and Polymer Science, vol 288, pp. 159-172.

Santander-Ortega, M.J., Jodar-Reyes, A.B., Csaba, N., Bastos-Gonzalez, D. & Ortega-Vinuesa, J. L. 2006, "Colloidal stability of Pluronic F68-coated PLGA nanoparticles: a variety of stabilisation mechanisms", Journal of Colloid and Interface Science, vol 302, pp. 522-529.

Santander-Ortega, M.J., Stauner, T., Loretz, B., Ortega-Vinuesa, J.L., Bastos-Gonzalez, D., Wenz, G., Schaefer, U.F. & Lehr, C. M. 2010, "Nanoparticles made from novel starch derivatives for transdermal drug delivery", Journal of Controlled Release, vol 141, pp. 85-92.

Santander Ortega, M.J., Peula-Garcia, J. M. Goycoolea, F. M. & Ortega-Vinuesa, J. L. 2009 "Chitosan based nanocapsules: effect of hydration forces on colloidal stability", III Reunion iberica de coloides e interfases (RICI). Extended communications. (ISBN: 978-84-338-5027-0), pp. 545-552.

Sorlier, P., Viton, C. & Domard, A. 2002, "Relation between solution properties and degree of acetylation of chitosan: Role of aging.", Biomacromolecules, vol. 3, pp. 1336-1342.

Valle-Gallego A., Goycolea, F.M., González L., Mallo F. and Alonso M.J. Chitosan-based nanocapsules for transmucosal delivery of Exendin-4.Submitted.

van Helden, A.K., Jansen, J.W. & Vrij, A. 1981, *J. Colloid Interface Sci.,* vol. 81, pp. 354.

Wang, Y.Y., Hong, C.T., Chiu, W.T. & Fang, J.Y. 2001, "In vitro and in vivo evaluations of topically applied capsaicin and nonivamide from hydrogels", *Interntaional Journal Pharmaceutics,* vol. 224, pp. 89-104.

Xing, F., Cheng, G., Yi, K. & Ma, L. 2005, "Nanoencapsulation of Capsaicin by Complex Coacervation of Gelatin, Acacia, and Tannins", *Journal of Applied Polmer Science,* vol. 96, pp. 2225-2229.

PARTE II

Antecedentes, hipótesis y objetivos

Artículo 2

Artículo 3

Discusión

ARTÍCULO 3

Chitosan-based nanocapsules for transmucosal delivery of exendin-4

Valle-Gallego A.[1,2], Goycoolea F.M.[1], González L.[2], Mallo F.[2] and Alonso M.J.[1*]

[1]*Departament of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Santiago de Compostela, Spain*

[2]*Laboratory of Endocrinology, Faculty of Biology, University of Vigo. Spain*

*Corresponding author: Prof. Alonso MJ.
Tel: + 34 981 594627
Fax: + 34 981 547148
E-mail: mariaj.alonso@usc.es

(Manuscript submitted to publication)

Abstract

Exenatide is a synthetic peptide derived from Exendin-4 (Ex4) currently approved for the treatment of diabetes mellitus type II. A major disadvantage for the use of exenatide in clinical practice is related to the necessity of being injected. In this work we have developed a new strategy intended for the nasal/oral administering of Ex4 based on the use of nanotechnologies. Namely, the peptide was entrapped in a core-shell nanocapsule consisting of an oily core and a chitosan (CS) coating. The resulting Ex4-loaded nanocapsules had a size in the range of ~189-226 nm and a positive zeta potential (ζ~ +36 to ~+53mV), diagnostic of CS allocation at the nanocapsules surface. The encapsulation efficiency (E.E) of Ex4 was high (E.E. > ~80%) and the nanocapsules had adequate stability in artificial simulated gastric (pH 1.2) and intestinal (pH 6.8) fluids and in acetate buffer (pH 4.0). The biologically active conformation of Ex4 was preserved during the formulation of peptide-loaded nanocapsules, as proved by i.v. injection of the released peptide to anesthetized rats. The efficacy of the nanocapsules as carriers for the nasal and oral administering of the peptide was evaluated in conscious rats. The results showed that the developed nanocapsules failed at improving the oral delivery of the peptide, however, they were efficient at enhancing its intranasal absorption. Overall, the results of this work evidence the potential of CS nanocapsules as carriers for improving the transport of the peptide across the nasal mucosa

Key words: exendin-4, nanocapsules, chitosan, transmucosal delivery, diabetes mellitus

.

1. Introducción

Exenatide, the synthetic product of exendin-4 (Ex4), has been recently approved in Europe and USA for the treatment of diabetes mellitus type 2 by subcutaneous injection. Ex4 is a 39-amino acid amidated peptide incretin mimetic that shares several glucoregulatory activities with the mammalian incretin hormone, glucagon-like peptide 1 (GLP-1). Well confirmed pharmacological effects of Ex4 produced as agonist GLP-1 receptor, include enhancement of glucose-dependent insulin secretion; glucose-dependent suppression of inappropriately high glucagon secretion; protective effects in the β-cell mass of the pancreas; slowing of gastric emptying; and reduction of food intake, hence, decrease in patient's body weight [1-3]. In a recent study, it has also been shown that Ex4 is a potentially useful and multipotent therapeutic agent for vascular diseases associated with high salt sensitivity [4]. Hence, as a result of its bioactivity, exenatide is an effective drug that reduces blood glucose levels in diabetic patients. Nevertheless, as for other drugs (i.e amphetamine), Ex4 has a dual actions. In healthy (non-diabetic) rats, the short-term response of Ex4 includes a paradoxical increase in the plasmatic glucose levels, interpreted as a consequence of the activation of the sympathetic nervous system, as we have recently shown [5]. In this work we have used this effect as an experimentally convenient short-term witness to probe the systemic absorption of bioactive Ex4.

The amino acid sequence of Ex4 responsible for the potency, the duration of the effect in vivo and the affinity for GLP-1 receptor is even

greater than those of GLP-1. More precisely, the affinity depends on the residues in the central part of the ligands Leu^{6}-Gly^{30} [6] and on the nine aminoacids C-terminal sequence [7], although the essential part for the interaction is the molecular conformation. Thus, the protection of Ex4 against pH changes (*pI* ~ 4.2) [8] and the enzymatic/chemical hydrolysis, plays an important role during the preparation of the formulation and the search of new pathways that avoid the subcutaneous route. In common with other proteins and peptides, Ex4 presents limited bioavailability (B.A) by intraduodenal (B.A. < 0.01 %) and nasal (B.A. < 1.7 %) route [9]. In light of this, recent efforts have been made aiming to modifying the structure of Ex4 [10] and GLP-1 [11, 12] to increase the oral absorption or improve the half-lives for reducing number of injections [13-15].

To overcome these problems, nanotechnological drug delivery platforms might represent an attractive alternative. In this respect, so far, only helodermine, a peptide of the GLP-1 family, has been loaded into PLGA nanospheres thus improving the *in vitro* absorption by M cells [16], though their *in vivo* efficacy was not evaluated. Nevertheless, previous studies with CS-based nanocapsules, have accounted for their potential application as effective vehicles for oral [17, 18] and nasal [19] administering of poorly absorbable peptides (e.g. salmon calcitonine, sCT) in a rat model. Moreover, this nanocapsule system has shown to be an effective intracellular delivery vehicle for taxanes [20], and for the intranasal administering of vaccines, i.e. Hepatitis B surface antigen [21].

In a parallel study, we have also succeeded to encapsulate 72-96 % of capsaicin, a liposoluble drug currently used in pain therapy [22].

Based on the above, in this work we have developed a new strategy intended for the nasal and oral administering of Ex4 based on the application of CS nanocapsules that preserve the bioactivity and transport the peptide across the epithelia in healthy conscious rats.

2. Materiales y métodos

2.1. Materials

Two chitosan (CS) samples were the same batches as those that have been used in parallel studies, namely (HDP-DA56: degree of N-acetylation (D.A) ~56% and M_w 266 kDa; HDP-DA1: D.A. ~1.6% and M_w ~124 kDa). Both samples were prepared and purified from a batch of squid pen chitosan (from Mathani, France) and the D.A. and M_w were characterized by ^{1}H NMR spectroscopy and HPLC-MALLS-DRI, respectively, as described elsewhere [22]. Miglyol 812® was donated by Sasol Germany GmbH (Witten, Germany); lecithin (Epikuron 145V) was donated by Cargill Texturizing Solutions, S.A (Barcelona, Spain); Ex4 was from Bachem Distribution Services GmbH (Weil am Rhein, Germany); acetonitrile HPLC gradient grade (≥99.9%) was from Merck (Darmstadt, Germany) and trifluoroaetic acid (≥99.0%) was from Sigma-Aldrich (Madrid, Spain); Milli-Q water was used throughout.

2.2. Preparation of nanocapsules

The protocol used to prepare unloaded (blank) nanocapsules was by solvent displacement according with the protocol previously described by Prego et al [19]. This protocol was slightly modified to formulate Ex4-loaded nanocapsules. Briefly, Ex4 was previously dissolved (1 mg/mL) in ethanol into which lecithin (80 mg/mL) was also dissolved and added with Miglyol 812® to yield a miscible solution into which acetone was added. The organic phase thus obtained was poured into an aqueous solution of CS (0.5 mg/mL) previously dissolved in water containing 5% stochiometric excess of acetic acid. Ex4-loaded nanocapsules for nasal and oral studies were prepared with HDP-DA56 and HDP-DA1, respectively.

2.3. Physical characterization

The Z-average size and polydispersity index of the nanocapsules was determined by dynamic light scattering with noninvasive back scattering (DLS-NIBS) technology and the values of zeta potential (ζ) were determined by mixed laser Doppler electrophoresis and phase analysis light scattering (M3-PALS) using a Malvern Zetasizer NanoZS (ZEN 3600, Malvern Instruments, Worcestershire, U.K.) fitted with a red laser light beam (λ = 632.8 nm). All measurements were made in triplicate.

2.4. Encapsulation efficiency

The encapsulation efficiency (E.E) of Ex4 in the nanocapsules was determined indirectly by quantifying free Ex4 contents by HPLC (Agillent Technologies, Germany) according with the method described elsewhere [23] and using fluorescent light detector (FLD).

2.5. Stability in simulated gastric and intestinal fluids

The colloidal stability of Ex4-loaded nanocapsules was evaluated during incubation at 37°C in simulated gastric (SIG, pH=1.2) and intestinal (SIF, pH=6.8) fluids (both containing enzymes [24]) and in acetate buffer (pH 4).

2.6. In vitro release studies

In vitro release studies were performed by incubating 40 μL of isolated Ex4-loaded nanocapsules in 1 mL of SIF without enzymes under horizontal shaking at 37°C. After 1h of incubation had elapsed, free Ex4 was separated from the nanocapsules by ultracenfrifugation at ~29000*g* for 2 h at 15°C. Free Ex4 contents were measured using a RIA kit (Phoenix Pharmaceuticals Inc., USA). The intra-assay coefficients of variation (CVs) were 0.15% (200 pg/ml), 0.35% (400 pg/ml), and 0.10 % (800 pg/ml).

2.7. In vivo studies

2.7.1 Animals

Healthy adult male Sprague–Dawley rats were housed in cages (five animals each) for at least 3 days before the experiments at 21±1°C in a 12:12 h light:dark cycle (all experiments were performed in the light cycle) and were fed and watered *ad libitum*. Changes in plasma glucose levels determined by Glucose RTU™ kit (BioMerieux® SA, France) were taken to indicate the effects of bioactive amounts of Ex4. In all cases plasmatic glycaemia was assessed prior to the administration of the treatments. Blood samples were taken from the jugular or tail veins in experiments conducted in anesthetized or conscious rats, respectively.

2.7.2. In vivo evaluation of the bioactivity of Ex4 after nanoencapsulation

Anaesthetized rats (300-400 g) were used to test the following treatments by intravenous administration: 1) Ex4 previously released from loaded nanocapsules in SIF without enzymes (n=6); 2) untreated Ex4 dissolved directly in SIF (n=3) used as positive control; 3) medium released from blank nanocapsules in SIF without enzymes (n=6); and 4) SIF (n=6). Treatments 3) and 4) were negative controls. The dose of Ex4 administered in treatments 1) and 2) was ~ 10 μg/kg while the volume administered was 200μl.

2.7.3. In vivo efficacy after nasal administering of Ex4 nanocapsules

Conscious rats (210-303 g) were fed and watered *ad libitum* during the whole experiment. Four groups of rats were treated as follows: 1) Isolated Ex4-loaded nanocapsules (n=8); 2) Isolated blank nanocapsules (n=5); 3) Buffer solution of acetate pH 4 (n=8) and 4) Ex4 (n=8). All the treatments were dissolved in acetate buffer pH 4.3. The dose of Ex4 administered in treatments 1) and 4) was ~ 11.2 μg/kg. Treatments were instilled into the nostrils in a total volume of 100μL per treatment with a micropipette: 25μL to each nostril, twice at 5 min time intervals.

2.7.4. In vivo efficacy after oral administering of Ex4 nanocapsules

Conscious animals (320-370 g) were fasted during 12 h before each experiment and watered *ad libitum* during the whole experiment. The following treatments were assayed: 1) Ex4-loaded nanocapsules (n=8); 2) Blank nanocapsules (n=6); 3) Saline (n=6); 4) Ex4 dissolved in saline. Treatments 1) to 3) were administered intragastrically using a catheter (stainless steel curved feeding needles from Harvard apparatus) while treatment 4) was injected by intraperitoneal route (n=3) as a positive control. The Ex4 dose in treatments 1) and 4) was 150 μg/rat and 5μg/kg of body for oral and i.p route, respectively. Volume administered was ~2.1mL.

2.8. Statistical analysis of data

The data of glycaemia from experiments of i.v. injection of Ex4 released from nanocapsules were analysed by the non-parametric Mann-Whitney

test for comparison between two independent groups. While data from the nasal and oral assays were analyzed by non parametric Kruskall-Wallis test [25]. We used the package of statistical software SPSS 15.0 (SPSS Inc. Chicago, IL). The level of significance was accepted with $p<0.05$. Data are represented as mean ± standard error of the mean.

3. Results and discussion

In this work we have addressed the preparation of Ex4 nanocapsules formulated with different types of CS, and have evaluated their efficacy after *in vivo* administering by oral and nasal route to conscious healthy rats. As in previous studies, the rationale behind has been to utilize CS-based nanocapsules aiming to preserve the bioactivity of the peptide as well promoting its absorption during transmucosal administering [26]. To this end, two types of CS of high (HDP-DA56) and low D.A (HDP-DA1) have been used to produce Ex4-loaded nanocapsules intended for nasal and oral administering, respectively. The selection of the type of CS in both cases was done on the basis of the expected degradation kinetics and stability of the nanocapsules relevant to each administering route. In the case of intranasally administered nanocapsules, the criterion for selection of CS of high D.A. (HDP-DA56) was the greater expected rate of degradation by lysozyme occurring in the nasal mucosal secretions [27, 28]. It was expected that a rapid degradation of the CS shell would favour the fast release of Ex4 from the nanocapsules core, thus effectively, overcoming the rapid mucocilliary clearance. In turn, for systems

intended for the oral route, the major criteria to select the optimal CS sample were: i) adequate stability shown in SGF and SIF and ii) increased susceptibility to degradation by proteases [29, 30]. For both types of administering routes it was aimed that the NCs would lead to enhancement of drug transport across the epithelia, in line with previous *in vitro* studies on human Caco-2 cells [31] and also contrasted in vivo [18, 19].

