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Doctoral thesis

Rational design of nanocarriers for oral peptide administration (Reduced Version)

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Report:

That the experimental dissertation entitled: "**Rational design of nanocarriers for oral peptide administration**" presented by **Zhigao Niu** was conducted under their supervision at the Department of Pharmaceutical Technology at the University of Santiago de Compostela. Being completed, they authorize its presentation and evaluation by the assigned jury members.

And for the record, they issue and sign the present certificate in Santiago de Compostela, March 10th, 2016.

Prof. María José Alonso Fernández Dr. Manuel Jesús Santander Ortega

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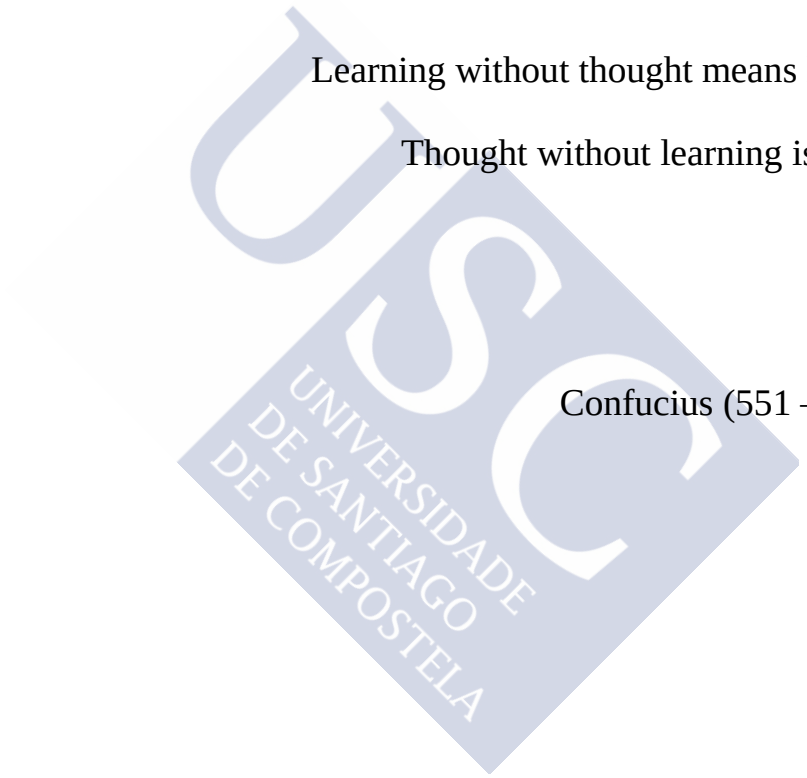
Learning without thought means labor lost.

Thought without learning is perilous.

Confucius (551 – 479 BC)



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Indice

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Resumen – Abstract

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Resumen

A pesar de su gran potencial, el uso terapéutico de los fármacos peptídicos está limitado por el hecho de presentarse como formas inyectables. La posibilidad de administrar estas moléculas complejas por vía oral podría incrementar enormemente su valor terapéutico. No obstante, el tracto gastrointestinal presenta importantes barreras biológicas que hacen de la administración oral de péptidos un gran reto. El objetivo de esta tesis ha sido el diseño y desarrollo de nanotransportadores poliméricos de péptidos capaces de subsanar los problemas de baja estabilidad y pobre absorción intestinal asociados a estas frágiles moléculas. Más allá de este ambicioso objetivo, se espera que el conocimiento generado en esta tesis doctoral contribuya de manera significativa a la comprensión de los procesos involucrados en la interacción de estas nanoestructuras con las barreras del tracto gastrointestinal.

En un primer abordaje, se diseñaron, en base a criterios racionales, nanocapsulas compuestas por un núcleo oleoso rodeado por una capa de poliarginina (PARG NCs), y explorado su potencial para la administración de péptidos por vía oral. La elección de la PARG vino dada por su conocida capacidad para abrir las uniones intimas intercelulares. Por otro lado, como componentes del núcleo se eligieron el ácido oleico y el desoxicolato de sodio, con el objeto de potenciar la capacidad de la PARG como promotor de la absorción celular. Las NCs resultantes presentaron un tamaño de partícula de 180 nm, una baja polidisdersidad (PDI < 0,2), una alta eficiencia de asociación de la insulina (> 80%), y una buena estabilidad coloidal tanto en fluidos intestinales simulados, así como, durante el almacenamiento a largo plazo. Además, en fluido intestinal simulado (FaSSIF-V2), estas NCs mostraron una capacidad para controlar la liberación de insulina. Estudios *in vitro* llevados a cabo en modelos celulares de Caco-2 mostraron la capacidad de estas nanocápsulas para aumentar la permeabilidad epitelial (mediante una reducción temporal de la TEER), y facilitar así el transporte de la insulina a través del epitelio (3.54%). Finalmente, los estudios in vivo en ratones mostraron la permanencia de la formulación asociada a la mucosa intestinal hasta al menos 24h después de su administración.

En un segundo abordaje, se diseñaron nanocomplejos del polimero A/polímero B con insulina. Estos nanocomplejos presentaron un tamaño de unos 200 nm, una estrecha distribución de tamaños (PDI 0.1) y una excelente eficiencia de asociación de la insulina (100%). El sistema presentó una buena estabilidad coloidal en fluidos intestinales simulados, protegiendo a la insulina de la degradación enzimática, así como en condiciones de almacenamiento a largo plazo. Estudios in vitro con células Caco-2 mostraron la capacidad de la formulación de los nanocomplejospara incrementar de manera remarcable el transporte de la insulina (47.59% internalización celular y 2.11% transporte).

En resumen, los resultados de esta tesis poner de relieve la posibilidad de diseñar nanotransportadores capaces de superar las multiples barreras biológicas asociadas a la administración oral de péptidos.

Abstract

Despite the increasing therapeutic potential of peptide drugs, their exploitation has been greatly hampered due to their necessity of being injected. The possibility to deliver these complex molecules by the oral route would highly increase their therapeutic value. However, the gastrointestinal tract (GIT) has several biological barriers that make the administration of peptides a very important challenge. The objective of this thesis has been to design and develop polymer-based peptide nanocarriers with the potential to overcome the low stability and poor intestinal permeability of these fragile macromolecules. Beyond this ambitious objective, this thesis is expected to significantly contribute to the understanding of the processes involved in the interaction of the nanostructures with the GIT barriers.

In a first approach, we rationally designed nanocapsules composed of an oily core surrounded by a shell made of polyarginine (PARG NCs) and explored their potential for oral insulin delivery. PARG was selected because of its reported capacity to open the intercellular tight junctions. The oily core consisted of oleic acid, which was intended to contribute to the penetration enhancing capacity of the surrounding PARG shell. The NCs had an average size of 180 nm, a narrow size distribution (PDI<0.2), a high insulin association efficiency (over 80%), and a good colloidal stability upon exposure to stimulated intestinal fluids, as well as, over long-term storage. In addition, these NCs showed a capacity to control insulin release upon incubation in fasted-state simulated intestinal fluid (FaSSIF-V2). *In vitro* studies using the Caco-2 cell model, showed that PARG NCs enhanced the epithelial permeability (transient TEER reduction), thereby facilitating the transport of insulin (3.54%). Finally, in vivo studies in mice showed that the formulation was retained along the intestinal mucosa up to 24h after administration.

In a different approach, we engineered a nanocomplexes of polymer A/polymer B and insulin. These nanocomplexes exhibited an average particle size of 200 nm, a narrow size distribution (PDI 0.1), and a very high insulin association efficiency (100%). The system displayed good colloidal stability in simulated intestinal fluids and also under long-term storage, protecting insulin from enzymatic degradation. *In vitro* Caco-2 cell

studies showed the capacity of the nanocomplexes to remarkably enhance insulin transport (47.59% cell uptake and 2.11% transport).

Overall, the results of these thesis underline the possibility to engineer nanocarriers that are able to overcome the multiple biological barriers associated to the oral peptide administration.

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Introducción

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Introducción

El reto asociado a la administración oral de péptidos

En las últimas décadas se ha visto claramente cómo, en general, la terapia basada en el uso de péptidos y proteínas podría verse claramente beneficiada mediante el diseño de plataformas que posibiliten su administración por vía oral. Sin embargo, las barreras biológicas que los péptidos han de superar para lograr su absorción sistémica tras su administración por vía oral suponen un gran reto tecnológico. Entre estas moleculas se encuentra la insulina, probablemente uno de los fármacos más complejos en cuanto a su formulación en formas de administración oral (BCS III). Descubierta en el año 1921 (Banting y Best), el uso terapéutico de la insulina se vio particularmente incrementado al lograr suproducción masiva mediante técnicas de ADN recombinante (1).