3.1. Nanocapsules physical properties

Table 1 summarizes the physical data for the blank and Ex4-loaded nanocapsules. No differences in Z-average size among the various systems were observed associated with the type of CS nor to the loading of Ex4. As expected, the nanocapsules (blank and Ex4-loaded) coated with HDP-DA56 exhibited a lower ζ value than did those comprising HDP-DA1 (i.e. bearing greater charge density). The values for size and ζ are within the range of those previously found for blank and capsaicin-loaded nanocapsules [22].

Table 1: Physico-chemical properties of blank and Ex4-loaded chitosan-based nanocapsules (NC).

Route	NC	D.A (%)[a]	Size (nm)	P.D.I	ζ (mV)	E.E (%)[b]	Ex-4 released[c]
Oral	Blank	1.6	189±14	0.19±0.02	+48±8	--	--
Oral	Loaded	1.6	206±8	0.13±0.03	+53±1	82.7±3.2	40.5±4.6
Nasal	Blank	56	208±13	0.14±0.03	+36±4	--	--
Nasal	Loaded	56	226±20	0.20±0.03	+45±5	84.9±1.9	--

[a] Degree of acetylation
[b] Encapsulation efficiency
[c] Percentage of released Ex4 in simulated intestinal medium (pH 6.8) after 1 h of incubation, at 37ºC .

3.2. Ex4 encapsulation efficiency (E.E.)

Table 1 also shows that the peptide exhibited a high association efficiency (E.E. >80%) regardless of the composition of the nanocapsules surface. Such high E.E. found for Ex4 contrasts with that for sCT in CS-coated nanocapsules with efficiencies in the range 41-54%. As it was argued, the CS or PEG-CS used to coat the nanoemulsion displaced sCT from it, thus effectively decreasing the encapsulation efficiency [18, 32]. The contribution of hydrophobic domains of Ex4 [6] along with its low *pI* (~4.2) [33] (i.e. thus favouring electrostatic interactions with oppositely charged CS) are thought to have been determinant for the efficient retention of the peptide at the lipidic core [34].

3.3. Stability of the nanocapsules

Before proceeding with the *in vivo* studies, it was necessary to confirm that the developed nanosystems were stable in conditions that simulate those found in the biological environment of the oral and nasal mucosae. To this end, the evolution of the Z-average diameter of the nanocapsules intended for oral route was evaluated in SGF (pH 1.2) and SIF (pH 6.8). Whereas those developed for the nasal route were tested in acetate buffer (pH 4.3) in line with previous studies [19, 35]. As shown in Figure 1, in all cases the formulations were stable for up to 120 min in the various tested buffers with no discernible signs of elevation in the Z-average diameter of the colloidal species. The stability of CS nanocapsules in SIF seems to contrast with the findings of previous studies by *Prego et al*, in which CS-based NCs aggregated after 1 h except those formed with CS-PEG 1% [32]. In our study, the nanocapsules formed with CS HDP-DA1 are believed to remain stable in SIF due to the high charge density of CS coating.

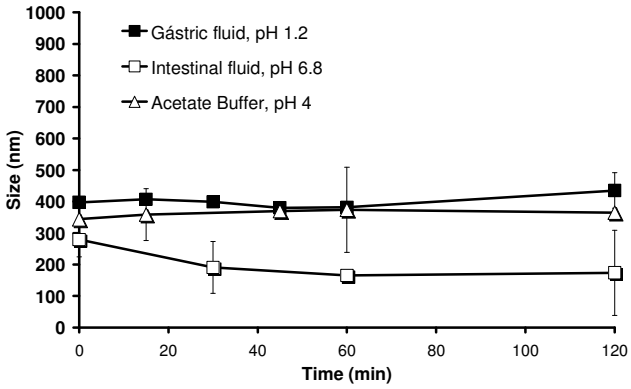


Figure 1: Evolution of the Z-average diameter for Ex4-loaded nanocapsules comprising chitosan of HDP-DA1 (squares) in simulated gastric fluid pH 1.2 (filled) and in simulated intestinal fluid pH 6.8 (empty) and for system comprising chitosan of HDP-DA 56 in acetate buffer medium pH 4.0 (empty triangle). All measurements were conducted at 37 °C (n=3).

3.4. In vivo experiments

As mentioned above, Ex4 induces an increase of glycaemia at short-term in healthy rats [5]. Therefore, in our *in vivo* assays, the glycaemia was utilized as a the metabolic response to systemic absortption of Ex4

3.4.1. Ex4 bioactivity after nanoencapsulation in healthy rats

A major concern regarding the *in vivo* assays was to confirm that the bioactive conformation of Ex4 was not severely altered during the nanoencapsulation process, induced by the contact of the peptide with ethanol and/or with lecithin in the organic phase [34]. Therefore, before such assays were conducted, an experiment was devised by virtue of which Ex4 was first released from peptide-loaded nanocapsules in SIF

(pH 6.8) and thereafter the released peptide was injected intravenous to healthy rats. The amount of Ex4 released after 1 h in SIF was quantified to be 40.5±4.6%. Inspection of Figure 2 reveals that the glycaemia time profile for released Ex4 from loaded nanocapsules was much the same as that of the positive control. In both cases, glucose plasma levels increased after 30 min post administering, and hyperglycemia was maintained during 120 min. This result was considered as experimental evidence that the bioactive conformation of Ex4 was preserved during the nanoencapsulation process and after its further release in artificial intestinal fluid.

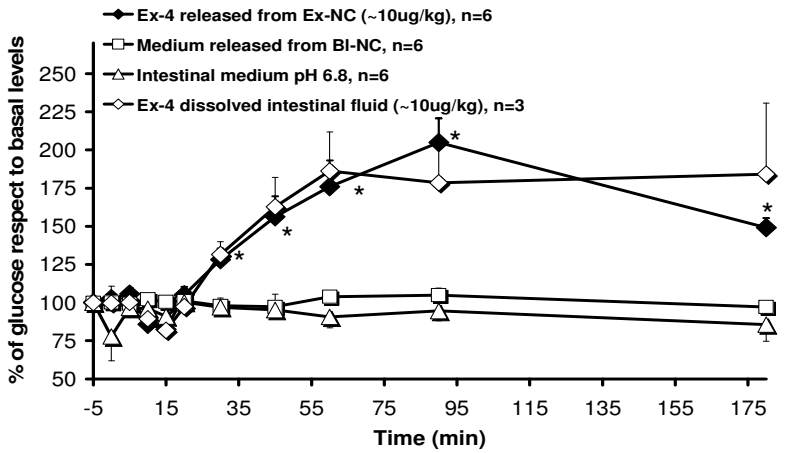


Figure 2: Relative plasma glycemic levels after i.v administering to healthy rats of released-Ex4 from loaded nanocapsules n=6 (filled diamonds). As control groups, medium released from blank nanocapsules n=6 (empty squares), intestinal medium n=6 (empty triangles) and untreated Ex4 (empty diamonds). (Mean ± S.E.M.).* $p < 0.05$ Statistical significant differences with respect to blank NC and intestinal medium

3.4.2. In vivo efficacy after nasal administering of Ex4 nanocapsules

In preceding studies, we have shown the ability of CS-based nanocapsules to increase the absorption of sCT post nasal administering in healthy rat [19]. In the present work, we have addressed the use of this nanosystem to transport Ex4 across the nasal epithelium in the same animal model. Figure 3 shows the time evolution on plasmatic glucose levels following nasal administering of Ex4-loaded nanocapsules in comparison with the corresponding control treatments. Notice that the group treated with Ex4-loaded nanocapsules showed greater hyperglycaemic response than the control groups treated with Ex4 in buffer solution and acetate buffer solution alone ($p \leq 0.05$). Moreover, the observed response was prolonged during a period of 120 min, proving the efficiency the potential of CS nanocapsules as carriers for improving the transport of Ex4 across the nasal barrier. The group of blank nanocapsules showed an unexpected increase on glucose levels that it could be produced by an irritation of the nasal mucosa during the administering.

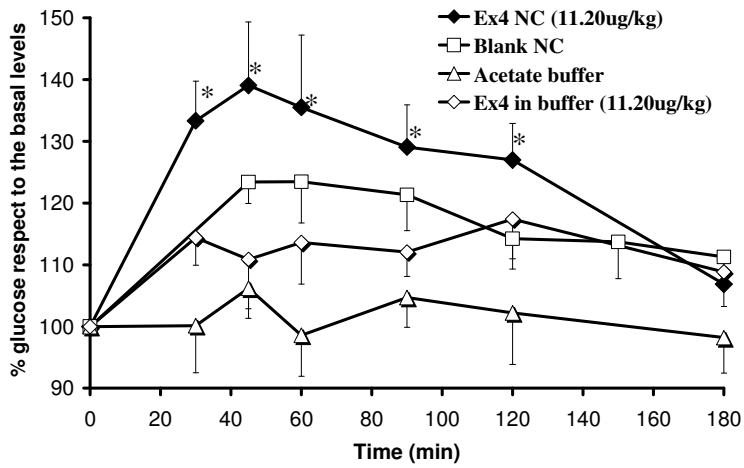


Figure 3: Relative plasma glucose levels achieved in healthy rats after oral administering of Ex4-loaded nanocapsules n=8 (filled diamonds). As control groups were used: Blank nanocapsules n=6 (empty squares), acetate buffer n=6 (empty triangles) and free Exendin-4 dissolved in acetate buffer n=6 (empty diamonds). (Mean ± S.E.M.). *$p \leq 0.05$ Statistical significant differences respect to free Ex4 and acetate buffer.

The obtained results are in good keeping with previous studies. As hypothesized, the presence of CS with high D.A. at the NC's surface favoured the systemic absorption of Ex4, as previously found after the administering of CS-based nanosystems (nanoparticles or NCs) loaded with peptides or antigens after nasal administration in various animal models [19, 35-38]. As it has been discussed in previous works, the ability of nanocapsules to increase the absorption of peptides across the nasal epithelium could be explained as the expected consequence of a greater bioadhesion of the nanocapsules at the nasal mucosa leading to a greater accumulation of CS in the epithelium, thus effectively, favouring the opening of tight junctions concomintant with the delivery of Ex4. This hypothesis has been supported by the observed ability of CS-based

nanosystems (nanocapsules and nanoparticles) to reversibly decrease the transepithelial resistance (TEER) in intestinal and air-way epithelial cell cultures when applied at sufficiently high doses [26] and agrees also with the conclusion that CS increases cell permeability by affecting paracellular and intracellular pathways [39]. There is another possibility that a fraction of nanocapsules could be taken up directly by epithelial cells and deliver Ex4 intracellularly. In this regards, recent studies have evidenced that CS-based nanocapsules are able to deliver taxanes intracellularly in cancer cell lines MCF7 and A549 [20]. Fluorescence microscopy images have shown that a dye encapsulated in the core of CS nanocapsules is localized as vesicles in the cytosol after 2 h incubation of both cell lines with the nanocapsules, thus effectively, suggesting that nanocapsules are internalized by the cells via an endocytic pathway [20]. The improved uptake could be related to a favoured interaction of the CS coated surface with the cells [40]. Similar results have also been found for other non cancer cell types, such as those of corneal epithelium [41].

3.4.3. In vivo efficacy after oral administering of Ex4 nanocapsules

In previous works addressing the performance of CS-coated nanocapsules as transmucosal carriers of the peptide sCT across the intestinal mucosa, it has been reported the ability of these systems to enhance the systemic absorption and prolong the hypocalcemic pharmacological response for up to 24 h [18, 32, 42]. A general conclusion of these studies was that the nanocapsules could exert a long-

term controlled delivery *in vivo* as a consequence of their capacity to associate to the intestinal mucosa.

Based on such previous studies, in the present work it was expected that CS-based nanocapsules will also enhance the intestinal absorption of Ex4 when administered orally. In Figure 4 are shown the pharmacological responses registered after oral administering Ex4-loaded nanocapsules along with the appropriate control treatments. Clearly, only when Ex4 was injected i.p. the treated group showed increment in glycaemia achieving its maximum after ~45 min and that persisted during up to 120 min, while the group treated with Ex4-loaded nanocapsules did not show any appreciable differences with respect to the controls. This lack of effectiveness of the tested nanocapsules to deliver Ex4 by oral administering seems to be in contrast with previous studies that have shown the ability of CS-coated nanocapsules to enhance the systemic absorption of sCT in the same rat model [18, 32, 42]. To account for this discrepancy, it can be suggested that the pharmacological response of Ex4-loaded NCs administered might have been prolonged beyond the time spanned during the performed assays. Indeed, in previous studies centered on sCT-loaded nanocapsules, the low dose of NCs led to a pulsatile pharmacological profile response, characterized by 2 peaks. The first peak (1-2 h) was releated with certain amount of peptide retained in the chitosan coating, whereas the second and maximum response was found between 12-24h and was due to the peptide encapsulated at the core and that required longer time to be released [42]. In our study, the hydrophobic character of Ex4 increased its affinity with the oily core of

the NC, thus, improving its encapsulation efficiency. As a consequence of this along with the short-term response of Ex4 on the glycaemia in healthy rats could effect of Ex4-nanocapsules by oral route might have been hindered.

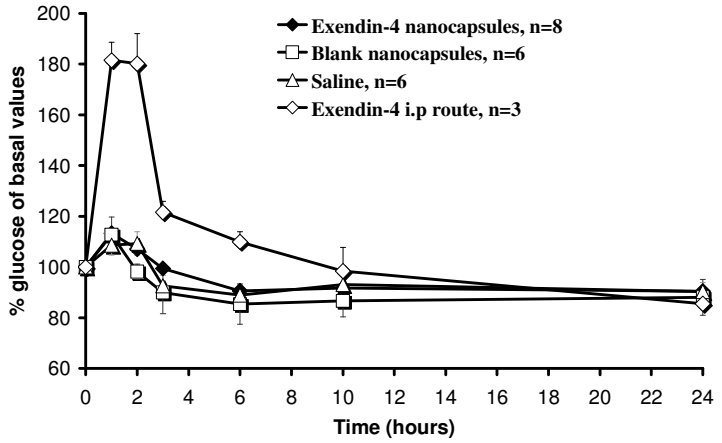


Figure 4: Glycemic levels after oral administering to healthy rats: Ex4-loaded nanocapsules n=8 (filled diamonds). As control groups were used: Blank nanocapsules n=6 (empty squares), saline n=6 (empty triangles) and untreated Ex4 dissolved in saline, by i.p route, n=3 (empty diamonds).

On the other hand,In studies conducted on insulin-loaded nanocapsules comprising isobutylcyanoacrylate, the hypoglycemic effects started to appear 48h after intragastric administering [43]. This delayed absorption of insulin after oral administering with nanocapsules formed with poly(isobutilcyanoacrilate) has been also reported previously by Abobakar et al [44], who evidence by fluorescence microscopy that the nanocapsules reached the ileum one hour after being administered

it could be possible that the oral absorption was delayed. Abobakar et al [43], who evidence the delayed absorption with poly(isobutilcyanoacrilate)

nanocapsules by fluorescence microscopy that they reached the ileum one hour after being administered.

4. Conclusión

Ex4 has been efficiently encapsulated and released from CS-coated nanocapsules in its bioactive form. This nanocarrier system enhances a rapid nasal absorption of Ex4 peptide in healthy rat model, constituting an efficient nanomedicine to promote the nasal absorption. However, it was not possible to demonstrate a short-term response when Ex4-loaded nanocapsules were administered orally. This may be due to a delayed release of the peptide leading to a longer pharmacological response than the time span of the present studies. Hence, further studies are needed so as to clarify these issues.

Acknowledgements

Financial support of the European Union from the Nanobiosaccharides project. (Ref No. 013882 of call FP6–2003–NMP–TI–3–Main) is gratefully acknowledged as well as to "Galician Research Network for the study of the Obesity" for a scholarship contract to A.V.-G. We are grateful to Rafael Romero for his help during in vivo studies.

References

1. Nielsen, L.L., et al., *Pharmacology of exenatide (synthetic exendin-4): A potential therapeutic for improved glycemic control of type 2 diabetes.* Regulatory Peptides, 2004. 117(2): p. 77-88.
2. Buse, J.B., et al., *Effects of exenatide (exendin-4) on glycemic control over 30 weeks in sulfonylurea-treated patients with type 2 diabetes.* Diabetes Care, 2004. 27(11): p. 2628-2635.
3. Kendall, D.M., et al., *Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulfonylurea.* Diabetes Care, 2005. 28(5): p. 1083-1091.
4. Hirata, K., et al., *Exendin-4 has an anti-hypertensive effect in salt-sensitive mice model.* Biochemical and Biophysical Research Communications, 2009. 380(1): p. 44-49.
5. Pérez-Tilve D., et al.., *Exendin-4 increases blood glucose levels acutely in rats by the activations of the sympathetic nervous system.* Am J Physiol Endocrinol Metab, Accepted 2009.
6. Runge, S., et al., *Differential structural properties of GLP-1 and exendin-4 determine their relative affinity for the GLP-1 receptor N-terminal extracellular domain.* Biochemistry, 2007. 46(19): p. 5830-5840.
7. Doyle, M.E., et al., *The importance of the nine-amino acid C-terminal sequence of exendin-4 for binding to the GLP-1 receptor and for biological activity.* Regulatory Peptides, 2003. 114(2-3): p. 153-158.
8. Andersen, N.H., et al., *Medium-dependence of the secondary structure of exendin-4 and glucagon-like-peptide-1.* Bioorganic and Medicinal Chemistry, 2002. 10(1): p. 79-85.
9. Gedulin, B.R., et al., *Pharmacokinetics and pharmacodynamics of exenatide following alternate routes of administration.* International Journal of Pharmaceutics, 2008. 356(1-2): p. 231-238.
10. Su, Y.C., et al., *Preparation, characterization, and application of biotinylated and biotin-PEGylated glucagon-like peptide-1 analogues for enhanced oral delivery.* Bioconjugate Chemistry, 2008. 19(1): p. 334-341.
11. Youn, Y.S., et al., *Improved peroral delivery of glucagon-like peptide-1 by site-specific biotin modification: Design, preparation, and biological evaluation.* European Journal of Pharmaceutics and Biopharmaceutics, 2008. 68(3): p. 667-675.

12. Lee, Y.H., et al., *Regional oral absorption, hepatic first-pass effect, and non-linear disposition of salmon calcitonin in beagle dogs.* European Journal of Pharmaceutics and Biopharmaceutics, 2000. 50(2): p. 205-211.
13. Kim, J.G., et al., *Development and characterization of a glucagon-like peptide 1-albumin conjugate the ability to activate the glucagon-like peptide 1 receptor in vivo.* Diabetes, 2003. 52(3): p. 751-759.
14. Kong, J.H., et al., *Long acting hyaluronate - exendin 4 conjugate for the treatment of type 2 diabetes.* Biomaterials, 2010.
15. Son, S., et al., *Preparation and structural, biochemical, and pharmaceutical characterizations of bile acid-modified long-acting exendin-4 derivatives.* Journal of Medicinal Chemistry, 2009. 52(21): p. 6889-6896.
16. des Rieux, A., et al., *Helodermin-loaded nanoparticles: Characterization and transport across an in vitro model of the follicle-associated epithelium.* Journal of Controlled Release, 2007. 118(3): p. 294-302.
17. Prego, C., et al., *The potential of chitosan for the oral administration of peptides.* Expert Opinion on Drug Delivery, 2005. 2(5): p. 843-854.
18. Prego, C., et al., *Chitosan nanocapsules as carriers for oral peptide delivery: Effect of chitosan molecular weight and type of salt on the in vitro behaviour and in vivo effectiveness.* Journal of Nanoscience and Nanotechnology, 2006. 6(9-10): p. 2921-2928.
19. Prego, C., et al., *Chitosan nanocapsules: A new carrier for nasal peptide delivery.* Journal of Drug Delivery Science and Technology, 2006. 16(5): p. 331-337.
20. Lozano, M.V., et al., *Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers.* Biomacromolecules, 2008. 9(8): p. 2186-2193.
21. Vicente, S.S., et al. *EUFEPS Workshop on Opportunity and Challenges in Vaccine Delivery*. 2008.
22. Goycoolea F.M.,et al., *Chitosan–Based Nanocapsules: Characterization of physical properties and application in drug delivery.* in *Proceedings of the 9th International Conference of the European Chitin Society*. 2009. San Servolo island, Venice, Italy.
23. Eng, J., et al., *Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas.* Journal of Biological Chemistry, 1992. 267(11): p. 7402-7405.
24. Pharmacopeia, U.S., *USP 31 - NF 29*. 1st ed. Vol. 1. 2008. 898.

25. Sidney Siegel, N.J.C.J., *Nonparametric Statistics for The Behavioral Sciences* 2nd Edition ed. Chapter 8: The case of k Indepent Samples. 1999, Sidney: McGrarw-Hill.
26. Prego, C., et al., *Transmucosal macromolecular drug delivery.* Journal of Controlled Release, 2005. 101(1-3 SPEC. ISS.): p. 151-162.
27. Wilson, M., *Microbial inhabitants of humans: their ecology and role in health and disease.* Vol. Chapter 4: The respiratory system and its indigenous microbiota. 2005, Cambridge: Cambridge University 450.
28. Nordtveit, R.J., et al., *Degradation of partially N-acetylated chitosans with hen egg white and human lysozyme.* Carbohydrate Polymers, 1996. 29(2): p. 163-167.
29. Kumar, A.B.V. and R.N. Tharanathan, *A comparative study on depolymerization of chitosan by proteolytic enzymes.* Carbohydrate Polymers, 2004. 58(3): p. 275-283.
30. Roncal, T., et al., *High yield production of monomer-free chitosan oligosaccharides by pepsin catalyzed hydrolysis of a high deacetylation degree chitosan.* Carbohydrate Research, 2007. 342(18): p. 2750-2756.
31. Schipper, N.G.M., et al., *Chitosans as absorption enhancers for poorly absorbable drugs. 1: Influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells.* Pharmaceutical Research, 1996. 13(11): p. 1686-1692.
32. Prego, C., et al., *Chitosan-PEG nanocapsules as new carriers for oral peptide delivery: Effect of chitosan pegylation degree.* Journal of Controlled Release, 2006. 111(3): p. 299-308.
33. Skovgaard, M., et al., *Using Evolutionary Information and Ancestral Sequences to Understand the Sequence-Function Relationship in GLP-1 Agonists.* Journal of Molecular Biology, 2006. 363(5): p. 977-988.
34. Neidigh, J.W., et al., *Exendin-4 and glucagon-like-peptide-1: NMR structural comparisons in the solution and micelle-associated states.* Biochemistry, 2001. 40(44): p. 13188-13200.
35. Fernández-Urrusuno, R., et al., *Enhancement of nasal absorption of insulin using chitosan nanoparticles.* Pharmaceutical Research, 1999. 16(10): p. 1576-1581.
36. Calvo, P., et al., *Development of positively charged colloidal drug carriers: Chitosan-coated polyester nanocapsules and submicron-emulsions.* Colloid and Polymer Science, 1997. 275(1): p. 46-53.

37. Goycoolea F.M., et al., *Chitosan-coated nanocapsules: Physical characterization, capsaicin encapsulation efficiency and stability in biological media.* . Submitted.
38. Teijeiro-Osorio, et al, *New generation of hybrid poly/oligosaccharide nanoparticles as carriers for the nasal delivery of macromolecules.* Biomacromolecules, 2009. 10(2): p. 243-9.
39. Dodane, V., et al., *Effect of chitosan on epithelial permeability and structure.* International Journal of Pharmaceutics, 1999. 182(1): p. 21-32.
40. Garcia-Fuentes, M., et al., *New surface-modified lipid nanoparticles as delivery vehicles for salmon calcitonin.* International Journal of Pharmaceutics, 2005. 296(1-2): p. 122-132.
41. De Campos, A.M., et al., *The effect of a PEG versus a chitosan coating on the interaction of drug colloidal carriers with the ocular mucosa.* European Journal of Pharmaceutical Sciences, 2003. 20(1): p. 73-81.
42. Prego, C., et al., *Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery.* Pharmaceutical Research, 2006. 23(3): p. 549-556.
43. Michel, C., et al., *The effect of site of administration in the gastrointestinal tract on the absorption of insulin from nanocapsules in diabetic rats.* Journal of Pharmacy and Pharmacology, 1991. 43(1): p. 1-5.
44. Aboubakar, M., et al., *Insulin-loaded nanocapsules for oral administration: In vitro and in vivo investigation.* Drug Development Research, 2000. 49(2): p. 109-117.

PARTE II

Antecedentes, hipótesis y objetivos

Artículo 2

Artículo 3

Discusión

DISCUSION - PARTE II

Los materiales y métodos empleados para la realización de este trabajo están descritos en los artículos correspondientes.

Recientemente, el grupo de investigación Nanobiofar de la Universidad de Santiago de Compostela ha desarrollado un sistema nanocapsular formado a base de quitosano (CS) y lecitina (LEC), para la administración de péptidos, como la calcitonina de salmón (sCT), mejorando la absorción a través de las mucosas. Teniendo en cuenta la versatilidad de este sistema y tomando como referencia los resultados que se han obtenido por vía nasal [97] y oral [126, 127], el principal objetivo ha sido la evaluar el potencial de las nanocápsulas de quitosano para la administración de exendina-4 (Ex4) por estas vías. Para ello, el péptido ha sido asociado eficazmente en el núcleo oleoso de las nanocápsulas diseñadas específicamente para cada vía de administración.

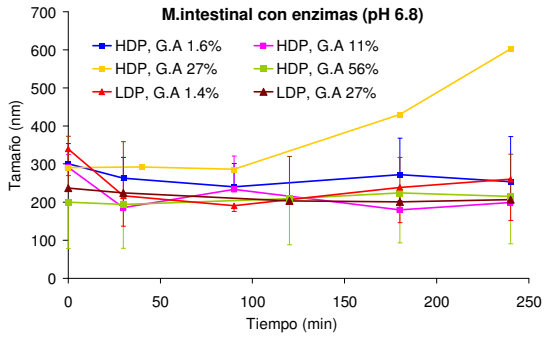
Selección del quitosano

La selección de los quitosanos para la formación de las nanocápsulas de quitosano cargadas con Ex4 (NC-Ex4), se hizo teniendo en cuenta la vía de administración. Previamente a la encapsulación del péptido, se prepararon nanocápsulas sin carga o "blancas" (NC-Bl) con diferentes variedades de quitosanos, de alto y bajo grado de polimerización (HDP, LDP respectivamente) y distinto grado de acetilación (GA) (Artículo 2). Preferiblemente, se seleccionaron quitosanos con un peso molecular similar al quitosano empleado en los estudios previos realizados con nanocápsulas, en este caso HDP. Para la vía nasal se pensó en un CS fácilmente degradable en el

epitelio nasal. Se espera que la lisozima presente en la cavidad nasal degrade el CS [128, 129], y ello contribuya a la rápida liberación de Ex4 de las nanocápsulas para ser absorbida evitando el aclaramiento mucociliar. Por tanto, seleccionamos el CS con alto Grado de polimerización y G.A. alto (HDP-GA 56) que facilita la degradación por lisozima [128].

Sin embargo, para la administración de Ex4 por vía oral se requiere que el sistema permanezca estable en el medio gastrointestinal y que posea una alta capacidad mucoadhesiva intestinal [46]. Para ello, previamente se evaluó la estabilidad *in vitro* de las nanocápsulas formadas con diferentes tipos de CS en fluidos intestinal (pH 6.8) y gástrico (pH 1.2) simulados (Figura 1). El CS con HDP-G.A 1 fue seleccionado en base a: i) su adecuada estabilidad gastrointestinal, ii) incremento de la resistencia frente a proteasas [106, 107]. Para ambas vías de administración, se demostró que las nanocápsulas de CS podrían favorecer el paso de sustancias a través del epitelio intestinal. Esta capacidad mucoadehsiva se demostró mediante estudios i*n vitro,* en cultivos de células humanas Caco-2 [130], y contrastado posteriormente con estudios *in vivo* [97, 126, 127].

a)



b)

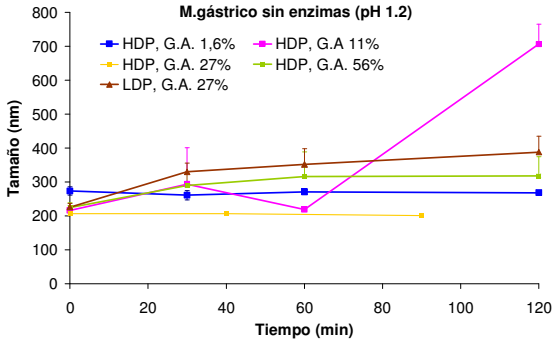


Figura 1: Estabilidad de las nanocápsulas sin carga formadas con diferentes tipos de quitosano en: a) medio intestinal simulado, pH 6.8 y b) medio gástrico simulado, pH 1.2.

Preparación y caracterización físico-química de las nanocápsulas de quitosano

Para la producción de las nanocápsulas se empleó la técnica del desplazamiento del solvente, previamente desarrollada en nuestro laboratorio [131].

En la Tabla 1, se resumen las características de las dos formulaciones de nanocápsulas de Ex4. Los tamaños de partícula fueron similares en ambas formulaciones mientras que como era esperado, el potencial zeta de la formulación desarrollada para la vía oral presentó un valor superior al de la formulación para vía nasal debido a la mayor densidad de carga del CS con el menor G.A. Los valores de tamaño y de potencial zeta (ζ) están dentro del rango encontrado en estudios previos con nanocápsulas blancas y cargadas con capsaicina [132] (artículo 2).

Independientemente del tipo de quitosano, la encapsulación de Ex4 fue alta (>80%) en ambos casos. La alta encapsulación de la Ex4 contrasta con los valores de encontrados previamente para la sCT, donde el rango oscila entre 41-54%. Posiblemente esto fue debido a la contribución de los dominios hidrofóbicos de la Ex4 y a su bajo punto isoeléctrico (*p.I* ~4.2) que favorecen la interacción con las cargas opuestas del CS [133] y por tanto, la retención de la Ex4 en el corazón lipídico [133].