La posibilidad de administrar insulina por vía oral es particularmente atractiva, ya que además de una mayor aceptación por parte del paciente, la absorción de insulina a través del intestino podría imitar la circulación enterohepática de insulina endógena, haciendo posible un efecto sostenido del fármaco. En conjunto, estas ventajas representarían un gran beneficio para el tratamiento de una enfermedad crónica como la diabetes. No obstante, esta posibilidad está aún lejos de ser alcanzada debido a las diferentes barreras biológicas que presenta el tracto gastrointestinal (GIT). Estas barreras incluyen los fluidos intestinales ricos en enzimas, que pueden degradar al péptido, la capa de moco que protege el epitelio intestinal y el propio epitelio, que no permite el paso de macromoléculas hidrófilas (2-4). Mientras que el problema de la estabilidad podría abordarse mediante el uso de recubrimientos entéricos, la baja permeabilidad de la mucosa intestinal sigue siendo la limitación de las formulaciones desarrolladas hasta el momento para su paso a clínica (5). Las diferentes estrategias utilizadas para resolver estos problemas incluyen la modificación química del péptido, la co-administración de promotores de la absorción e inhibidores de enzimas,

encapsulación del péptido en sistemas de liberación adecuados (3). Desafortunadamente, se ha visto que hasta ahora estas estrategias poseen ciertas limitaciones que podrían justificar su limitado desarrollo clínico. Por ejemplo, la modificación química de péptidos puede inducir cambios en el perfil de actividad/toxicidad de la molécula original (6). La co-administración de insulina con inhibidores enzimáticos y promotores de la absorción puede producir efectos secundarios no deseados tales como una digestión lenta, o la absorción intestinal de compuestos no deseados. El desarrollo de nuevos nanotransportadores capaces de ayudar a los péptidos a superar las barreras indicadas supone un objetivo atractivo, no carente de desafíos técnicos significativos.

Nanovehículos y sistemas lipídicos para administración oral de fármacos

A día de hoy la nanotecnología ha permitido la administración oral de fármacos tanto solubles como insolubles, así como la administración dirigida a células intestinales específicas y una mejor absorción intestinal del fármaco a través de las vías para- y transcelular (7). Diferentes nanotransportadores, entre los que se incluyen nanopartículas poliméricas y lipídicas, liposomas, micro / nanoemulsiones, sistemas auto-emulsionables micro / y nanocápsulas han sido diseñados para la administración oral de insulina. La mayoría de los cuales se han tratado en el Capítulo 1 de esta tesis, titulado "Lipid-based nanocarriers for oral peptide delivery". Haciendo uso de las propiedades específicas de los polímeros, lípidos y otros excipientes auxiliares, ha sido posible diseñar nanovehículos que exhiben una buena estabilidad coloidal en el entorno GIT, además de poseer la capacidad de proteger a la insulina frente al ataque enzimático, así como de controlar su liberación y favorecer suabsorción a través del epitelio intestinal. Sin embargo, hay muy pocos estudios centrados en el estudio de la interacción de estos sistemas con las barreras biológicas asociadas a la vía oral de una manera sistémica. Es por esto, quizás, que existe una variabilidad significativa y una reproducibilidad limitada en los resultados *in vivo* publicados hasta el momento. En general, este escenario hace evidente la necesidad de llevar a cabo estudios

mecanísticos que nos ayuden a entender el mecanismo de interacción entre los nanotransportadores de fármacos y las barreras biológicas asociadas a la vía oral.

Entre los enfoques tecnológicos explorados para lograr la administración de insulina por vía oral, los que emplean excipientes lipídicos son particularmente prometedores debido a su gran diversidad, biocompatibilidad y una funcionalidad específica. La mayoría de los excipientes lipídicos son derivados de aceites o grasas consumidos normalmente en la dieta, que confieren, además de la biodegradabilidad y por tanto la baja toxicidad, la capacidad de (i) aumentar la permeabilidad de la membrana intestinal, (ii) reducir la degradación proteolítica y (iii) aumentar transporte linfático intestinal. En este campo, diversos nanotransportadores lipídicos han sido estudiados para la administración oral de péptidos, incluyendo nanopartículas lipídicas sólidas, liposomas, microemulsiones, nanoemulsiones, sistemas de administración de fármacos autoemulsionantes y las micro / nanoemulsiones recubiertas por un polímero, es decir, las nanocápsulas. Debido a su naturaleza hidrofóbica un reto a superar ha sido la baja eficiencia de encapsulación de la insulina en estos nanosistemas. Varias estrategias se han explorado para solucionar esta limitación. Una opción muy común ha sido el uso de ácidos grasos de cadena media y sus glicéridos, que se dispersan fácil en medios acuosos y son capaces de solubilizar péptidos (8, 9). Otras estrategias de formulación incluyen (i) la formación de emulsiones W/O/W (10, 11), (ii) la hidrofobización de los péptidos para promover la formación de micelas inversas (12, 13) o la complejación / conjugación con restos lipófilos (14) y (iii) la interacción electrostática con los lípidos o agentes tensioactivos (10). Todas estas alternativas han llevado a una mejora significativa de la capacidad de encapsulación de los sistemas lipídicos. Los mecanismos involucrados en la liberación del péptido desde la matriz lipídica incluyen (i) la difusión simple a través de los canales que contiene la matriz de lípidos (15), (ii) la disociación iónica seguida por una difusión a través de los canales (16, 17), (iii) la degradación de la matriz lipídica mediada por lipasas (15), y (iv) Hinchado-rotura de los nanosistemas por fuerzas osmoticas (18). A parte de la

liberación controlada de fármacos, los lípidos generalmente tienen una alta afinidad por la membrana celular, y muchos de los ingredientes lipídicos de estos nanosistemas han mostrado propiedades como promotores de la absorción, i.e. los triglicéridos de C10 y C8 (19, 20). Finalmente, estos sistemas podrán mejorar su comportamiento *in vivo* mediante el uso de una cubierta polimérica (dando lugar a la formación de nanocápsulas) que mejorase la estabilidad de los nanotransportadores en condiciones fisiológicos.

Poliarginina

Los polipéptidos y poliaminoacidos ricos en arginina, tales la poliarginina, han sido ampliamente estudiados en el desarrollo de nanovehículos para la administración oral de insulina debido a sus excelentes propiedades como promotores de la absorción celular. Estudios mecanísticos en los que se compararon moléculas ricas en arginina con moléculas ricas en lisina, mostraron que las primeras lograban una mayor eficiencia de internalización, lo cual indica claramente la importancia de los grupos guanidinio de la cadenas laterales de poli/oligoarginina (21). Además de la alta ionización de la cadena lateral de guanidina (arginina pKa ~ 12.5) (22), la mayor eficiencia se atribuye al hecho de que los grupos ricos en guanidinio son capaces de formar puentes de hidrógeno en la superficie celular. La formación de estos enlaces convierte al péptido hidrófilo en un material más hidrófobo, reduciendo la energía necesaria para pasar a través de la bicapa lipídica de las células (23).

La poli-L-arginina (PARG), polipéptido que cuenta con un máximo de 554 residuos de arginina. El potencial de esta molécula para mejorar la absorción de fármacos hidrófilos se ha estudiado tanto a nivel de mucosas como a nivel intracelular (24-30). Estudios llevados a cabo en ratas han puesto de relación el efecto del peso molecular de la PARG en la absorción FD-4 (molécula modelo). En función de su capacidad para mejorar la absorción de FD-4 las diferentes PARG se ordenaron de la siguiente manera: PARG 92 kDa> PARG 45,5kDa> PARG 8,9 kDa (29). Interesante es también

el hecho de que todas las PARG estudiadas mostraron una mayor capacidad para promover la absorción que promotores clásicos como son el glicolato sódico o el taurocolato sódico (29). En esta línea, la PARG ha mostrado un gran potencial para mejorar la absorción de péptidos y proteínas (con un peso molecular igual o inferior a 20KDa) a través de mucosas (30).

Conclusiones and perspectivas

La PARG ha mostrado un gran potencial para mejorar el transporte de péptidos a través de las células epiteliales, ya sea a través de la vía endocítica o por una ruta paracelular. También se han realizado estudios centrados en el uso de nanotransportadores que contenían PARG para mejorar el transporte intracelular del fármaco encapsulado. No obstante, hay pocos estudios relacionados con su uso para la administración oral de péptidos.

En general, los estudios publicados en los últimos años señalan la posibilidad de combinar de forma sinérgica la nanotecnología con el uso de polímeros ricos en arginina, con el objeto de mejorar el transporte de macromoléculas a través de la barrera intestinal. Ésta ha sido la base principal del trabajo desarrollado en esta tesis. Esta idea se ha materializado mediante la combinación de macromoléculas ricas en arginina con otros promotores de la absorción (sales biliares, aceites), en forma de nanoestructuras capaces de encapsular insulina y mejorar su biodisponibilidad tras su administración oral.

Esta breve introducción se complementa con la revisión presentada en esta tesis (Capítulo 1) titulada "Lipid-based nanocarriers for oral peptide delivery", recientemente enviada para su publicación en la revista Advanced Drug Delivery Reviews.