Tabla 1: Propiedades físico-químicas de las nanocápsulas cargadas con exendina-4

Vía	CS GA (%)[a]	Tamaño (nm)	I.P [b]	ζ (mV)	E.E (%)[c]	Ex-4 liberada (%)[d]
Oral	1	206±8	0.13	+53±1	82.7±3.2	40.5±4.6
Nasal	56	226±20	0.20	+45±5	84.9±1.9	--

[a]Grado de acetilación (G.A) del quitosano (CS)
[b]Indice de polidispersión
[c] Eficacia de encapsulación E.E = [(Total exendina4 – exendina4 libre)/Total exendina4] × 100

[d]Exendina liberada tras la incubación de las nanocápsulas en medio intestinal simulado sin enzimas durante 1 h a 37ºC.

Estabilidad en fluidos biológicos simulados

Ambos tipos de nanocápsulas resultaron ser estables en fluidos biológicos simulados relevantes a la vía de administración. Las nanocápsulas desarrolladas para la vía oral fueron evaluadas en medio gástrico e intestinal con presencia de enzimas. Sin embargo, las nanocápsulas diseñadas para la vía nasal se incubaron en el vehículo de administración, tampón acetato pH 4.0, empleado en estudios previos en nuestro laboratorio como medio para la administración de péptidos por vía nasal [11, 40, 42, 97].(Fig. 2). Como se muestra en la figura 2, en todos los casos las nanocápsulas fueron estables durante 120 min en los distintos medios de incubación. La estabilidad de en medio gastrointestinal contrasta con los estudios previos de nanocápsulas de CS-PEG 1% que agregaban tras 1 h de incubación [127]. Sin embargo en nuestro estudio las nanocápsulas permanecieron estables en medio intestinal debido a la alta densidad de carga en la cubierta de CS.

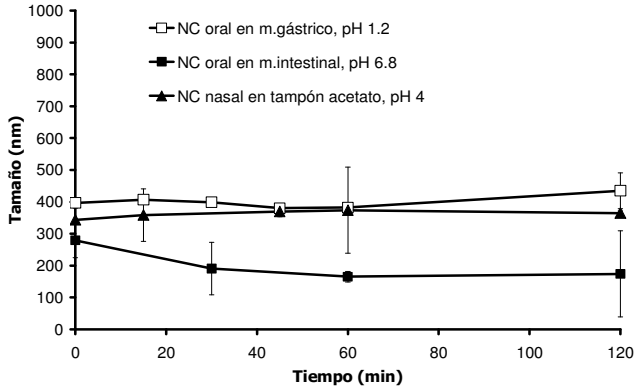


Figura 2: Evolución del tamaño medio de partícula de: nanocápsulas cargadas con exendina-4 formadas por quitosano HDP-GA 1 en fluido gástrico simulado pH 1.2 (cuadrados blancos) y fluido intestinal simulado (cuadrados negros) y nanocápsulas formadas con quitosano HDP-GA56 en

tampón acetato pH 4.0 (triangulos negros). Todas las medidas fueran realizadas a 37°C bajo agitación horizontal (n=3)

Estudios in vivo

Evaluación de la bioactividad de la Ex4

Previamente a la administración de las nanocápsulas por vía oral y nasal, se llevó a cabo un experimento para asegurar que la actividad biológica de la Ex4 no había sido alterada durante el proceso de encapsulación y liberación. Para ello, la Ex4 liberada tras la incubación de las nanocápsulas cargadas con el péptido en medio intestinal (sin enzimas) fue administrada a ratas control por vía intravenosa, y se comparó la respuesta con la obtenida en animales a los que se les administró solo el vehículo y el medio de liberación. En la Figura 3, se observa que la Ex4 liberada de las nanocápsulas incrementa los niveles de glucosa de forma idéntica que la Ex4 sin tratar. En ambos grupos, los niveles de glucosa se alzaron 30 min tras la administración y se mantuvieron elevados durante al menos 120 min [22]. Este estudio confirma que la respuesta biológica de la Ex4 no está alterada durante los procesos de encapsulación y liberación.

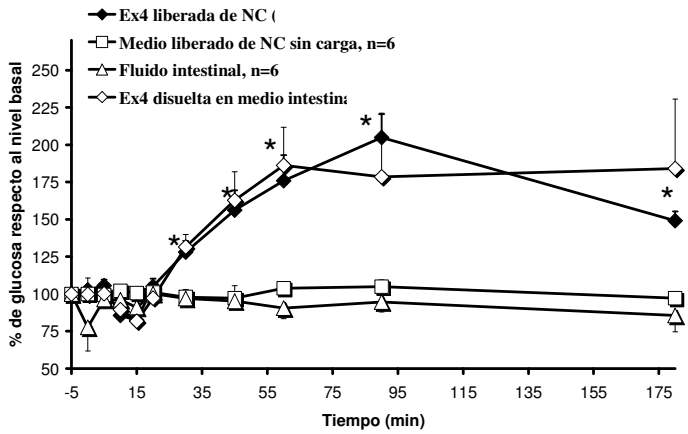


Figura 3: Evolución de los niveles de glucosa tras administración i.v a ratas sanas: Ex4 liberada de las nanocápsulas cargadas (NC-Ex4), n=6 (diamantes negros). Como grupos controles, medio de liberación de las nanocápsulas sin carga (NC-Bl) n=6 (cuadrados blancos), medio intestinal n=6 (triangulos blancos) y Ex4 del stock disuelta en fluido intestinal (diamantes blancos). * $p < 0.05$ diferencias estadísticamente significativas respecto al grupos tratados con NC-Bl y medio intestinal. Dosis de Ex4 ~10µg/Kg

Respuesta farmacológica tras la administración nasal de nanocápsulas de quitosano en la rata.

Una vez comprobada la actividad biológica de la Ex4 durante los procesos de encapsulación y liberación, investigamos la eficacia de las nanocápsulas cargadas con Ex4 (NC-Ex4))mediante vía nasal en la rata. La Figura 4 representa la evolución de los niveles plasmáticos de NC-Ex4 tras la administración nasal comparando con sus respectivos controles: nanocápsulas blancas (NC-Bl), tampón acetato y Ex4 disuelta en tampón acetato. Se observa que el grupo tratado con las NC-Ex4 presenta un incremento en los niveles de glucemia a corto plazo correspondiente a la activación del sistema nervioso simpático y la glándula adrenal por la Ex4 en ratas no diabéticas [22]. Este aumento se prolonga durante al menos 120 min tras la

administración nasal presentando diferencias estadísticamente significativas ($p \leq 0.05$) respecto a los grupos controles tratados con tampón y Ex4 disuelta en tampón acetato. Sin embargo, en el grupo tratado con las nanocápsulas sin carga (blancas) se encontró un aumento inesperado de los niveles de glucosa. Este efecto podría ser causado por la posible irritación local de la mucosa nasal durante la administración, produciendo un aumento en los niveles de glucosa.

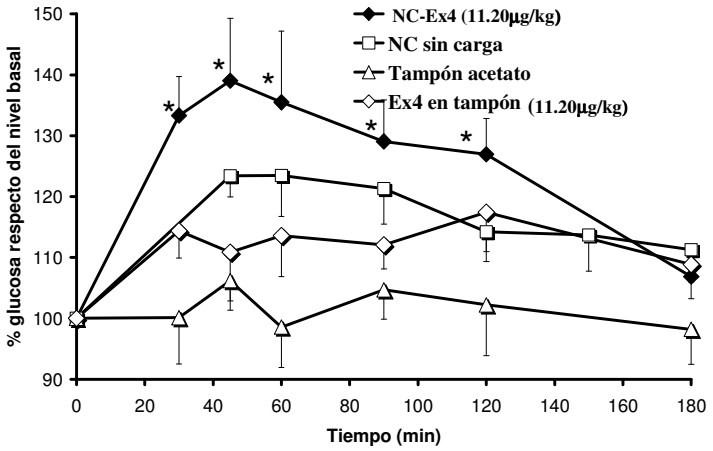


Figure 4: Evolución de los niveles de glucosa tras administración nasal en ratas sanas: nanocápsulas cargadas con Ex4 (NC-Ex4) n=8 (diamantes negros). Los grupos control: nanocápsulas sin carga (NC-Bl) n=6 (cuadrados blancos), Tampón acetato, n=6 (triángulos blancos) y Ex4 disuelta en tampón acetato n=6 (diamantes blancos). (Mean ± s.e.). *$p \leq 0.05$ diferencias estadísticamente significativas respecto a los grupos control tratados con tampón acetato y Ex4 disuelta en tampón.

Como estaba previsto, la presencia de CS con alto grado de acetilación favorece la absorción de Ex4. Estos resultados obtenidos se suman a los de estudios previos en los que nanocápsulas y nanopartículas de CS que aumentaron la absorción sistémica de péptidos y antígenos en varios modelos animales [11, 40, 42, 97,

134]. La capacidad del CS para incrementar la absorción de péptidos a través del epitelio nasal podría ser explicada como consecuencia de la bioadhesión de las nanocápsulas en la mucosa nasal, favoreciendo la apertura de las uniones íntimas junto con la concomitante liberación de Ex4. Esta hipótesis ha sido avalada por la observada habilidad de los sistemas de CS para disminuir reversiblemente la resistencia epitelial en cultivos de células nasales e intestinales cuando se aplica a determinadas dosis [135]. También ha sido avalada por la capacidad del CS para incrementar la permeabilidad celular por vía paracelular e intracelular [104]. Sin embargo, existe otra posibilidad en la que una fracción de las nanocápsulas podría ser capturada directamente por las células epiteliales y liberar la Ex4 intracelularmente. Un estudio reciente demuestra que los nanocápsulas de CS liberan taxanos intracelularmente en líneas de cáncer [136]. El incremento en la captura podría estar relacionado con la interacción del CS con la superficie de las células [137]. Resultados similares fueron encontrados en otro tipo de líneas celulares, como las células del epitelio corneal [138].

Respuesta farmacológica tras la administración oral de nanocápsulas de quitosano en la rata

Una vez demostrada la efectividad de las NC para la liberación de Ex4 por vía nasal, se evaluó su respuesta tras la administración oral. Basándonos en estudios previos desarrollados con nanocápsulas [126, 127] y nanopartículas lipídicas [137] que demostraron incrementar el transporte de péptidos debido a la mucoadhesividad del CS por las células intestinales en rata, inferimos que las

nanocápsulas serían también un vehículo eficaz para el transporte de Ex4 a través del epitelio intestinal.

La respuesta farmacológica tras la administración oral de las NC-Ex4 y de sus controles, que incluye un control de Ex4 por vía i.p, se muestra en la Figura 5. Claramente se observa que solo cuando la Ex4 es inyectada por vía intraperitoneal (i.p) se produce un incremento de los niveles plasmáticos de glucosa en el corto plazo de 1-2 h, mientras que la Ex4 contenida en las nanocápsulas administradas por vía oral no generó variaciones apreciables en la glucemia. La ausencia de respuesta por vía oral contrasta con los estudios anteriores que demostraron la capacidad de las nanocápsulas de CS para vehiculizar sCT en el mismo modelo animal [125-127]. Teniendo en cuenta esta discrepancia, cabe argumentar que es posible que la respuesta farmacológica de haberse producido no se haya detectado. Siguiendo los estudios previos de nanocápsulas por vía oral, se demostró que cuando la dosis de sCT administrada por vía oral es baja, la respuesta farmacológica no se produce de forma continua, sino de forma pulsátil. Caracterizado por el primer pico de efecto menor entre la 1-3h y el pico de mayor efecto entre las 12-24h tras la administración [125]. Este segundo efecto se relacionó con la liberación del péptido encapsulado en el núcleo de la nanocápsulas. En nuestro estudio, la Ex4, debido a sus regiones hidrofóbicas es factible que exhiba mayor afinidad por los componentes del núcleo respecto de la sCT, por ello aumenta la eficacia de encapsulación, pero retarda la liberación del péptido. Este retraso en la liberación implicaría un retraso en la respuesta farmacológica que sumado a la respuesta de corto plazo que produce la Ex4, es posible que hayan podido enmascarar el efecto glucémico de las nanocápsulas en los tiempos de muestreo del experimento.

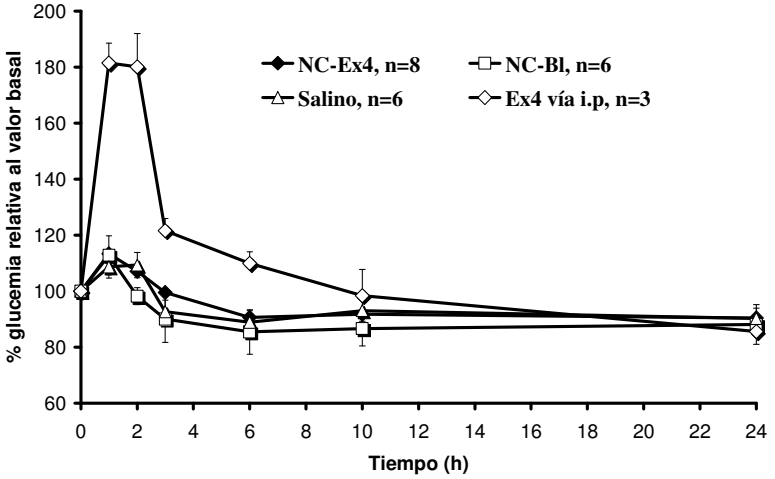


Figure 5: Niveles de glucosa tras administración oral a ratas sanas de nanocápsulas cargadas con Ex4 (NC-Ex4), n=8 (diamantes negros). Como grupos controles negativos se administraron: nanocápsulas sin carga (NC-Bl), n= 6 (cuadrados blancos), solución salina, n=6 (triángulo blanco). El control positivo se hizo con Ex4 por vía intraperitoneal (i.p), n=3 (diamante blanco).

Por otra parte, Estudios previos de nanocápsulas de isobutilcianoacrilato cargadas con insulina han revelado los efectos hipoglucémicos 48h después de su administración intragástrica [139]. Este retraso en la absorción se evidenció en los estudios de *Abobakar et al* mediante microscopio de fluorescencia, en los que las nanocápsulas alcanzaban el íleo una hora después de la administración [140].

CONCLUSIONES

CONCLUSIONES

El trabajo experimental desarrollado durante la presente tesis doctoral se ha dirigido al diseño de diferentes sistemas de tamaño nanométrico formados con lecitina y/o quitosano, destinados a la administración de péptidos para el tratamiento de la diabetes mellitus tipo 1 y 2 por vías no parenterales, como la vía oral y la vía nasal. De un modo más específico los resultados nos han permitido extraer las siguientes conclusiones:

Parte 1: "Desarrollo de nanosistemas a base de lecitina para la administración de insulina por vía oral para el tratamiento de la diabetes mellitus tipo 1".

1. Se han desarrollado dos sistemas capaces de asociar insulina utilizando la interacción iónica entre los componentes. La técnica de preparación es sencilla, no requiere energía y tampoco componentes orgánicos que puedan influir sobre la estabilidad de la insulina. Por una parte, se han obtenido nanocomplejos a partir de lecitina-insulina cuyas propiedades físicas están influenciadas por la composición dada de lecitina e insulina incorporadas, así como, por el grado de ionización de esta última. Por otra parte, se han establecido las condiciones para la formación de nanopartículas de lecitina-quitosano-insulina y estudiado sus propiedades en función de carga neta del péptido y de la cantidad de quitosano. La carga superficial de estos sistemas refleja diferencias que parecen estar relacionadas con su organización molecular.

2. El tamaño de partícula de las formulaciones de ambos tipos de sistemas está dentro del rango nanométrico (<~290 nm). El potencial zeta dependió claramente de la composición del sistema, mostrando valores altamente negativos en el caso del sistema formado únicamente con lecitina e insulina, en tanto que las formulaciones de nanopartículas con quitosano presentaron potenciales tanto positivos como negativos.

3. Los nanocomplejos de lecitina-insulina y las nanopartículas de lecitina-quitosano estudiados presentan una excelente capacidad de asociación de insulina (≥ 83 %). Además, ambos sistemas poseen adecuada estabilidad coloidal en fluido simulado gástrico e intestinal.

4. La administración por vía oral de los nanocomplejos de lecitina-insulina en rata diabética produce un ligero descenso en los niveles plasmáticos de glucosa durante las primeras horas. Además, esta respuesta fue estadísticamente significativa respecto de los grupos control.

5. La inclusión del quitosano, como biopolímero mucoadhesivo, para la formación de las nanopartículas de lecitina-quitosano cargadas con insulina, incrementa y prolonga el descenso de los niveles plasmáticos de glucosa respecto a los obtenidos con nanocomplejos de lecitina-insulina por vía oral. Las nanopartículas alcanzaron en ratón diabético tras 2 h de administración niveles normoglucémicos y prolongaron la hipoglucemia durante 12 h. Estos resultados fueron consistentes con aquellos obtenidos tras el estudio de los niveles de glucosa en orina.

6. Las nanopartículas de lecitina-quitosano cargadas con insulina administradas por vía oral en rata diabética, no presentan disminución de la glucemia cuando se evalúa el resultado del total de los animales. Sin embargo, el 30% de los animales tratados con nanopartículas cargadas con insulina muestran un pronunciado descenso en los niveles de glucosa equivalente a la administración de insulina por vía subcutánea.

Parte 2: "Aplicación de las nanocápsulas de lecitina-quitosano para la vehiculización transmucosal de exendina-4 para el tratamiento de la diabetes mellitus tipo 2"

1. Se han desarrollado nanocápsulas de quitosano empleando la técnica de desplazamiento del solvente a partir de quitosanos de diferentes grados de polimerización y acetilación. Específicamente, se han desarrollado dos prototipos de nanocápsulas formadas con quitosano de alto (~56 %) y bajo (~1 %) grado de acetilación para la administración de exendina-4 por vía nasal y oral, respectivamente.

2. Las formulaciones de nanocápsulas de quitosano con o sin carga de exendina-4, presentan tamaños nanométricos de aproximadamente 200 nm. Sin embargo, el potencial zeta varía con el grado de ionización del quitosano, mostrando mayor carga superficial positiva las nanocápsulas formadas con el quitosano de menor grado de acetilación. Asimismo, la exendina-4 se ha logrado encapsular eficazmente (> 82 %) en

ambas formulaciones de nanocápsulas incrementando el porcentaje de asociación respecto a otros péptidos.

3. Los estudios *in vivo* realizados en rata no diabética demuestran que la exendina-4 es encapsulada y liberada de la nanocápsulas de quitosano en su forma bioactiva. Posteriormente, los resultados de la eficacia farmacológica obtenidos tras la administración de las formulaciones de exendina-4 por vía nasal, revelan la capacidad de las nanocápsulas de quitosano de aumentar la absorción de la exendina-4. Sin embargo, no fue posible demostrar la respuesta de las nanocápsulas cargadas con exendina-4 cuando son administradas por vía oral.

REFERENCIAS

1. Farmaceúticos, C.O.d., *Catálogo de Medicamentos. Consejo Plus.* 1ª ed, ed. C.G.d.C.O.d. Farmaceúticos. 2009.

2. Casanueva, F.F., *Diabetes mellitus: conceptos básicos. Endocrinología*, ed. C.P.F. Arce V.M., Mallo F. 2006, Universidade de Santiago de Comostela: Universidade, Servizo de Publicacions e Intercambio Científico da Universidade de Santiago de Compostela. 417.

3. Shaikh, I.M., et al., *Advanced approaches in insulin delivery.* Current Pharmaceutical Biotechnology, 2005. 6(5): p. 387-395.

4. Ortiz, C., et al., *Identification of insulin variants using Raman spectroscopy.* Analytical Biochemistry, 2004. 332(2): p. 245-252.

5. Wintersteiner, O. and H.A. Abramson, *The isoelectric point of insulin: electrical properties of adsorbed and crystalline insulin.* J. Biol. Chem, 1933. 99: p. 741-753.

6. Gedulin, B.R., et al., *Pharmacokinetics and pharmacodynamics of exenatide following alternate routes of administration.* International Journal of Pharmaceutics, 2008. 356(1-2): p. 231-238.

7. Damgé, C., C.P. Reis, and P. Maincent, *Nanoparticle strategies for the oral delivery of insulin.* Expert Opinion on Drug Delivery, 2008. 5(1): p. 45-68.

8. Babu, V.R., et al., *Developments in polymeric devices for oral insulin delivery.* Expert Opinion on Drug Delivery, 2008. 5(4): p. 403-415.

9. S.A., F.T. *Flamel's Solution: FT-105.* 2009 [cited February 2010]; Available from: http://www.flamel.com/techAndProd/medusa_products.

10. Krauland, A.H., et al., *In vivo evaluation of a nasal insulin delivery system based on thiolated chitosan.* Journal of Pharmaceutical Sciences, 2006. 95(11): p. 2463-2472.

11. Fernández-Urrusuno, R., et al., *Enhancement of nasal absorption of insulin using chitosan nanoparticles.* Pharmaceutical Research, 1999. 16(10): p. 1576-1581.

12. Bowsher, R.R., et al., *Sensitive RIA for the specific determination of insulin lispro.* Clinical Chemistry, 1999. 45(1): p. 104-110.

13. Komatsu, Y., et al., *C-type natriuretic peptide (CNP) in rats and humans.* Endocrinology, 1991. 129(2): p. 1104-1106.

14. DeFronzo, R.A., *Pharmacologic therapy for type 2 diabetes mellitus.* Annals of Internal Medicine, 1999. 131(4): p. 281-303.
15. St. Louis : Facts and Comparisons, c., *Drug Facts and Comparisons*. Vol. 1. 2008: Advisory Panel.
16. Parkes, D., *Pharmacokinetic actions of exendin-4 in the rat: Comparison with glucagon-like peptide-1.* Drug Development Research, 2001. 53(4): p. 260-267.
17. Eng, J., et al., *Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas.* Journal of Biological Chemistry, 1992. 267(11): p. 7402-7405.
18. Nielsen, L.L., et al., *Pharmacology of exenatide (synthetic exendin-4): A potential therapeutic for improved glycemic control of type 2 diabetes.* Regulatory Peptides, 2004. 117(2): p. 77-88.
19. Hirata, K., et al., *Exendin-4 has an anti-hypertensive effect in salt-sensitive mice model.* Biochemical and Biophysical Research Communications, 2009. 380(1): p. 44-49.
20. Botana, M., *Nuevos tratameintos de la diabetes y susmanifiestaciones específicas: estimuladores del receptor GLP-1*, XXI Congreso de la Sociedad Gallega de Endocrinología Nutrición y Metabolismo, Editor. 2009, Auditorio Municipal: Ourense.
21. Jin, C.H., et al., *A new orally available glucagon-like peptide-1 receptor agonist, biotinylated exendin-4, displays improved hypoglycemic effects in db/db mice.* Journal of Controlled Release, 2009. 133(3): p. 172-177.
22. Pérez-Tilve D., et al., *Exendin-4 increases blood glucose levels acutely in rats by the activations of the sympathetic nervous system.* Am J Physiol Endocrinol Metab, Accepted 2009.
23. Khan Ghilzai, N.M., *New developments in insulin delivery.* Drug Development and Industrial Pharmacy, 2003. 29(3): p. 253-265.
24. Takei, I. and T. Kasatani, *Future therapy of diabetes mellitus.* Biomedicine and Pharmacotherapy, 2004. 58(10): p. 578-581.
25. Khafagy, E.S., et al., *Current challenges in non-invasive insulin delivery systems: A comparative review.* Advanced Drug Delivery Reviews, 2007. 59(15): p. 1521-1546.

26. Hugues B, et al., *Laboratory animals in endocrinology. Biomodels of type I diabetes mellitus.* Rev. cuba. endocrinol., 2001. 12(3): p. 168-177.
27. Gordon Still, J., *Development of oral insulin: Progress and current status.* Diabetes/Metabolism Research and Reviews, 2002. 18(SUPPL. 1).
28. Klingler, C., et al., *Insulin-micro- and nanoparticles for pulmonary delivery.* International Journal of Pharmaceutics, 2009. 377(1-2): p. 173-179.
29. Al-Qaddi S., et al., *Chitosan nanoparticle-based inhalable dry-powders for protein lung delivery: IN vivo evaluation of microencapsulated insulin-loaded nanoparticles in rats*, E.e.c. society), Editor. 2009: San Servolo, 2009 , Italia.
30. Aungst, B.J. and N.J. Rogers, *Site dependence of absorption-promoting actions of Laureth-9, Na salicylate, Na2EDTA, and aprotinin on rectal, nasal, and buccal insulin delivery.* Pharmaceutical Research, 1988. 5(5): p. 305-308.
31. Lee, Y.C., et al., *Review on the systemic delivery of insulin via the ocular route.* International Journal of Pharmaceutics, 2002. 233(1-2): p. 1-18.
32. Sintov, A.C. and U. Wormser, *Topical iodine facilitates transdermal delivery of insulin.* Journal of Controlled Release, 2007. 118(2): p. 185-188.
33. Maroni, A., et al., *Feasibility, stability and release performance of a time-dependent insulin delivery system intended for oral colon release.* European Journal of Pharmaceutics and Biopharmaceutics, 2009. 72(1): p. 246-251.
34. Bayat, A., et al., *Nanoparticles of quaternized chitosan derivatives as a carrier for colon delivery of insulin: Ex vivo and in vivo studies.* International Journal of Pharmaceutics, 2008. 356(1-2): p. 259-266.
35. Aungst, B.J. and N.J. Rogers, *Site dependence of absorption-promoting actions of Laureth-9, Na salicylate, Na2EDTA, and aprotinin on rectal, nasal, and buccal insulin delivery.* Pharmaceutical Research, 1988. 5(5): p. 305-308.
36. Shaikh, I.M., et al., *Advanced approaches in insulin delivery.* Current Pharmaceutical Biotechnology, 2005. 6(5): p. 387-395.
37. Richardson, J.L., et al., *Vaginal absorption of insulin in the rat: Effect of penetration enhancers on insulin uptake and mucosal histology.* Pharmaceutical Research, 1992. 9(7): p. 878-883.

38. Khafagy, E.S., et al., *Current challenges in non-invasive insulin delivery systems: A comparative review.* Advanced Drug Delivery Reviews, 2007. 59(15): p. 1521-1546.
39. Golomb, G., et al., *A new route of drug administration: Intrauterine delivery of insulin and calcitonin.* Pharmaceutical Research, 1993. 10(6): p. 828-833.
40. Goycoolea, F.M., et al., *Chitosan-alginate blended nanoparticles as carriers for the transmucosal delivery of macromolecules.* Biomacromolecules, 2009. 10(7): p. 1736-1743.
41. Wang, X., et al., *Chitosan-NAC nanoparticles as a vehicle for nasal absorption enhancement of insulin.* Journal of Biomedical Materials Research - Part B Applied Biomaterials, 2009. 88(1): p. 150-161.
42. Teijeiro-Osorio, D., et al., *New generation of hybrid poly/oligosaccharide nanoparticles as carriers for the nasal delivery of macromolecules.* Biomacromolecules, 2009. 10(2): p. 243-249.
43. Zhang, X., et al., *Nasal absorption enhancement of insulin using PEG-grafted chitosan nanoparticles.* European Journal of Pharmaceutics and Biopharmaceutics, 2008. 68(3): p. 526-534
44. Jain, A.K., et al., *Effective insulin delivery using starch nanoparticles as a potential trans-nasal mucoadhesive carrier.* European Journal of Pharmaceutics and Biopharmaceutics, 2008. 69(2): p. 426-435.
45. Dyer, A.M., et al., *Nasal delivery of insulin using novel chitosan based formulations: A comparative study in two animal models between simple chitosan formulations and chitosan nanoparticles.* Pharmaceutical Research, 2002. 19(7): p. 998-1008.
46. Woitiski, C.B., et al., *Strategies toward the improved oral delivery of insulin nanoparticles via gastrointestinal uptake and translocation.* BioDrugs, 2008. 22(4): p. 223-237.
47. Crane, C.W. and G.R. Luntz, *Absorption of insulin from the human small intestine.* Diabetes, 1968. 17(10): p. 625-627.
48. Jung, T., et al., *Biodegradable nanoparticles for oral delivery of peptides: Is there a role for polymers to affect mucosal uptake?* European Journal of Pharmaceutics and Biopharmaceutics, 2000. 50(1): p. 147-160.

49. Lane, M.E., et al., *Quantitative estimation of the effects of bile salt surfactant systems on insulin stability and permeability in the rat intestine using a mass balance model.* Journal of Pharmacy and Pharmacology, 2005. 57(2): p. 169-175.
50. Mesiha, M.S., et al., *Oral and subcutaneous absorption of insulin poly(isobutylcyanoacrylate) nanoparticles.* International Journal of Pharmaceutics, 2005. 288(2): p. 289-293.
51. Uchiyama, T., et al., *Enhanced permeability of insulin across the rat intestinal membrane by various absorption enhancers: Their intestinal mucosal toxicity and absorption-enhancing mechanism of n-lauryl-Î²-D-maltopyranoside.* Journal of Pharmacy and Pharmacology, 1999. 51(11): p. 1241-1250.
52. Morishita, M., et al., *The dose-related hypoglycemic effects of insulin emulsions incorporating highly purified EPA and DHA.* International Journal of Pharmaceutics, 2000. 201(2): p. 175-185.
53. Marschütz, M.K. and A. Bernkop-Schnürch, *Oral peptide drug delivery: Polymer-inhibitor conjugates protecting insulin from enzymatic degradation in vitro.* Biomaterials, 2000. 21(14): p. 1499-1507.
54. Liu, H., et al., *Potential utility of various protease inhibitors for improving the intestinal absorption of insulin in rats.* Journal of Pharmacy and Pharmacology, 2003. 55(11): p. 1523-1529.
55. Agarwal, V., et al., *Oral delivery of proteins: Effect of chicken and duck ovomucoid on the stability of insulin in the presence of Î±-chymotrypsin and trypsin.* Pharmacy and Pharmacology Communications, 2000. 6(5): p. 223-227.
56. Tas, C., et al., *In vitro and ex vivo permeation studies of etodolac from hydrophilic gels and effect of terpenes as enhancers.* Drug Delivery, 2007. 14(7): p. 453-459.
57. Miyazaki, Y., et al., *In vitro and in vivo evaluation of mucoadhesive microspheres consisting of dextran derivatives and cellulose acetate butyrate.* International Journal of Pharmaceutics, 2003. 258(1-2): p. 21-29.
58. Prego, C., et al., *The potential of chitosan for the oral administration of peptides.* Expert Opinion on Drug Delivery, 2005. 2(5): p. 843-854.
59. Bernkop-Schnürch, A. and A. Greimel, *Thiomers: The next generation of mucoadhesive polymers.* American Journal of Drug Delivery, 2005. 3(3): p. 141-154.