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Introduction

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Introduction

The need of oral peptide delivery systems-the example of insulin

Over the last decades, it has been clearly recognized that, in general, the area of peptide/protein therapeutics would greatly benefit from the design of adequate oral delivery technologies. As a consequence, currently, one of the greatest challenges in the drug delivery field is to make feasible the oral administration of these complex molecules. Among these molecules, insulin represents the standard drug and, probably, one of the most challenging drugs with regard to its formulation in an oral delivery system. Insulin was discovered in 1921 (Banting and Best), and thanks to the DNA recombination techniques, this biomacromolecule and its analogs are widely used to treat diabetes (1).

Besides the oral route, other alternative routes of insulin administration have been drawing gradual attention in the past decades, including pulmonary, buccal, nasal and transdermal pathways (2-4). Among them, pulmonary insulin is the only one that has reached the market (5). However, the possibility to deliver insulin by the oral route is particularly attractive since, besides the high patient acceptance, the intestinal insulin absorption could mimic the enterohepatic circulation of endogenous insulin, and provide the possibility of sustained drug effect. All together, these advantages would be of great benefit for the treatment of a chronic disease like diabetes. Unfortunately, this possibility is still far from being reached due to the several biological barriers of the gastro-intestinal tract (GIT), which have been revealed so far to be extremely difficult to overcome for a drug such as insulin. These barriers include the enzyme-rich intestinal fluids, which may hamper the stability of this labile peptide, the mucus layer that protects the enterocytes and the enterocytes themselves, which do not allow the pass of hydrophilic macromolecules (6-8). While the stability issue could be addressed by the use of dosage forms with enteric coatings, the low

permeability of the intestinal mucosa, remains to be the main limitation for the clinical success of the formulations developed so far (9) Among the different strategies used to solve these issues, there is the one consisting on the chemical modification of the drug molecule, the one involving co-administration with permeation enhancers and enzyme inhibitors, and finally the entrapment of this peptide into adequate drug delivery systems (7). Unfortunately, all these strategies have been found to encounter significant hurdles in their clinical development process. For example, the drug chemical modification may impair changes in the activity/toxicity profile of the original molecule (10). The co-administration of insulin with enzyme inhibiters and penetration enhancers may also result in undesired side effects, such as a slowed digestion and the intestinal absorption of undesired compounds. The development of a drug carrier formulation that would help overcoming the indicated barriers is an attractive option, although not absent of significant technical challenges.

Impact of nanotechnology in the design of oral peptide delivery systems

As a booming technology in the last decades, nanomedicine have shed light on pharmaceutical sciences. In general, it allows the oral delivery of both aqueous-soluble and non-soluble drugs, as well as the targeted delivery to specific intestinal cells and an improved intestinal drug permeation via both, paracellular and transcellular pathways (11). Various nanocarriers have been engineered to load insulin for its oral administration, including polymeric nanoparticles, liposomes, solid lipid nanoparticles, micro/nanoemulsions, self-micro/nano-emulsifying drug delivery systems, and nanocapsules, most of which have been detailed in chapter 1 of this thesis entitled “Lipid-based nanocarriers for oral peptide delivery”. By combining the properties of polymers, lipids and other auxiliary excipients, it has been possible to design nanocarriers which exhibit a good colloidal stability in simulated GIT environment, the capacity to protect insulin against enzymatic attack and control its release, an adequate muco-diffusion profile and an enhanced permeation across the

intestinal epithelium. However, there are only very few studies addressing the performance of nanocarriers, in terms of dealing with the above-mentioned barriers in a systematic way. This maybe the reason why there is a significant variability and a limited reproducibility on the *in vivo* efficacy data reported so far. Overall, this scenario makes evident the necessity to develop comprehensive studies on the mechanistic interaction of the drug nanocarriers with the biological barriers.

Polyarginine

A wide range of biomaterials of both, natural and synthetic origin, have been involved in the fabrication of nanocarriers for oral insulin delivery. Generally regarded as non-toxic, biodegradable and biocompatible, poly amino acids are gaining significant attention in this field. An attractive feature of these molecules relies on the fact that their side chains can be functionalized, providing them with a versatility that could be used for different therapeutic aims (12).

Polyarginines, have been extensively studied due to their excellent cell permeation properties. In mechanistic studies comparing arginine with lysine molecules, arginines showed higher internalization efficiency, indicating the importance of guanidinium groups on polyarginine side chains (13). Beyond the stronger ionic force associated with the highly cationized guanidinium side chain (arginine pKa~12.5) (14), the higher efficiency showed by this peptide is also attributed to the fact that the guanidinium-rich groups are capable to form bidentate ionic hydrogen bond with anionic phosphate, sulfate and carboxylate groups located on the cell surface. The formation of these ionic pairs convert the water soluble peptide to a lipid soluble material, which reduced the energy required to pass through the lipid bilayer of the cells (15).

Poly-L-arginine (PARG), has shown the capacity to enhance the penetration of drugs

across the cell membrane and even across mucosal tissues (16-20). A comparative study *in vivo* aimed at determining the importance of the molecular weight (Mw) of PARG on its permeation enhancer capacity following nasal delivery (FD-4 as model drug) resulted in the following permeability ranking: PARG 92 kDa > PARG 45.5 kDa > PARG 8.9 kDa (21). Moreover, these authors showed that the permeation enhancing capacity of PARG polymers, was higher than that of classical permeation enhancers such as sodium glycocholate or sodium taurocholate (21). This capacity is in agreement with the enhancement of the transport of various protein drugs across nasal membrane (22). Additionally, it has been reported that the PARG penetration enhancing effect is dose-dependent (23).

PARG has a good safety profile, without causing damage to rat erythrocyte and isolated rabbit nasal mucosa (24). However, opposite to the ability displayed by most CPP to enhance endocytosis, PARG polymers are rather known to induce the transient opening of cellular tight junctions, thereby favoring the paracellular drug absorption in a molecular weight dependent fashion (24). As later revealed by mechanistic studies, these polymers can lead to transient internalization of tight junction proteins between the epithelium cells via clathrin-mediated endocytosis, which leads to the increase of the permeability of hydrophilic drugs (25-27). Finally, the internalized tight junction proteins are then recycled through endosome pathway, re-forming the intercellular tight junction. These features are similar to the paracellular permeation enhancing properties displayed by chitosan, which has been widely employed for pharmaceutical applications including drug delivery systems and turned out to be a great success (28).

PARG has been compared with regard to their capacity to interact with the anionic phospholipids of liposomes. The results showed that as the molecular weight rises from 8 up to ~550 arginine residues, the interaction with the cell membrane also increases. Incubation of PARG polymers, with around 300 arginine residues or more,

with lipid membranes resulted in the lipid membrane perturbation and the subsequent translocation across the phospholipid bilayers (29). These results are, somehow, in contradiction with those claiming that the ideal number of arginine residues is in the range of 7-15. Therefore, overall, the limited amount of literature regarding the use of arginine-rich polymers/polymers in the design of antigen delivery carriers suggest the interest to further investigate the potential of these promising biomaterials.

Conclusions and perspectives

PARG has exhibited a capacity to transport peptides across epithelial cells either through an endocytic or paracellular routes. Moreover, there are a few articles disclosing preliminary data about the potential of PLGA and lipid nanoparticles involving arginine for enhancing insulin absorption (30, 31). On the other hand, PARG-based nanocarriers have also been designed for intracellular drug delivery and few attempts have been directed towards their use for oral peptide delivery.

Overall, the background literature suggest that there are possibilities to synergically combine nanotechnology with the use of arginine-rich polymers with the final goal of improving the transport of macromolecules across the intestinal barrier (32), and this has been the main basis for the idea behind this thesis work. This idea was attempted to be materialized by combining polyarginine with other penetration enhancers (bile salts, oils) as well as protective PEGylated polymers.

This brief introduction is complementary to the review presented in thesis chapter 1 entitle “Lipid-based nanocarriers for oral peptide delivery”, recently submitted for publication in the Advanced Drug Delivery Reviews.

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Chapter 1

Lipid-Based Nanocarriers for Oral Peptide Delivery

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Abstract

This chapter is aimed to overview the lipid-based nanostructures designed so far for the oral administration of peptides and proteins, and to analyse the influence of their composition and physicochemical (particle size, zeta potential) and pharmaceutical (drug loading and release) properties, on their interaction with the gastro-intestinal environment, and the subsequent PK/PD profile of the associated drugs. The ultimate goal is to highlight and comparatively analyse the key factors that may be determinant of the success of these nanocarriers for oral peptide delivery. The article ends with some prospects on the challenges to be addressed for the intended commercial success of these delivery vehicles.