60. Roldo, M., et al., *Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: Synthesis and in vitro evaluation.* European Journal of Pharmaceutics and Biopharmaceutics, 2004. 57(1): p. 115-121.
61. Bernkop-Schnürch, A., C.E. Kast, and M.F. Richter, *Improvement in the mucoadhesive properties of alginate by the covalent attachment of cysteine.* Journal of Controlled Release, 2001. 71(3): p. 277-285.
62. Carvalho, E.L.S., et al., *Mucosal Delivery of Liposome–Chitosan Nanoparticle Complexes*. In Nejat Düzgüneş ed, ed. M.i. enzymology. Vol. 465. 2009 Burlington: Elsevier Inc. 289-312.
63. Sarmento, B., et al., *Oral bioavailability of insulin contained in polysaccharide nanoparticles.* Biomacromolecules, 2007. 8(10): p. 3054-3060.
64. Sarmento, B., et al., *Alginate/chitosan nanoparticles are effective for oral insulin delivery.* Pharmaceutical Research, 2007. 24(12): p. 2198-2206.
65. Rekha, M.R. and C.P. Sharma, *Synthesis and evaluation of lauryl succinyl chitosan particles towards oral insulin delivery and absorption.* Journal of Controlled Release, 2009. 135(2): p. 144-151.
66. Yin, L., et al., *Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery.* Biomaterials, 2009. 30(29): p. 5691-5700.
67. Elsayed, A., et al., *Formulation and characterization of an oily-based system for oral delivery of insulin.* European Journal of Pharmaceutics and Biopharmaceutics, 2009. 73(2): p. 269-279.
68. Lin, Y.H., et al., *Preparation and characterization of nanoparticles shelled with chitosan for oral insulin delivery.* Biomacromolecules, 2007. 8(1): p. 146-152.
69. Qian, F., et al., *Chitosan graft copolymer nanoparticles for oral protein drug delivery: Preparation and characterization.* Biomacromolecules, 2006. 7(10): p. 2722-2727.
70a. Bhumkar, D.R., et al., *Chitosan reduced gold nanoparticles as novel carriers for transmucosal delivery of insulin.* Pharmaceutical Research, 2007. 24(8): p. 1415-1426.
70b. Pan, Y., et al., *Bioadhesive polysaccharide in protein delivery system: Chitosan nanoparticles improve the intestinal absorption of insulin in vivo.* International Journal of Pharmaceutics, 2002. 249(1-2): p. 139-147.

70c. Pan, Y., et al., *Relationship between drug effects and particle size of insulin-loaded bioadhesive microspheres.* Acta Pharmacologica Sinica, 2002. 23(11): p. 1051-1056
71. Chalasani, K.B., et al., *A novel vitamin B12-nanosphere conjugate carrier system for peroral delivery of insulin.* Journal of Controlled Release, 2007. 117(3): p. 421-429.
72. Chalasani, K.B., et al., *Effective oral delivery of insulin in animal models using vitamin B12-coated dextran nanoparticles.* Journal of Controlled Release, 2007. 122(2): p. 141-150.
73. Salmaso, S., et al., *Biopharmaceutical characterisation of insulin and recombinant human growth hormone loaded lipid submicron particles produced by supercritical gas micro-atomisation.* International Journal of Pharmaceutics, 2009. 379(1-2): p. 51-58.
73b. Cui, F.D., et al., *Preparation of insulin loaded PLGA-Hp55 nanoparticles for oral delivery.* Journal of Pharmaceutical Sciences, 2007. 96(2): p. 421-427.
74 Foss, A.C., et al., *Development of acrylic-based copolymers for oral insulin delivery.* European Journal of Pharmaceutics and Biopharmaceutics, 2004. 57(2): p. 163-169.
74b. Carino, G.P., et al., *Nanosphere based oral insulin delivery.* Journal of Controlled Release, 2000. 65(1-2): p. 261-269.
75. Alonso-Sande, M., *Nanopartículas poliméricas modificadas con manosa: Nuevos sistemas destinados a la administracion oral de macromoléculas terapéuticas*, in *Departamento de Farmacia y Tecnología Farmaceútica*. 2006, Santiago de Compostela: Santiago de Compostela. p. 255.
75b Cui, F., et al., *Biodegradable nanoparticles loaded with insulin-phospholipid complex for oral delivery: Preparation, in vitro characterization and in vivo evaluation.* Journal of Controlled Release, 2006. 114(2): p. 242-250.
76. Watnasirichaikul, S., et al., *In-vitro release and oral bioactivity of insulin in diabetic rats using nanocapsules dispersed in biocompatible microemulsion.* Journal of Pharmacy and Pharmacology, 2002. 54(4): p. 473-480.
77. Damgé, C., et al., *Poly(alkyl cyanoacrylate) nanospheres for oral administration of insulin.* Journal of Pharmaceutical Sciences, 1997. 86(12): p. 1403-1409.

78. Graf, A., et al., *Oral insulin delivery using nanoparticles based on microemulsions with different structure-types: Optimisation and in vivo evaluation.* European Journal of Pharmaceutical Sciences, 2009. 37(1): p. 53-61.
79. Xiong, X.Y., et al., *Vesicles from Pluronic/poly(lactic acid) block copolymers as new carriers for oral insulin delivery.* Journal of Controlled Release, 2007. 120(1-2): p. 11-17.
80. Xia, C.Q., et al., *Hypoglycemic effect of insulin-transferrin conjugate in streptozotocin-induced diabetic rats.* Journal of Pharmacology and Experimental Therapeutics, 2000. 295(2): p. 594-600.
81. Damge, C., et al., *Nanocapsules as carriers for oral peptide delivery.* Journal of Controlled Release, 1990. 13(2-3): p. 233-239.
82. Zhenqing, H., et al., *Use of natural plant exudates (Sanguis Draxonis) for sustained oral insulin delivery with dramatic reduction of glycemic effects in diabetic rats.* Journal of Controlled Release, 2004. 97(3): p. 467-475.
83. Wang, T., et al., *Lyophilization of water-in-oil emulsions to prepare phospholipid-based anhydrous reverse micelles for oral peptide delivery.* European Journal of Pharmaceutical Sciences. 39(5): p. 373-379.
84. Kisel, M.A., et al., *Liposomes with phosphatidylethanol as a carrier for oral delivery of insulin: Studies in the rat.* International Journal of Pharmaceutics, 2001. 216(1-2): p. 105-114.
85. Iwanaga, K., et al., *Oral delivery of insulin by using surface coating liposomes. Improvement of stability of insulin in GI tract.* International Journal of Pharmaceutics, 1997. 157(1): p. 73-80.
86. Choudari, K.B., et al., *Liposomes as carrier for oral administration of insulin effect of formulation factors.* Journal of Microencapsulation, 1994. 11(3): p. 319-325.
87. Al-Achi, A. and R. Greenwood, *Intraduodenal administration of biocarrier-insulin systems.* Drug Development and Industrial Pharmacy, 1993. 19(11): p. 1303-1315.
88. Patel, H.M. and B.E. Ryman, *Oral administration of insulin by encapsulation within liposomes.* FEBS Letters, 1976. 62(1): p. 60-63.
89. Hargrove, D.M., et al., *Biological activity of AC3174, a peptide analog of exendin-4.* Regulatory Peptides, 2007. 141(1-3): p. 113-119.

90. Son, S., et al., *Preparation and structural, biochemical, and pharmaceutical characterizations of bile acid-modified long-acting exendin-4 derivatives.* Journal of Medicinal Chemistry, 2009. 52(21): p. 6889-6896.
91. Hirai, S., T. et al., *Effect of surfactants on the nasal absorption of insulin in rats.* International Journal of Pharmaceutics, 1981. 9(2): p. 165-172.
92. Tengamnuay, P. and A.K. Mitra, *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.* Pharmaceutical Research, 1990. 7(4): p. 370-375.
93. Drejer, K., et al., *Intranasal administration of insulin with phospholipid as absorption enhancer: Pharmacokinetics in normal subjects.* Diabetic Medicine, 1992. 9(4): p. 335-340.
94. Hinchcliffe, M. and L. Illum, *Intranasal insulin delivery and therapy.* Advanced Drug Delivery Reviews, 1999. 35(2-3): p. 199-234.
95. Pringels, E., et al., *Influence of deposition and spray pattern of nasal powders on insulin bioavailability.* International Journal of Pharmaceutics, 2006. 310(1-2): p. 1-7.
96. Magdassi, S., et al., *Formation of positively charged microcapsules based on chitosan-lecithin interactions.* Journal of Microencapsulation, 1997. 14(2): p. 189-195.
96b. Sonvico, F., et al., *Formation of self-organized nanoparticles by lecithin/chitosan ionic interaction.* International Journal of Pharmaceutics, 2006. 324(1): p. 67-73.
97. Prego, C., et al., *Chitosan nanocapsules: A new carrier for nasal peptide delivery.* Journal of Drug Delivery Science and Technology, 2006. 16(5): p. 331-337.
98. Mady, M.M., et al., *Biophysical studies on chitosan-coated liposomes.* European Biophysics Journal, 2009. 38(8): p. 1127-1133.
99. Pharmacopeia, U.S., *USP 31 - NF 29*. 1st ed. Vol. 1. 2008. 898.
100. Sugawara T, et al., *Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human intestinal cells.* J Nutr 2001. 131: p. 2921-7.
101. Lakshminarayana R, et al., *Enhanced lutein bioavailability by lyso-phosphatidylcholine in rats.* Mol Cell Biochem, 2006. 281:: p. 103-10.

102. Sorlier, P., et al., *Relation between the degree of acetylation and the electrostatic properties of chitin and chitosan.* Biomacromolecules, 2001. 2(3): p. 765-772.
103. Thanou, M., et al., *Chitosan and its derivatives as intestinal absorption enhancers.* Advanced Drug Delivery Reviews, 2001. 50(SUPPL. 1).
104. Dodane, V., et al., *Effect of chitosan on epithelial permeability and structure.* International Journal of Pharmaceutics, 1999. 182(1): p. 21-32.
105. Vårum, K.M., et al., *In vitro degradation rates of partially N-acetylated chitosans in human serum.* Carbohydrate Research, 1997. 299(1-2): p. 99-101.
106. Kumar, A.B.V. and R.N. Tharanathan, *A comparative study on depolymerization of chitosan by proteolytic enzymes.* Carbohydrate Polymers, 2004. 58(3): p. 275-283.
107. Roncal, T., et al., *High yield production of monomer-free chitosan oligosaccharides by pepsin catalyzed hydrolysis of a high deacetylation degree chitosan.* Carbohydrate Research, 2007. 342(18): p. 2750-2756.
108. Trombotto, S., et al., *Chemical preparation and structural characterization of a homogeneous series of chitin/chitosan oligomers.* Biomacromolecules, 2008. 9(7): p. 1731-1738.
109. Hayashi, K. and M. Ito, *Antidiabetic action of low molecular weight chitosan in genetically obese diabetic KK-Ay mice.* Biological and Pharmaceutical Bulletin, 2002. 25(2): p. 188-192.
110. Kondo, Y., et al., *Low molecular weight chitosan prevents the progression of low dose streptozotocin-induced slowly progressive diabetes mellitus in mice.* Biological and Pharmaceutical Bulletin, 2000. 23(12): p. 1458-1464.
111. Miura, T., et al., *Hypoglycemic and hypolipidemic effect of chitosan in normal and neonatal streptozotocin-induced diabetic mice.* Biological and Pharmaceutical Bulletin, 1995. 18(11): p. 1623-1625.
112. Yao, H.T., et al., *A comparative study on hypoglycemic and hypocholesterolemic effects of high and low molecular weight chitosan in streptozotocin-induced diabetic rats.* Food and Chemical Toxicology, 2008. 46(5): p. 1525-1534.
113. Calvo, P., et al., *Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers.* Journal of Applied Polymer Science, 1997. 63(1): p. 125-132.

114. Perry, M.C., et al., *The interaction of insulin with phospholipids.* Biochemical Journal, 1971. 125(1): p. 179-187.
115. Chuah, A.M., et al., *Formation of biocompatible nanoparticles via the self-assembly of chitosan and modified lecithin.* Journal of Food Science, 2009. 74(1).
116. Hafner, A., et al., *Melatonin-loaded lecithin/chitosan nanoparticles: Physicochemical characterisation and permeability through Caco-2 cell monolayers.* International Journal of Pharmaceutics, 2009. 381(2): p. 205-213.
116b Barbieri, S., et al., *Chitosan/Lecithin Autoassembled nanoparticles: a new carrier for oral delivery.* 9th International Conference of the European Chitin Society. EUCHIS. , 2009.
117. Batzri, S. and E.D. Korn, *Single bilayer liposomes prepared without sonication.* BBA - Biomembranes, 1973. 298(4): p. 1015-1019.
118. Pons, M., et al., *Liposomes obtained by the ethanol injection method.* International Journal of Pharmaceutics, 1993. 95(1-3): p. 51-56.
119. Gerelli, Y., et al., *Structure of self-organized multilayer nanoparticles for drug delivery.* Langmuir, 2008. 24(20): p. 11378-11384.
120. Gerelli, Y., et al., *Structure and organization of phospholipid/polysaccharide nanoparticles.* Journal of Physics Condensed Matter, 2008. 20(10).
121. Rowland, R.N. and J.F. Woodley, *The stability of liposomes in vitro to pH, bile salts and pancreatic lipase.* Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism, 1980. 620(3): p. 400-409.
122. Rinaudo, M., et al., *Solubilization of Chitosan in Strong Acid Medium.* International Journal of Polymer Analysis and Characterization, 1999. 5(3): p. 267-276.
123. Sorlier, P., et al., *Relation between solution properties and degree of acetylation of chitosan: Role of aging.* Biomacromolecules, 2002. 3(6): p. 1336-1342.
124. Il'ina, A.V. and V.P. Varlamov, *Effect of the Degree of Acetylation of Chitosan on Its Enzymatic Hydrolysis with the Preparation Celloviridin G20kh.* Applied Biochemistry and Microbiology, 2003. 39(3): p. 239-242.
125. Prego, C., et al., *Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery.* Pharmaceutical Research, 2006. 23(3): p. 549-556.

126. Prego, C., et al., *Chitosan nanocapsules as carriers for oral peptide delivery: Effect of chitosan molecular weight and type of salt on the in vitro behaviour and in vivo effectiveness.* Journal of Nanoscience and Nanotechnology, 2006. 6(9-10): p. 2921-2928.
127. Prego, C., et al., *Chitosan-PEG nanocapsules as new carriers for oral peptide delivery: Effect of chitosan pegylation degree.* Journal of Controlled Release, 2006. 111(3): p. 299-308.
128. Nordtveit, R.J., et al., *Degradation of partially N-acetylated chitosans with hen egg white and human lysozyme.* Carbohydrate Polymers, 1996. 29(2): p. 163-167.
129. Wilson, M., *Microbial inhabitants of humans: their ecology and role in health and disease.* Vol. Chapter 4: The respiratory system and its indigenous microbiota. 2005, Cambridge: Cambridge University 450.
130. Schipper, et al., *Chitosans as absorption enhancers for poorly absorbable drugs. 1: Influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells.* Pharmaceutical Research, 1996. 13(11): p. 1686-1692.
131. Calvo, P., et al., *Development of positively charged colloidal drug carriers: Chitosan-coated polyester nanocapsules and submicron-emulsions.* Colloid and Polymer Science, 1997. 275(1): p. 46-53.
132. Goycoolea F.M., et al., *Chitosan-coated nanocapsules: Physical characterization, capsaicin encapsulation efficiency and stability in biological media .* Submitted.
133. Neidigh, J.W., et al., *Exendin-4 and glucagon-like-peptide-1: NMR structural comparisons in the solution and micelle-associated states.* Biochemistry, 2001. 40(44): p. 13188-13200.
134. Calvo, P., et al., *Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines.* Pharmaceutical Research, 1997. 14(10): p. 1431-1436.
135. Prego, C., et al., *Transmucosal macromolecular drug delivery.* Journal of Controlled Release, 2005. 101(1-3 SPEC. ISS.): p. 151-162.
136. Lozano, M.V., et al., *Highly efficient system to deliver taxanes into tumor cells: Docetaxel-loaded chitosan oligomer colloidal carriers.* Biomacromolecules, 2008. 9(8): p. 2186-2193.

137. Garcia-Fuentes, M., et al., *A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery.* European Journal of Pharmaceutical Sciences, 2005. 25(1): p. 133-143.
138. De Campos, A.M., et al., *The effect of a PEG versus a chitosan coating on the interaction of drug colloidal carriers with the ocular mucosa.* European Journal of Pharmaceutical Sciences, 2003. 20(1): p. 73-81.
139. Michel, C., et al., *The effect of site of administration in the gastrointestinal tract on the absorption of insulin from nanocapsules in diabetic rats.* Journal of Pharmacy and Pharmacology, 1991. 43(1): p. 1-5.
140. Aboubakar, M., et al., *Study of the mechanism of insulin encapsulation in poly(isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization.* Journal of Biomedical Materials Research, 1999. 47(4): p. 568-576.

ANEXOS

ANEXOS

Histología hepática y necropsia

Artículo 4

HISTOLOGÍA HEPÁTICA.

Una vez obtenidos los resultados tras la administración oral de las nanocápsulas, se realizaron estudios morfológicos en busca de posibles señales que demostraran la absorción de la exendina-4 y/o las nanocápsulas a través del epitelio intestinal. En general, las sustancias cuando se absorben en el intestino delgado acceden al hígado a través de la vena porta. Por lo tanto, los estudios histológicos del hígado podrían revelar algún signo relacionado con la absorción de la exendina-4 y/o las nanocápsulas.

Para desvelar diferentes aspectos relacionados con la morfología y función hepática, los cortes histológicos fueron teñidos utilizando la tinción de Masson, Perls, Halls, tricrómico de Masson, Fontana y Sudán negro. Sin embargo, sólo la tinción general hematoxilina-eosina mostró anomalías en la morfología. Se observaron depósitos de grasa extracelulares relacionados con esteatosis aguda de gota pequeña en las muestras de ratas tratadas con exendina-4 y/o con nanocápsulas (ver Fig. 6). Posteriormente, se confirmaron las mismas alteraciones hepáticas en animales tratados con solución salina. Estos resultados se relacionaron con la alimentación a base de piensos con elevado contenido en grasa. Por tanto, las nanocápsulas y/o la exendina-4 administradas por vía oral, si acceden al hígado, no inducen alteraciones morfológicas o funcionales a corto plazo.

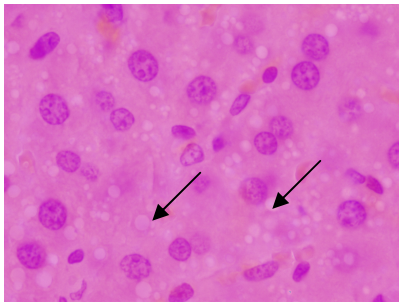


Figura 1: Corte histológico del hígado a 100x con tinción general hematoxilina-eosina. Se observan depósitos de grasa extracelulares.

NECROPSIA

Concluidos los experimentos in vivo, los animales fueron examinados post-mortem. En todos los experimentos se evaluó el estado general, aunque de forma particular se dedicó especial atención a los órganos relacionados con la vía de administración. Los animales que recibieron el tratamiento por vía oral, se les examinó en detalle el estómago, intestino delgado y grueso e hígado. Mientras que los animales que recibieron tratamiento por vía nasal se les examinó los pulmones.

Ratas tratadas con nanocomplejos de lecitina-insulina y nanocápsulas de quitosano por vía oral.

Las ratas diabéticas y sanas tratadas con nanocomplejos de lecitina-insulina y nanocápsulas de quitosano por vía oral, respectivamente, no presentaron ningún signo de alteración en el tracto gastrointestinal respecto al grupo de animales control tratados con salino.

Roedores diabéticos tratados con nanopartículas de lecitina-quitosano por vía oral

Por otra parte, los animales (ratón/rata) conscientes tratados con las nanopartículas con o sin carga, presentaban en el interior del estómago una gran masa. Mientras que en los roedores tratados con agua o salino, el estómago no presentaba ningún contenido. En ambos casos, el interior del intestino delgado fue independiente del tipo de tratamiento recibido, mostrándose vacio.

Ratas diabéticas anestesiadas tratadas con nanopartículas de lecitina-quitosano por vía oral

De los tres grupos que recibieron tratamiento, únicamente el grupo tratado con agua no presentaba ningún signo de alteración, en estómago e intestino delgado. Mientras que las ratas tratadas con nanopartículas de lecitina-quitosano con o sin carga, presentaban el estómago e intestino llenos de sangre.

ANEXOS

Histología hepática y necropsia

Artículo 4

ARTÍCULO 4

Chitooligosaccharides (COS) do not modify the short-term glucose plasma levels of healthy and streptozotocin (STZ)-induced diabetic rats

Valle-Gallego A[1,2], Goycoolea F.M[1*].,Mallo F.[2],Ladavière C.[3], Trombotto S.[3] and M.J. Alonso [1]

[1]*Departement of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of Santiago de Compostela, Spain*

[2]*Laboratory of Endocrinology, Faculty of Biology, University of Vigo. Spain*

[3]*Laboratoire des Matériaux Polymères et Biomatériaux, Ingénierie des Matériaux Polymères (UMR 5223), CNRS, Université Claude Bernard Lyon 1, France*

*Corresponding author: Francisco M. Goycoolea
E-mail: fm.goycoolea@usc.es

(Manuscript to be submitted for publication)

Abstract

The aim of this work was to evaluate the effect of a single dose (35mg/rat) of chitosan oligomers (COS) by i.p route to normal and STZ-induced diabetic rats on the acute short-term glycaemia response. The results evidence that COS (DP 2-10) do not modify the plasma glucose response in both models over a period of 24h.

Introduction

Chitooligosaccharides (COS) are a group of oligosaccharides obtained by chemical or enzymatic hydrolysis of chitosan (CS) or chitin [1]. Generally, COS refer to chitosan or chitin with a degree of polymerization DP < ~20. The potential to improve food quality and human health has been emphasized [2]. In addition, recent studies have claimed that COS exhibit a number of biological activities in mammals: increase the insulin secretion [3], improve the glucose tolerance [3, 4], stimulate the proliferation and viability of pancreatic islet cells in STZ-induced diabetic rats [4], lower the blood cholesterol, lower the high blood pressure, protect effects against infections, control the arthritis, enhance antitumor properties [2] and prevent the diabetic cardiomyopathy [3].

The role of CS and COS on the glucose metabolism has been addressed in a number of studies. The CS ability to modify the glucose levels in a long-term response after an oral administering has been reported in several experiments on diabetic and normal animals. Miura *et al.* (1995), first showed that the incorporation of CS in the diet (5% food admixture) during 4 weeks reduced the glycaemic levels significantly in neonatal normal and streptozotocin (STZ)-induced diabetic mice with hypoinsulinemia [5]. In another studies, low molecular weight CS (LMWCS) was added to drinking water (0.2 or 0.8% water solution) during several weeks and found to reduce the glycemia and insulinemia in a dose-dependent manner in non fasting genetically obese diabetic KK-A^y mice with hyperinsulinemia [6] and to prevent the progression of low

dose STZ-induced slowly progressive non insulin dependent diabetes mellitus (NIDDM) in mice [7]. Recently, other study compared two types of CS (high and low molecular weight CS, HMWCS and LMWCS, respectively) incorporated in food during one month for STZ-induced diabetic rats [8]. HMWCS proved a significant reduction on the glycemic levels *versus* the control group. However LMWCS, obtained by chemical hydrolysis from HMWCS, did not decrease the glucose plasma. In the same study, the group of normal rats fed with both types of CS (5% food admixture) during 4 weeks, did not modify their glucose levels with respect to the control group fed without CS [8]. Nevertheless, this effect is at odds with the effects observed by Miura *et al.* [5].

COS have also been documented to perform activity on the glucose regulation in two studies. Lee *et al.* (2003), included COS in drinking water (0.3% water) of neonatal STZ-induced NIDDM rats, decreasing the glucose levels in fasting rats and improving the glucose tolerance in fasting and feeding rats after 4 weeks of treatment [3]. Meanwhile, in normal rats, COS increased the glucose tolerance but did not lead to a significant modification of the glycaemia [3]. In a recent study, Liu *et al.* (2007) administered COS with different doses (250-500-1500mg/ml) by oral gavage in STZ-induced diabetic rats. After 9 days, low doses of COS stimulated the proliferation and viability of pancreatic islet cells, increasing the insulin secretion. After 8 weeks, any dose improved the tolerance glucose after 1 hour decreasing the glucose response [4].

Nowadays, CS has been utilized to produce nano- and microparticles CS-based systems as carriers for drug delivery [9-11]. Major efforts have been made for the application of these nanomedicine systems in the diabetes therapy (exendin-4 or insulin). In order to elucidate the bioactivity of CS-based carriers on the glucose regulation, we have evaluated in this work the short-term response after treatment with COS produced by enzymatic hydrolysis of LMWCS in normal and STZ-induced diabetic rats.

Material and methods

The CS sample (degree of *N*-acetylation (DA): 11 %; M_W: 122100 Da) was previously prepared and purified from a batch of squid pen chitin (from Mahtani, India). The DA and MW were characterized by ^{1}H NMR spectroscopy and a SEC-MALLS-DRI system, respectively, as described elsewhere [12]. Streptozotocin (STZ) and Chitosanase (CSase) from Streptomyces griseus lyophilized powder (>50U/mg protein) were supplied by Sigma-Aldrich Chemie (Steinheim, Germany).

1.-Preparation of COS

To obtain COS, CS was hydrolyzed using CSase. CS (56.14mg) was dissolved in acetate buffer pH 4.5. It was incubated with 10.8U of CSase dissolved in acetate buffer in pH 5, during 4 h at 37ºC. To separate the enzyme, the solution was centrifuged with Amicon filters (3kDa) during 20min at 3200rpm and 15ºC; the COS filtered solution was collected for further studies.

2. - Characterization of COS

2.1. – Size exclusion chromatography

CS and COS samples were analyzed by size exclusion chromatography (SEC) using a column Waters Ultrahydrogel (7.8x300mm) and a guard column Waters Ultrahydrogel (6x400mm). The SEC system was composed of an isocratic pump SpectraSeries P100 (ThermoQuest, San Jose, CA) and a light scattering detector Waters 2424 ELS. The mobile phase was composed of a 100 mM sodium acetate buffer (pH 4.0) at a flow rate of 1.0 mL/min.

2.2. - MALDI-TOF Mass Spectrometry

The mass spectrum was acquired with a Voyager-DE STR (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser emitting at 337 nm with a 3 ns pulse. The instrument was operated in the reflector mode. Ions were accelerated to a final potential of 20 kV. The positive ions were detected in all cases. Spectrum was the sum of 200 shots and an external mass calibration of mass analyzer was used (mixture of peptides from Sequazyme standards kit, Applied Biosystems, Framingham, MA). A strong cation-exchange resin (DOWEX 50W-X8, Supelco, Bellefonte, PA) was added to the sample solution to decrease the salt quantity. Matrix and COS solutions were mixed at a volume ratio of 1:1.The matrix used for all experiments was 2,5-dihydroxybenzoic acid (DHB) purchased from Sigma-Aldrich (St Louis, MO) and used directly

without further purification. The solid matrix and the COS sample were dissolved at 10 g/L and 3 g/L in water, respectively. 20 μL of the matrix solution were then mixed with 20 μL of the COS solution. An aliquot of 0.5 μL of the resulting solution was spotted onto the MALDI sample plate and air-dried at room temperature.

3. - In vivo studies

3.1. – Animals

Healthy adult male Sprague–Dawley rats were housed in cages (five animals each) for at least 3 days before the experiments at 21±1°C in a 12:12 h light:dark cycle (all experiments were performed in the light cycle) and were fed and watered *ad libitum* during the whole experiment.

3.2. -STZ-induced diabetic rats

To induce diabetes mellitus in rats, STZ (70mg/kg) in 0.1M citrate buffer (pH 5) was administered at once by i.p. The control group received only saline. Diabetes was confirmed after 24 h by detecting glucose in urine. The diabetic rats were maintained with an insulin dose of 1-1.25IU/12 hours during one week before the experiment, while normal rats were treated with saline. To monitor the progression of diabetes, glucose in urine and the body weight were quantified daily.

3.3. –Blood samples

To assess plasmatic glycaemia, blood samples were collected 30 min before administering the treatments to establish basal glucose levels and at different times after dosing. The blood was collected from the tail vein in Eppendorf tubes with 10µL EDTA and the plasma was separated by centrifugation at ~1000g for 7 min at 4ºC. The samples were preserved at -50ºC in a freezer. Glucose in plasma was determined by the glucose–oxidase method (Glucose RTU™, BioMerieux® SA, France).

3.4. - Experimental procedure

The animals were divided into the following groups: 1) Streptozotocine (STZ)-induced diabetic rats treated with COS (35mg/kg) (n=5) ; 2) Normal (healthy, non-diabetic) rats treated with COS (35mg/kg) (n=6); 3) as control, Streptozotocine (STZ)-induced diabetic rats treated with saline (n=6). One dose was administered by i.p route.