Abbreviations:

BA: bioavailability, BSA: bovine serum albumin, FDA: Food and Drug Administration, GMO: glyceryl monostearate, GRAS: Generally recognized as safe, HLB: hydrophilic-lipophilic balance, LBDDS: lipid-based drug delivery system(s), LC: long chain, LCT: long chain triglycerides, LFCS: lipid formulation classification system, MC: medium chain, MCT: medium chain triglycerides, MW: molecular weight, NC: nanocapsules, OVA: ovalbumin, PACA: poly(alkylcyanoacrylate), p/p: peptides and proteins, PA: pharmacological bioavailability; PD: pharmacodynamics, PEG: poly(ethylene glycol), PK: pharmacokinetics, SA: stearic acid, SC: subcutaneously, sCT: salmon calcitonin, SDC: sodium deoxycholate, SGC: sodium glycocholate, SGF: simulated gastric fluid, STC: sodium taurocholate, SLN: solid lipid nanoparticles, SEDDS: self-emulsifying drug delivery systems, SMEDDS: self-microemulsifying drug delivery systems, SNEDDS: self-nanoemulsifying drug delivery systems, TG: triglycerides, TP: tripalmitin.

1. Introduction

Since the launch of recombinant human insulin, protein/peptide therapeutics have gained increasing attention as an alternative to conventional small organic drug molecules. With hundreds of peptide/proteins molecules now commercialized, it is estimated that the market value of protein/peptide therapeutics will be €180 billion 2018 (1, 2). Despite this commercial success, it is widely acknowledged that the full clinical potential of these potent macromolecules has been greatly hampered by their necessity of parenteral administration. As a consequence, the search for new strategies, which may enable the oral delivery of peptide/proteins, is one of the main challenges in the drug delivery field.

Among the technological approaches explored to achieve efficient oral peptide delivery, those employing lipid excipients are particularly promising because of their wide diversity, favourable biocompatibility and specific functionality. In particular, the fact that the majority of lipids excipients are derived from dietary oils/fats, confers the advantages both in terms of biodegradability and the capacity to cross the intestinal barrier.

Starting with an overview of the physicochemical properties of the lipid materials available for drug delivery, this chapter will comparatively analyse the state-of-the-art of nanostructured lipid-based systems intended for oral peptide delivery. The focus will be in four distinct categories of nanocarriers: (i) solid lipid nanoparticles (SLN), (ii) micro and nanoemulsions, including self-emulsifying systems, (iii) liposomes and (iv) hybrid lipid-polymer systems, e.g. nanocapsules. These delivery carriers will be analysed with regard to their capacity to associate and control the release of peptides, and to overcome the multiple biological barriers associated with oral administration. These barriers include (i) the intestinal fluids, which may compromise the stability of the nanocarriers and also that of the associated peptides, (ii) the mucus layer, which may hinder the access of the nanocarriers to the absorbing epithelium and, finally, (iii)

the intestinal epithelium. At the end, an attempt will be made to establish a relationship between the nanocarrier biopharmaceutical properties and the effectiveness in terms of PK/PD of the associated peptide

2. Lipids used in the formulation of oral peptide delivery systems

Defined by their intrinsic natural origin, lipids constitute a family of molecules largely exploited in the pharmaceutical field due to their favourable physicochemical and biopharmaceutical properties, as described in the following sections.

2.1 Lipids selection

A fundamental understanding of the chemistry and physicochemical properties of the lipids is a prerequisite for successful formulation of peptides and proteins. Criteria for the selection of lipids include purity and chemical stability, solvent capacity, water miscibility, digestibility and fate of digested products, safety and regulatory profile (3). Lipids are often "used as received" and due to the diverse array of commercial lipid raw material suppliers, special attention must be given to the datasheet specifications as well as to the batch-to-batch reproducibility (4).

Figure 1 and table 1 display the chemical structure and characteristics of lipids commonly used in the formulation of lipid-based nanocarriers intended for oral peptide delivery (5, 6). These include fatty acids (FA), fatty alcohols, long chain (LC) and medium chain (MC) monoglycerides, diglycerides and triglycerides (TG) and phospholipids. Structurally, these amphiphillic molecules possess a hydrophobic region, which is composed of one, two or three hydrocarbon chain(s) of different lengths, and a differentiated polar head. The number and length of the hydrocarbon chain, as well as the degree of unsaturation (double bonds) determine the hydrophobicity of the molecule. The polar nature is associated to the carboxyl- or alcohol group in the case of fatty acids or fatty alcohols, respectively and to the sterification of the fatty acids with glycerol groups, in the case of the glycerides:

Finally, in the case of phospholipids, in addition to the glycerol, there are phosphate and the choline groups (Figure 1).

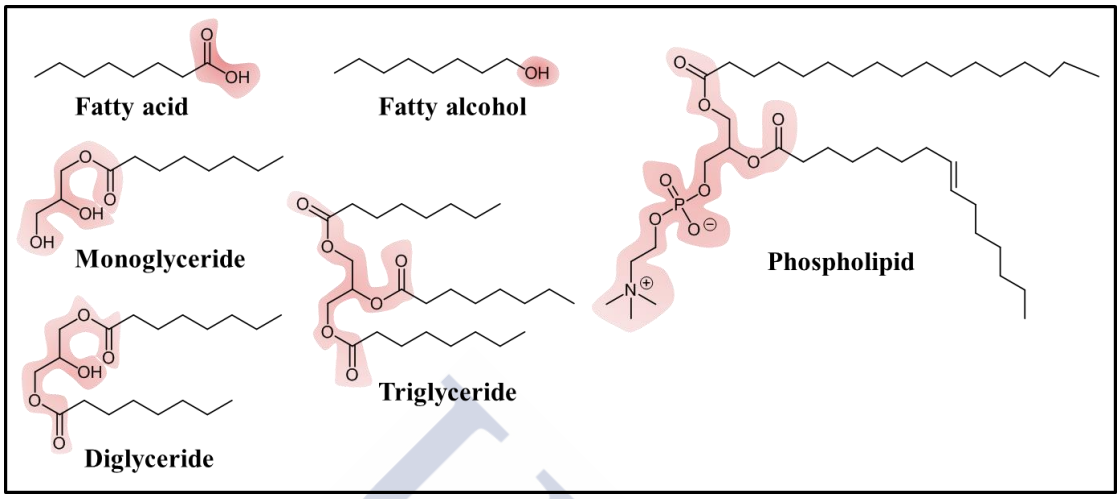


Figure 1. Chemical structure of some common lipids used in nanocarriers for the oral administration of peptides. The polar section in each structure is shadowed.

Given their amphiphillic properties, lipid excipients are commonly employed in the formation of emulsion-type of systems. For such systems, the use of phase diagrams has been a classical approach to identify suitable mixing ratios of different lipids, surfactants and drugs in order to achieve an optimal solubilisation capacity of hydrophobic drugs (7). This approach has also been adopted in the case of hydrophilic peptides and proteins for the formulation of water-in-oil-in water emulsions (8, 9). In these emulsion-type systems, the oil phase is usually composed of triglycerides or mixed glycerides (a mixture of mono-, di- and triglycerides) consisting of long-chain and/or medium-chain fatty acids. Triglycerides are of vegetable origin and can be presented as pure or complex mixtures of triglycerides, substituted with fatty acids of variable length chain and degree of unsaturation. The lower hydrophobicity of MCT as compared to the LCT could be expected to facilitate the interaction with hydrophilic peptides, as well as their dispersion and processing in in biological media (10). The capacity of triglycerides to entrap proteins could also be enhanced by increasing the polarity of the oil phase through the incorporation mono- and

diglycerides (11) and/or higher proportions of hydrophilic surfactants. Partial hydrolysis of triglycerides yields a wide range of mixed glyceride excipients, which contain variable proportions of monoglycerides, diglycerides and triglycerides. Although glycerides they are classified as neutral lipids in food science and lipid chemistry (10), their polarity increases as: triglycerides < diglycerides < monoglycerides.

In addition to glycerides, free fatty acids, fatty alcohols and phospholipids (12) are also frequently used in the design of lipid-based delivery carriers, because of their surface active and penetration enhancing properties, as well as their capacity to self-assemble.

Class		Examples	Characteristics
Triglycerides	**Long chain (LCT)**	Castor oil, soybean oil, tripalmitin, triolein	GRAS status, easily digested, and absorbed. Low self-dispersing capacity.
	Medium chain (MCT)	Triglycerides of caprylic/ capric acid, Miglyol® 812, Captex355	GRAS status. Higher chemical stability than LCT. Good self-dispersing ability and better solvent capacity for hydrophilic molecules than LCT.
Mono-, di-glycerides		Glycerol monostearate, glycerol monooleate, glyceryl palmitostearate, mono/di-glycerides of caprylic acid	GRAS status. Better self-dispersing ability than LCT and MCT due to their amphiphilic nature.
Fatty acids		Stearic acid, oleic acid, linoleic acid, palmitic acid (subproducts of TG degradation)	GRAS status. High water insolubility, surfactant properties and self-dispersing ability. Longer carbon chain hinders hydrophilic molecules encapsulation but enhance colloidal stability and protection against enzymatic attack.
Fatty alcohols		Stearyl alcohol	High water solubility, surfactant properties and self-dispersing ability. Used in mixtures with fatty acids to decrease lipids recrystallinisation.
Phospholipids		Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, dipalmitoyl-glycero-hosphocholine, dimyristoyl-phosphatidylcholine, distearoylphosphatidylethanolamine-PEG	Ionic amphiphiles, emulsifying and dispersion agents, self-assembling capacity into well-defined structures and bilayers.

Table 1. Examples of lipids used for oral p/p delivery systems and their relevant characteristics.

Finally, there are a range of other amphiphilic components used in the formulation of peptides and proteins, which includes a variety of synthetic surfactants, synthetic lipids (e.g. combining PEG and hydrolysed oils), and bile salts (13, 14), whose roles are not solely to facilitate oil dispersion and to improve the entrapment of peptides but also to fluidise biological membranes, thereby facilitating the penetration of the associated peptides.

The increasing awareness of the merits of lipid excipients in promoting intestinal absorption is reflected by their inclusion in several emerging peptide delivery technologies currently undergoing clinical trials. For example, the results from Phase III trials of Octreolin®, an enteric coated capsule technology containing Octreotide in an oily suspension consisting of C8 lipids (sodium caprylate), are particularly noteworthy (13, 14). Similarly, Merrion Pharma's Gastro-Intestinal Permeation Enhancement Technology (GIPET®), which employs C8 and C10 lipids as absorption enhancers, reported promising clinical studies as an oral peptide delivery technology (15). Novo Nordisk, who licensed the GIPET® technology, has successfully completed Phase I trials for a novel oral insulin (NN1956) and also for a proprietary GLP-1 analogue (NN9928) (16). Phase II trials comparing novel oral insulin to Insulin glargine subcutaneously (SC) are currently on-going (17). These emerging technologies underline the importance of lipid materials excipients combined with additional excipients, in order to overcome multiple barriers associated to the oral peptide delivery.

2.2. Functionality of lipid materials for oral peptide delivery

Lipid excipients have been shown to influence the intestinal absorption of drugs by a variety of mechanisms including reducing intestinally mediated proteolysis, increasing membrane permeability and promoting intestinally lymphatic uptake.

2.2.1 Increased intestinal membrane permeability

The inherent permeability enhancing abilities of various lipid excipients, including endogenous bile salts and phospholipids, are well known (18, 19). Lipids may enhance the permeability of the intestinal mucosa via a number of different mechanisms, including altered membrane fluidity, opening of tight junctions and other mechanisms, such as the inhibition of efflux mechanisms (20-23).

- **Fluidisation of intestinal epithelial membranes**

In general lipid-based amphiphilic molecules such as fatty acids, bile salts and surfactants are known to enhance transcellular transport by causing a transient disruption to the lipid bilayer (24-26). For example, bile salt and fatty acid mixed micelles have been shown to increase permeability of insulin in an *in situ* rat intestinal perfusion model (27). A number of oral peptide delivery technologies under clinical development are making use of this capacity of lipids, among them Peptelligence® (Enteris Biopharma) incorporating sodium taurodeoxycholate (28) and POD® (Oramed Pharmaceuticals) incorporating omega-3 fatty acids (29, 30).

- **Modulation of tight junctions**

A range of lipid digestion products, particularly medium chain fatty acids (C8-12), as well as various surfactants, either of natural, i.e. bile salts (21, 25) or synthetic origin, such as Labrasol® (31), have been shown to increase paracellular peptide drug transport, by altering the tight-junctions (21, 32). In the case of the fatty acids, such as C10 fatty acid sodium caprate, it has been indicated that the opening of tight junctions is associated with stimulation of calmodulin-dependent contraction of actin fibres (33). However, it has also been suggested that these fatty acids may additionally increase the transcellular flux of peptides (34). Again, there are a number of oral peptide delivery technologies that attempt to exploit C8-C10 fatty acid mediated permeation enhancement such as GIPET® (Merrion Pharma) (35) and Octreolin® (Chiasma Inc) (13).

- **Other mechanisms of enhanced transport**

Another postulated mechanism by which lipids act to increase transcellular absorption is by formation of a lipid-protein carrier complex (36, 37). Lipid 'carrier' molecules are diverse in structure and the mechanisms by which the protein-carrier complex enhances absorption are still unclear (36). For example, Eligen® (Emisphere) is an oral delivery technology based on the derivatives of the lipid carrier, sodium N-(8-(2-hydroxybenxyl)amino) caprylate (SNAC). SNAC has been shown to increase insulin permeability approximately 10 fold across Caco-2 cell monolayers (38). The increased insulin flux was mediated solely via the transcellular route, as tight junction integrity and paracellular flux was unaltered. In addition, a number of lipid excipients have shown inhibitory activities against the intestinal drug efflux transporters, thereby increasing drug transport across the enterocytes (39). For example sodium taurocholate, Cremophor® EL, polysorbate 80 and Solutol® have all been shown to inhibit P-gp mediated efflux in *in vitro* cell lines (40, 41).

2.2.2 Reduced proteolytic degradation

Bile salts, such as sodium glycocholate (SGC), have been shown to inhibit intestinal peptidase mediated protein degradation (27, 42). In an *in vivo* study comparing five protease inhibitors, Yamanoto *et al* demonstrated that 20mM SGC was comparable to camostat mesilate and bacitracin, both specific intestinal protease inhibitors, in terms of hypoglycemic effect following administration to the large intestine of rats (43).

2.2.3 Increased intestinal lymphatic transport

Lipids, in particular long chain fatty acid, have been frequently utilised to enhance the lymphatic absorption of lipophilic drugs (44). The majority of drugs that have been shown to undergo significant intestinal lymphatic transport are highly lipophilic, and this transport has been associated with chylomicrons synthesized within enterocytes. Because of this, it has been suggested that the lipidization of peptides, for example in the form of a fatty acid prodrug, could enhance the association with chylomicrons,

thereby facilitating the lymphatic transport of peptides such as calcitonin and insulin (45). Alternatively, large molecular weight proteins and peptide-carrier constructs that are absorbed intact across the intestine, may be selectively taken up via the lymphatics because their macromolecular size favours uptake via the leakier structure of the lymphatics vessels, as compared to blood capillaries (46). The intestinal lymphatics also play a key role in antigen sampling and presentation within the Gut-associated lymphoid tissue (GALT). In this sense, it has been widely reported that peptide loaded micro- and nanoparticles are similarly transported via this pathway, and while there is considerable debate regarding the extent of nanoparticles transported via this route, in some cases, substantial increases in oral bioavailability via this pathway have been reported (47-50).

3. Analysis of performance of lipid-based nanocarriers for oral peptide delivery

3.1. Peptide/protein loading and controlled release capacity of lipid-based nanocarriers

A variety of lipid-based systems have been explored with regard to their potential for enhancing oral peptide bioavailability. Interestingly, with the exception of the solid lipid nanoparticles (SLN), a common ingredient to most of them is the use of medium chain fatty acids and their glycerides. As indicated in Table 2, specific advantages of these lipids include their easy dispersibility in aqueous media and their capacity for peptide solubilization. However, this capacity is still limited and, hence, the use of specific formulation strategies, based on the formation of w/o/w emulsions or the hydrophobilization of the peptides by reverse-micellization or their complexation/conjugation with lipophilic ingredients, have been critical for the achievement of a significant peptide loading in lipid based systems (Figure 2). In addition, the formation of liquid-crystal phases (lamellar, hexagonal, cubic phases, etc.) is also proposed as a strategy to enhance peptide solubility in lipids (51, 52), with the added advantage of controlling the rate of release. In fact, there are a number of liquid-crystal gels that have shown their capacity to control the release of numerous

peptide drugs (53-56), however, the transformation of these gels into nanometric structures that could work as peptide carriers is still in a premature stage.

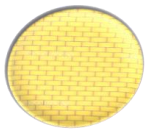
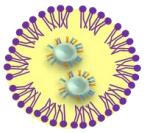
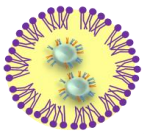
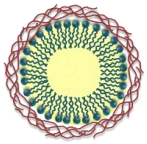
	TYPE OF LIPID INVOLVED	PROFITABLE LIPID PROPERTIES
SOLID LIPID NANOPARTICLES		
	Long chain triglycerides, fatty acids and phospholipids.	Triglycerides slower than other glycerides, decelerating peptide release. Structure-dependent lipolysis resistance promotes better stability. Tripalmitin owns high affinity for the cell membrane inducing endocytosis.
EMULSIONS, NANOEMULSION, MICROEMULSIONS		
	Long chain and medium chain glycerides and fatty acids	Long chain and medium chain fatty acids act as penetration enhancers. Unsaturated carbon chains have the ability to disrupt the cell membrane. Oleic acid increases mobility of phospholipids of the cell membrane.
SEDDS, SMEDDS, SNEDDS		
	Medium chain mono-, di- and triglycerides, long chain and medium fatty acids. High concentration of surfactants and optionally hydrophilic co-solvents are required in these systems.	Medium chain (eg. C8/C10) lipid derivates act as good penetration enhancers. Medium chain fatty acids help to peptide/protein solubilisation and favours emulsification.
NANOCAPSULES		
	Medium chain mono-, di- and triglycerides, long chain fatty acids.	
LIPOSOMES		
	Phospholipids, phosphatyl-glycerol derivates, saturated and unsaturated fatty acids.	Phospholipids form amphiphilic structures capable to self-assemble.