Results and disccusion

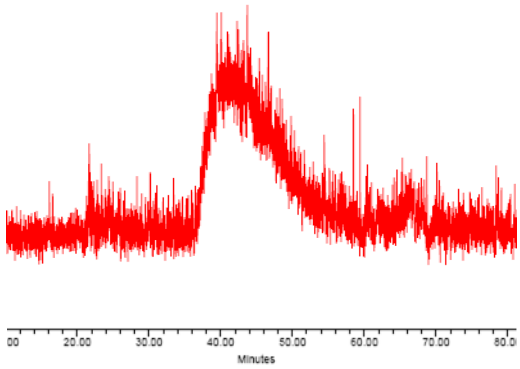
In numerous studies, CS (20KDa) and COS (1.2 kDa D.A. 10%) [4]have been found to exert long-term activity on glucose levels, insulin secretion and pancreatic cells health. Consequently, CS and COS have been proposed for the treatment of diabetes mellitus [6, 7]. However, the major concern regarding with the use of CS as a main component of antidiabetic drug delivery systems. For that reason, it was to confirm its

effect after intestinal absorption on the short-time bioactivity of CS on glucose regulation. The absorption mechanism of CS in the small intestine is not fully understood. It is generally believed that CS is absorbed after degradation into COS and D-glucosamine or *N*-acetyl D-glucosamine monosaccharides in the intestinal fluid. Unlike HMWCS, COS have been suggested to be easily absorbed through the intestine due to their high solubility in aqueous solution and, hence, to pass rapidly to the blood stream thus effectively producing systemic biological effects [3, 4, 6, 7]. We hypothesized that the hydrolysis of CS in the gastrointestinal track could occur by the actions of different enzymes, namely pepsin, protease, amylase and lipase A, producing different percentages of COS or LMWCS [13-15] which could be absorbed in the small intestine as oligosaccharides ingested in diet. In addition, it is known that exogenous D-glucosamine interferes with the signalling for a down regulation of the cellular glucose uptake, leading to hyperglycemia and insulin resistance [16]. Hence, we know that D-glucosamine is not produced by enzymatic hydrolysis of CS in the intestine due to in the studies in which CS was added in water/food, the results have shown a decrease in glucose levels [5-8]. Moreover, although it is not known the oral bioavailability of COS or CS, it is established that STZ causes morphological, functional, and metabolic alterations in the small intestine, increasing the activity of intestinal disaccharidases, which accelerates the absorption of glucose in the intestine [17]. Hence, in order to avoid the disadvantages associated with oral pathway, we preferred to administer COS by i.p instead of CS by oral route.

In vitro, COS are produced either by chemical or enzymatic methods. The enzymatic processes offer many advantages over chemical methods that involve long harsh treatments. According to their mode of enzymatic hydrolysis, chitosanase (CSase) enzymes can be divided into two types, exo- and endo-type. In this work, we selected an endo-CSase enzyme that releases a mixture composed predominantly of dimer, trimer, and oligomers from CS and maximizes the yield of COS. By contrast, exo-CSase liberates single D-glucosamine residues from the non-reducing terminal of CS and chitooligosaccharides [1].

In the present study, COS samples obtained from CS of DA 11% has been utilized to evaluate the *in vivo* short-term response on glucose metabolism. In Figure 1, it is represented the SEC chromatograms for CS and the corresponding COS obtained after incubation with CSase. It can be appreciated that after treatment with CSase, the native CS sample, characterized by a single elution peak (Fig 1A) transforms into COS as evidenced by the occurrence of several peaks eluting at longer (retention time of CS : 40-50 min *versus* COS : 60-70 min) retention times (Fig. 1B). In addition, COS structures were confirmed by MALDI-TOF analysis (Fig. 2, see also the assignments of peaks in supporting information), showing the distribution of oligomers between DP 2 and DP 10.

A)



.

B)

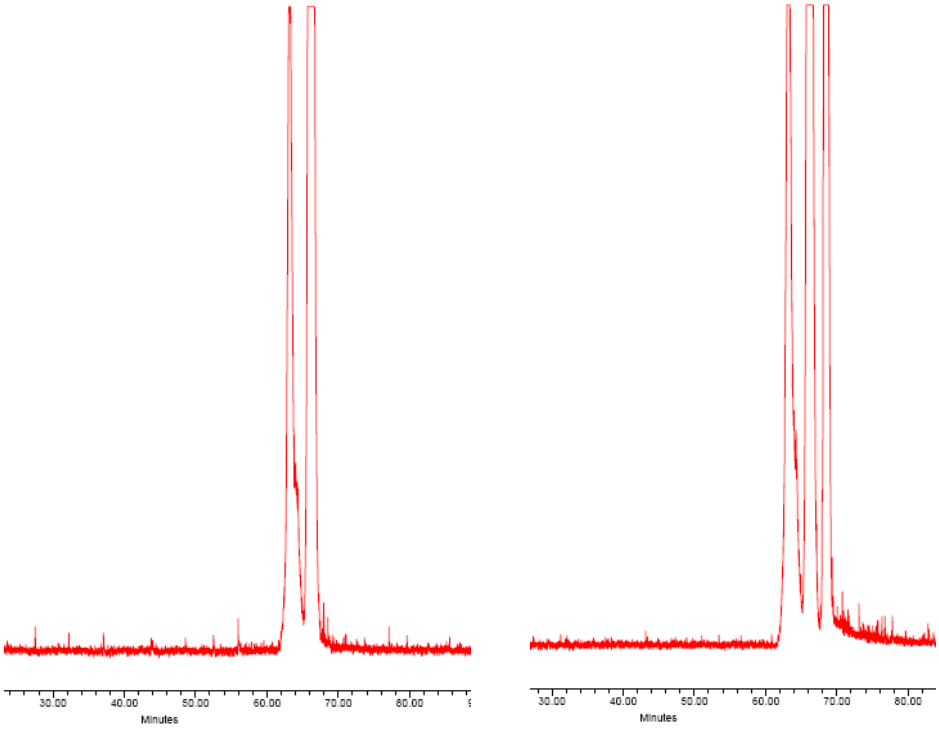


Figure 1: SEC Chromatograms. A) Chitosan DA 11 %; B) chitooligosaccharides from chitosan with DA 11 %, n=2.

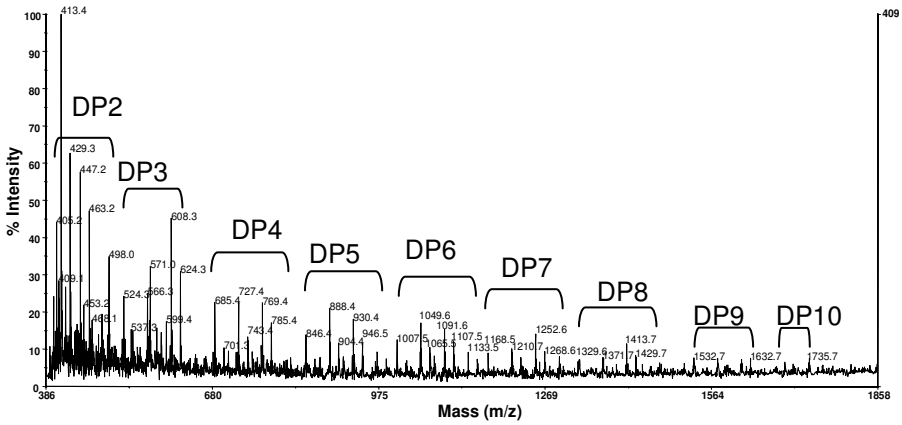


Figure 2: Positive ion MALDI-TOF mass spectrum of the COS sample in reflector mode (with DHB matrix). An assignment of peaks is given in supporting information

In Figure 3, it shows the time evolution on plasmatic glucose levels following i.p administering of COS (35mg/kg) in normal and STZ-induced diabetic rats. Inspection of the curves reveals that the glycaemia time profile for normal rats treated with COS was not modified during the experimental time. Our result is in accordance with the studies of long-term response (4 weeks) in normal rats [8] and neonatal normal rats [3]. In turn, the glucose reduction in STZ-induced diabetic rats treated with the same COS did not show appreciable differences from the corresponding negative control treatment (saline). The percentages respect to basal values showed the same profile for both groups of diabetic rats (see also figure 4). This result contradicts previous studies that evaluated the long-term glycaemic response to diabetic rats after administering 0.3% (w/w COS/water) COS of drinking water [3]. A possible interpretation to the discrepancies obtained between our results and those of other studies in similar animal models was understood

through the duration of the treatment. The increase of the insulin sensibility produced due to low triglycerides plasma levels was achieved after 4 weeks [3, 8]. Moreover, the enhance of the insulin secretion by the improvement of the viability and the growing of the -cells was showed after 9 days [4]. This indicates that the effect of COS on the glucose metabolism is a secondary effect as consequence over the changes in the action and production of insulin. Our study based in the short-term response of COS could not modified lipids plasma levels either proliferation and health of pancreatic cells with one dose of COS only.

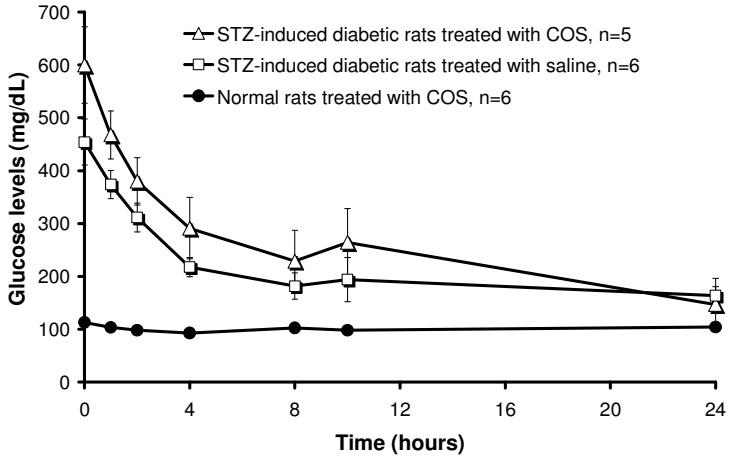


Figure 3: Glycemic levels (mg/dL) after i.p bolus administering. (empty triangles) STZ-induced diabetic rats treated with COS, n=5; (filled circles) Normal rats treated with COS, n=6, as control group; (empty square)STZ-induced diabetic rats treated with saline n=6. (Mean ± S.E.M).

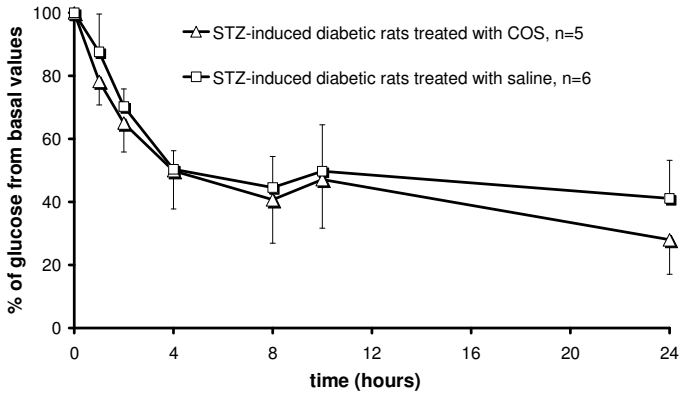


Figure 4: Percentage of glucose from basal values from STZ-induced diabetic rats (triangles) treated with COS, n=5; (squares) treated with saline, n=6.

In conclusion, the result of this study indicates that a single dose (short term) of COS DP 2-10 (35mg/kg) administered by i.p to normal and diabetic rats does not modify the short-term (24 h) glycaemic response. To corroborate the long term glycaemic response of this type of COS are needed further studies.

References

1. Jeon, Y.J., F. Shahidi, and S.K. Kim, *Preparation of chitin and chitosan oligomers and their applications in physiological functional foods.* Food Reviews International, 2000. 16(2): p. 159-176.
2. Kim, S.K. and N. Rajapakse, *Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review.* Carbohydrate Polymers, 2005. 62(4): p. 357-368.
3. Lee, H.W., et al., *Antidiabetic effects of chitosan oligosaccharides in neonatal streptozotocin-induced noninsulin-dependent diabetes mellitus in rats.* Biological and Pharmaceutical Bulletin, 2003. 26(8): p. 1100-1103.
4. Liu, B., et al., *Antidiabetic effects of chitooligosaccharides on pancreatic islet cells in streptozotocin-induced diabetic rats.* World Journal of Gastroenterology, 2007. 13(5): p. 725-731.
5. Miura, T., et al., *Hypoglycemic and hypolipidemic effect of chitosan in normal and neonatal streptozotocin-induced diabetic mice.* Biological and Pharmaceutical Bulletin, 1995. 18(11): p. 1623-1625.
6. Hayashi, K. and M. Ito, *Antidiabetic action of low molecular weight chitosan in genetically obese diabetic KK-Ay mice.* Biological and Pharmaceutical Bulletin, 2002. 25(2): p. 188-192.
7. Kondo, Y., et al., *Low molecular weight chitosan prevents the progression of low dose streptozotocin-induced slowly progressive diabetes mellitus in mice.* Biological and Pharmaceutical Bulletin, 2000. 23(12): p. 1458-1464.
8. Yao, H.T., S.Y. Huang, and M.T. Chiang, *A comparative study on hypoglycemic and hypocholesterolemic effects of high and low molecular weight chitosan in streptozotocin-induced diabetic rats.* Food and Chemical Toxicology, 2008. 46(5): p. 1525-1534.
9. Fernandez-Urrusuno, R., et al., *Chitosan nanoparticles: A new vehicle for increasing the nasal absorption of peptides.* Proceedings of the Controlled Release Society, 1999(26): p. 353-354.
10. Grenha, A., B. Seijo, and C. RemuÃ±Ã¡n-LÃ³pez, *Microencapsulated chitosan nanoparticles for lung protein delivery.* European Journal of Pharmaceutical Sciences, 2005. 25(4-5): p. 427-437

11. Goycoolea, F.M., et al., *Chitosan-alginate blended nanoparticles as carriers for the transmucosal delivery of macromolecules.* Biomacromolecules, 2009. 10(7): p. 1736-1743.
12. Goycoolea Francisco M., S.R., Menchicchi B., Valle-Gallego A., Santander-Ortega M.J., Peula-García José M. , Remuñán-López Carmen, Alonso María J. *Chitosan–Based Nanocapsules: Characterization of physical properties and application in drug delivery.* in *Proceedings of the 9th International Conference of the European Chitin Society*. 2009. San Servolo island, Venice, Italy.
13. Zhang, H., et al., *Preparation of chitooligosaccharides from chitosan by a complex enzyme.* Carbohydrate Research, 1999. 320(3-4): p. 257-260.
14. Roncal, T., et al., *High yield production of monomer-free chitosan oligosaccharides by pepsin catalyzed hydrolysis of a high deacetylation degree chitosan.* Carbohydrate Research, 2007. 342(18): p. 2750-2756.
15. Kumar, A.B.V. and R.N. Tharanathan, *A comparative study on depolymerization of chitosan by proteolytic enzymes.* Carbohydrate Polymers, 2004. 58(3): p. 275-283.
16. Stumpf, J.L. and S.W. Lin, *Effect of glucosamine on glucose control.* Annals of Pharmacotherapy, 2006. 40(4): p. 694-698.
17. Adachi, T., et al., *Morphological changes and increased sucrase and isomaltase activity in small intestines of insulin-deficient and type 2 diabetic rats.* Endocrine Journal, 2003. 50(3): p. 271-279.