Table 2. Most common lipids used in the design of lipid-based nanocarriers for oral peptide administration.

In the following subsections, the specific details regarding the encapsulation and controlled release properties of lipid nanostructures will be presented.

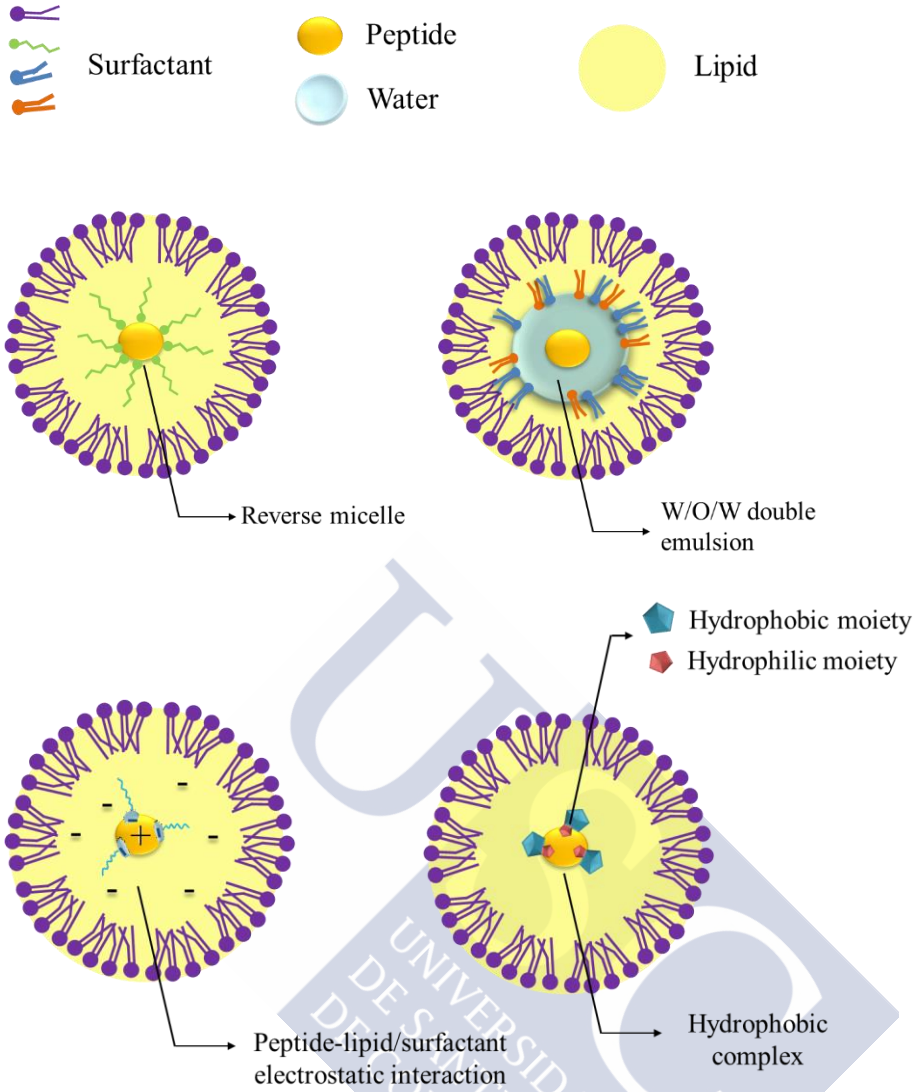


Figure 2. Illustration of some strategies developed to enhance drug loading capacity into lipid based nanocarriers.

3.1.1. *Solid Lipid Nanoparticles*

Solid lipid nanoparticles (SLN) are made of natural, semi-synthetic or synthetic lipids including triglycerides, partial glycerides, fatty acids, waxes, phospholipids and steroids. They additionally incorporate an emulsifier layer, which provides them with the adequate stability upon dispersion in water. SLN are characterized by the fact that they remain in the solid state at room and body temperatures (57). Introduced by Müller *et al*. and Gasco *et al*. over 25 years ago (58, 59), the intrinsic lipidic nature of SLN has conferred these systems with a number of interesting properties, namely, (i)

the capacity to protect drugs from degradation, owing to structure dependent lipolysis resistance (60, 61), (ii) the possibility to control drug release benefiting from the varied amount and type of lipid employed (61) (ii) a favourable biocompatibility (62, 63); and (v) ease of large scale production (64). As a consequence, a number of formulations, such as TrabiOral™, Rifamsolin™, Ocusolin™, Vansolin™, Zysolin™ are currently under early clinical development (65), however, the advances so far have been mainly focused on hydrophobic drugs, whereas the delivery of peptides and proteins is still at an early phase. Table 3 illustrates examples of SLNs intended for oral peptide delivery.

Among the techniques described for the production of SLN, only those that avoid the use of high temperature are, in principle, appropriate for protein/peptide encapsulation. These techniques involve the dissolution of the lipids in organic solvents followed by their diffusion (66-68) and evaporation (69). The formation of w/o/w double emulsions (70) have been the techniques most frequently reported for achieving significant loadings of peptides and proteins within SLN. For example, our group has previously reported the efficient encapsulation (90%) of salmon calcitonin (sCT) by the double emulsion method, profiting from the electrostatic interaction between the cationic peptide, dissolved in the internal aqueous phase, and the anionic lipids, tripalmitin (TP) and lecithin (71). Other authors have observed similar entrapment for sCT (90%) and insulin (AE 98%, drug loading 18.92%) using the w/o/w technique, however, the high loading was attributed to the formation of sodium cholate reverse micellar structures that enveloped the drug (72, 73). The hydrophobic ion pairing (HIP) technique has also been employed to enhance peptide hydrophobicity, and, thus, its entrapment within the lipid matrix. Yuan *et al*. obtained 75% leuprolide entrapment by complexing with stearic acid (SA) or sodium stearate (SS) prior to the encapsulation within SLN (74). Overall, a number of studies, (Table 3) have achieved good entrapment of peptide drugs making use of the strategies described above. The final loading of the SLN has been found to be dependent on the liposolubility of the

peptide (e.g. 33 % loading for levothyroxine (75), but also on the entrapment strategy, having reached loading values in the range of 10-20% for sCT and insulin (72, 73).

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Peptide	Lipids	Size and Z-potential	Drug entrapment	*In vitro* drug release	Ref.
Insulin	GMO or cetyl palmitate	180nm-1.5μm -7 to -37mV	AE up to 84% DL up to 1.23%	~ 20% burst release in 0.01M HCl; slower release in pH 7.4 buffer	(66-68, 76)
	Cetyl palmitate	350nm	AE >43%	Biphasic, initial burst and prolonged release (data not shown)	(77)
	Stearic acid, palmitic acid, SPC	110-119nm -49 to -54mV	AE 97.8% DL 18.9%	1h release < 30% in PBS; sustained release up to 144h	(73)
	SA-r8, soybean phospholipids	162nm +29.9mV	AE 76% DL 3.19%	-	(78)
	Stearic acid	58-75nm -13 to -15mV	AE 18-40%	-	(48)
	Tripalmitin	111-395nm -17 to -39mV	-	45% release in pH 7.4 buffer in 1h	(70)
	Hydrogenated castor oil	397-513nm -23 to -30mV	AE 78-86% DL 1.58-1.71%	Incorporation of PLGA into the SLN offered controlled drug release	(79)
sCT	Stearic acid & tripalmitin/ trimyristin/ trilaurin	154-243nm -60 to -45mV	AE 88-95% DL 4.87-10.70%	-	(72)
	SA-PEG2k-CSK/ SA-PEG2k-IRQ, tripalmitin,SPC	244-410nm, -20 to -29mV	AE 51-59% DL1.87-2.20%	Release induced by drug diffusion and SLN skeleton dissolution	(80)
	Tripalmitin or tripalmitin with C8/C10 TG	200-537nm -50 to +29mV	AE 31 to >90%	Initial burst release ~ 20% followed by sustained release in pH 4 buffer	(71, 81-83)
Gonadorelin	GMO	422nm -21mV	AE 50 - 69%	Biphasic release, 24.4% release in 6h, sustained release in 12 days (first 2h in 0.1N HCl, followed by pH6.8 buffer)	(84)
Thymopentin or insulin	GMO/GP/GT/GB/stearic acid/ octadecyl alcohol/cetyl palmitate	214-449nm	INS AE 33-76% Thy AE 47-76%	4h INS release 33-76% (first 2h in pH 1.2, later in pH 6.8 media); low burst with GP; hydrophobicity & viscosity influence AE and release	(85)
	GP	305nm,-18mV	INS AE 57% Thy AE 62%	10% (Thy) & 15% (INS) release at 4h (first 2h in pH 1.2, later in pH 6.8 media)	(86)
Leuprolide	Stearic acid, Sodium Stearate	~ 400nm -46mV	AE 28 - 75% DL 0.28-0.76%	8.3% or 23.7% release in 2h in PBS, depending on preparation method	(74)
Levothyroxine	Tripalmitin, palmitic acid	188nm -23mV	AE > 99% DL 33.17%	Slowed release following initial burst in pH 7.4 medium	(75)
Lysozyme	TG14/TG18/DG/MG	-	-	In fasted state simulated intestinal fluid, lipase-mediated release for TG14, TG18 and DG, not for MG	(87)

C8/C10 TG: Miglyol 812N (Caprylic/capric triglycerides); CSK: goblet cell ligand CSKSSDYQC; DL: drug loading; DG: diglycerides; GB: glyceryl behenate; GMO: glyceryl monostearate; GP: glyceryl palmitostearate; GT: glyceryl tripalmitate; IRQ: cell-penetrating peptide IRQRRRR; MG: monoglycerides; OAA: octadecyl alcohol; SA-PEG2k: PEG-40 stearate; sCT: salmon Calcitonin; SPC: soybean phosphatidylcholine; TG14: trimyristin; TG18: tristearin.

Table 3. Examples of compostions of SLN for oral delivery of peptide/protein drugs: the lipids involved, physicochemical properties, drug entrapment, and *in vitro* release profile.

With regard to the release properties of SLN, the controlled release capacity is highly dependent on composition. For example, Christophersen and co-workers studied the impact of the glycerides chain on the release profile of the model protein, lysozyme (87). It was concluded that a lipase-mediated degradation mechanism was the main trigger for peptide release from SLN composed of triglycerides. Interestingly the peptide delivery profile clearly matched the release profile of free fatty acids. In the case of diglyceride-based SLN, the drug release mechanism involved both a lipolysis mediated and peptide diffusion mechanisms. Finally, the release from monoglyceride-based SLN was found to occur by simple diffusion through the lipid channels created in the imperfect matrix. The study concluded that peptide release rate followed the trend: monoglyceride > diglyceride > triglyceride, suggesting that it is possible to control the protein release by using the adequate mixture of lipids. On the other hand, in other studies, it has been reported that the incorporation of surfactants, liquid-crystal forming ingredients, i.e. glyceryl monostearate (GMO) and the co-entrapment of hydrogels may significantly contribute to the controlled release of the associated protein (66, 67, 84, 86).

In addition, to the above-indicated diffusion and degradation mediated mechanism of release, the specific ionic/hydrophobic interaction of the peptide molecules with the lipid components is expected to influence the release profile. As indicated, the cationization of the peptide drug has been a strategy to increase its entrapment due to the ionic interaction with anionic lipids and, such interaction, was found to impact the subsequent release. Interestingly, depending on the nature of the peptide and its ability to interact with the counter-ion, different effects have been observed. For instance, the incorporation of anionic lipid lecithin into SLN led to a gradual release of the cationic peptide sCT (up to 40% in 6h) (71). In a separate study, the incorporation of increasing amounts of lecithin contributed to the sustained release of vascular endothelial growth factor (VEGF) for over 45 days (88). A very different effect was observed for insulin-SC reverse-micelle loaded SLN. In this case, insulin was initially positively charged in an acidic medium in order to promote the entrapment within the SC micelles. However, once the system reached the physiological pH, the charge of insulin was inverted and the repulsion between the drug and bile salts broke down the systems, thus giving rise to burst drug release (73).

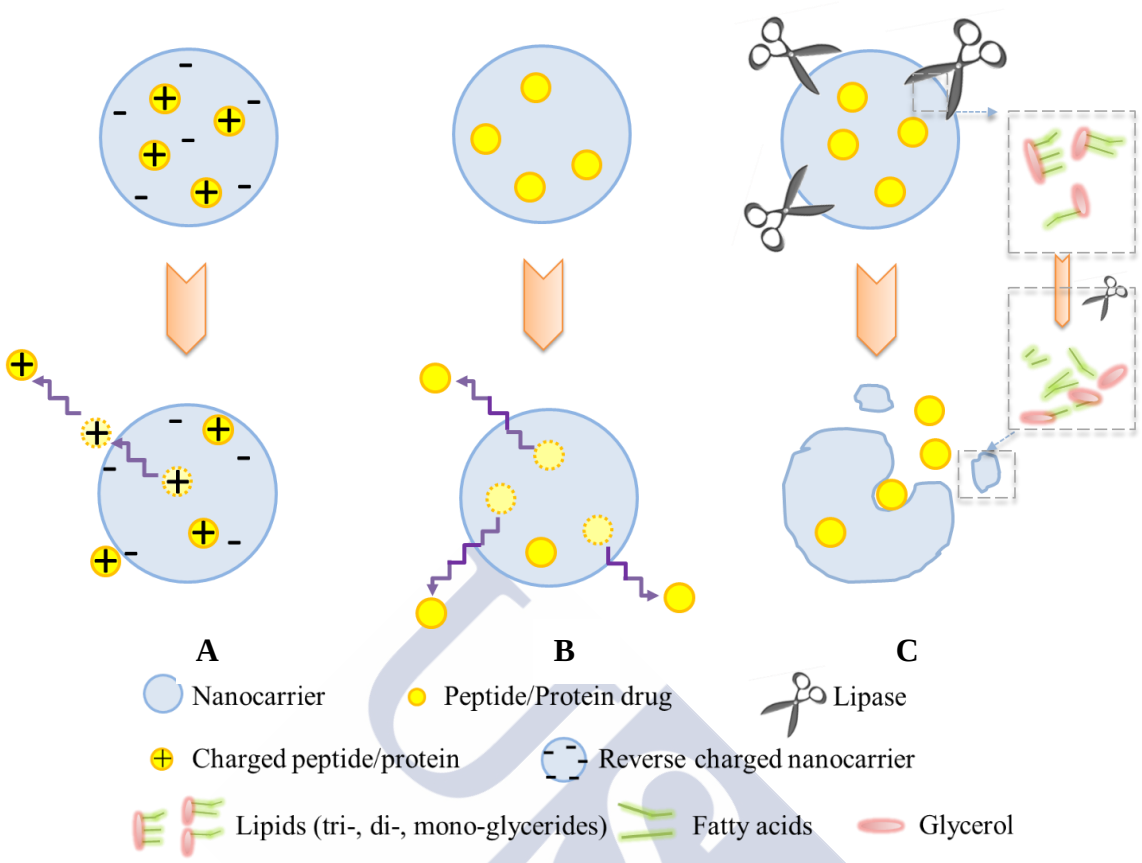

Figure 3. Peptide release mechanisms based on (A) ionic disassociation followed by diffusion through channels of the lipid matrix, (B) simple diffusion through channels of the lipid matrix and (C) lipase-mediated degradation of the lipid matrix.

In conclusion, the SLN formulation approaches used so far have involved the use of counter ions to increase the hydrophobicity of the peptide prior to encapsulation or in the process of encapsulation, the formation of peptide loaded reverse-micelles, as well as the use of the double emulsion approach to facilitate the physical entrapment of the peptide molecules. On the other hand, peptide controlled release can be achieved using lipidic mixtures with lipids owning distinct degradation profiles and also through the control of the porosity of the lipid matrices.

3.1.2. *Microemulsions and nanoemulsions*

Microemulsions are a thermodynamically stable and isotropically transparent dispersion of two or more immiscible liquids stabilized by one or more suitable surfactants (89, 90). Both water-in-oil (w/o) and oil-in-water (o/w) microemulsions may form, although the interpretation is complex, since microemulsions are not true emulsions but colloidal solutions with solubilised water or solubilised oil microemulsions are characterized by an ultra-low

interfacial tension between oil and water phases brought about by the selected surfactants and while they may have complex and diverse microstructures varying from micro-droplet, to bicontinuous and solution types, they are generally considered to exhibit a size below 100 nm. Microemulsions can form spontaneously, given the low/limited energy required to produce a thermodynamically stable system. Nanoemulsions, on the other hand, are not thermodynamically stable and require high energy input to produce a kinetically stable system of droplets less than 200nm (91, 92).

With regard to the preparation techniques, microemulsions are prepared by mixing the oily components plus the surfactant/s and co-surfactant/s in adequate proportions (93), whereas nanoemulsions may be obtained using high-energy processes (homogenizer, microfluidizers or ultrasonicator) to manufacture emulsions (94-96), or lower-energy approaches including spontaneous emulsification (solvent displacement) or phase inversion temperature (PIT) methods (92, 97-100). The former method successfully avoided high temperature that may hamper the bioactivity of peptides, and the second approach omitted the usage of organic solvent.

From drug delivery perspective, micro/nanoemulsions are attractive due to the existence of microdomains of different polarity within the same single phase solution, which can facilitate solubilisation of either hydrophilic or lipophilic materials. The encapsulation of the hydrophilic peptides into micro/nanoemulsions has been achieved through the formation of simple w/o emulsion or multiple w/o/w emulsions (92). Additionally, since medium chain lipids can be more easily mixed with aqueous phase compared to long chain lipids, they are also commonly used in emulsions to facilitate the solubilisation and encapsulation of hydrophilic drugs (101, 102). Finally, the potential interaction of the peptides with specific components, either lipids or surfactants, is expected to significantly contribute to the loading of peptides and their release mechanism. Overall, the efforts dedicated to these peptide formulations, have culminated in over 80% entrapment efficiency for various peptidic drugs such as insulin, sCT and BSA (Table 4).

Peptide	Lipids and emulsifiers	Emulsion Type	Size and Z-potential	Drug entrapment	Ref.
Insulin	Triacetin; DMAB, propylene glycol	W/O	161nm	AE 85%	(103)
	Oleic acid; polyglyceryl-6-dioleate, PEG-8 C8/C10 glycerides	W/O	108nm, Insulin-CS complex +30mV	Insulin complex AE 79%	(104)
	Soybean oil; HCO-60, L-1695, sodium cholate glycerin	S/O/W	1.1μm	-	(105, 106)
	Soybean oil or Medium chain TG; Cetyl PEG/PPG- 10/1 dimethicone (W/O); Tween 80 (W/O/W)	W/O/W	1-20μm	AE> 95%	(107)
	Triolein., oleic acid, DHA, EPA, stearic acid, linoleic acid, linolenic acid, EPC, PEA; Span® 80 (W/O); Tween® 80 (W/O/W)	W/O/W	-	-	(108)
	Soybean oil, triolein, trilinolein, oleic acid, linoleic acid, EPC,PEA; Span® 80 (W/O); Tween® 80 (W/O/W),	W/O/W	-	-	(109)
	Oleic acid, EPC, PEA; Span® 80 (W/O), Tween® 80 (W/O/W),	W/O/W	263 to 591nm	-	(110)
	Lauric acid, palmitic acid, stearic acid, linoleic acid, palmitoleic acid; SGC	W/O	-	-	(111)
Insulin & aprotinin	Olive oil, C8/C10 TG, PEG-HCO; SPC	W/O	<200nm	-	(112)
sCT & Aprotinin	Liquid paraffin; cholesterol, Arlacel 1689, Atlas SCS 2054 (W/O); Synperonic PE/F127 (W/O/W)	W/O/W	14μm	AE 92.1%	(113)
TAT (TAMRA labelled)	C8/C10 TG; Polyethoxylated castor oil	W/O	21.4nm	-	(8)
BSA	Isopropyl myristate; polyethoxylated castor oil, propylene glycol, Tween®20	W/O	21.8nm, -24.8mV	AE > 90%	(114)
EFE-d	C8/C10 TG; Caprylocaproyl macrogol-8 glycerides; Polyglyceryl-3 dioleate	W/O	6.86nm	-	(115)

SK&F106760	Captex355; Capmul® MCM, Tween® 80	W/O	15.2nm	-	(116)

Arlacel 1689: sorbitan oleate (and) polyglyceryl-3 polyricinoleate; Captex355: glyceryl tricaprylate/tricaprate; Capmul® MCM: mono-diglyceride of medium chain fatty acids (mainly caprylic and capric); DHA: docosahexaenoic acid; DMAB: didoceyldimethylammonium bromide; EFE-d: earthworm fibrinolytic enzyme; EPA: eicosapentaenoic acid; EPC: egg phosphatidylcholine; HCO-60: polyoxyethylene hydrogenated castor oil; L-1695: sucrose lauric ester; PEG-HCO: PEG-7 hydrogenated castor oil; PEA: phosphatidylethanolamine.

Table 4. List of emulsion, microemulsions and nanoemulsions for oral delivery of p/p drugs: lipids involved, emulsion type, physicochemical properties and drug entrapment.

There are very few studies on the mechanism of release of peptides from micro/nanoemulsions. Overall, as in the case of the loading, the specific interaction of the peptides with lipid constituents and surfactants is expected to influence the partition between the oily phase and the external aqueous medium. In addition, the degradation of the oil in external media is expected to trigger drug release. In a study with emulsions that contains medium chain triglycerides/soybean oil, surfactants Tween 80® and cetyl PEG/PPG-10/1 dimethicone, the authors found an osmotically-driven swelling effect that caused the breakdown of the emulsions and further leads to drug release (107) (Figure 4). In other studies, the authors proposed to avoid undesired peptide release by protecting the emulsions from degradation (112, 113). Moreover, from a product development perspective, there is the possibility to incorporate emulsions, either in a liquid or dry form in pH-sensitive pharmaceutical dosage-forms (105, 106).

All these activities on micro/nanoemulsion-based formulations has led to significant advancements for enhancing oral peptide delivery (30, 117-119). Further promising development in microemulsions also has given rise to self-emulsifying drug delivery systems (SEDDS, SMEDDS and SNEDDS) and similar structures, as described below.

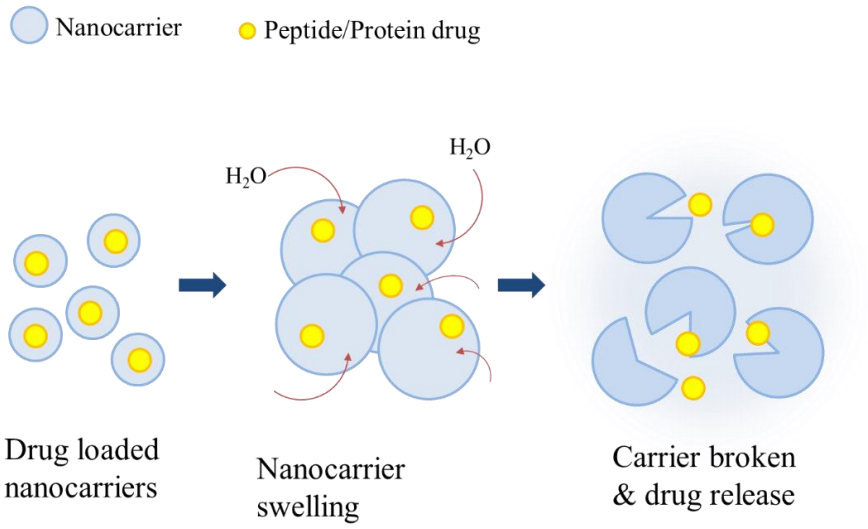

Figure 4. Osmotically swelling-breakdown based peptide release mechanism.

3.1.3. *Self-emulsifying drug delivery systems (SEDDS, SMEDDS, SNEDDS)*

Lipid based formulations that self-emulsify on gentle dispersion in gastrointestinal fluids, to form emulsions (SEDDS) or microemulsions (SMEDDS), are of particular interest to the pharmaceutical industry, given that such 'ME pre-concentrates' can be packaged into gelatin capsules allowing precise and convenient unit oral dosage forms. SMEDDS are usually composed by an oil phase (<20% w/w), high concentration of surfactant (often 30 to 60 %), and in many cases a hydrophilic co-surfactant (eg. propylene glycol, polyethylene glycols) and optionally a co-solvent and/or an aqueous phase. These formulations are distinguished from SEDDS (oil phase 40-80%) by smaller emulsion droplets produced on dilution, producing transparent or translucent stable nano-emulsions with droplet sizes less than 200 nm. While traditionally the term SMEDDS was employed, more recently there is a trend to describe many of these systems as self-nano-emulsifying systems (SNEDDS) (120).

Most of the commercially available SMEDDS are employed for poorly water soluble drugs (121). However, given the merits of lipids excipients on enhancing stability and permeability of peptides, there has been a renewed interest in their use for oral peptide delivery (50, 122, 123). As in the case of the regular micro/nanoemulsions, the loading of the peptide within these systems may be limited by its hydrosolubility.

However, this may be overcome by the addition of polar solvents (eg. co-solvents) or small quantities of water to produce a W/O microemulsion. In this regard, it is important to keep in mind that any change in the components or their relative concentrations in the SMEDDS can lead to a change or even to the destruction of the system. Because of this, the selection of components and their concentrations is key and it is determined using ternary phase diagram and described in detail elsewhere (5). An alternative strategy to increase peptide loading has been reported whereby the lipophilic surfactants are used to coat the peptide, which is then dispersed in the SMEDDS, to produce solid-in-oil suspension/dispersions (124, 125).

A key consideration in the use of SMEDDS for oral peptide delivery is the impact of dilution *in vivo* in gastrointestinal fluids, which can affect the drug release or leakage following oral administration. On initial dilution with aqueous media, an o/w microemulsion may form, which on further dilution may then undergo phase inversion to either a w/o or w/o/w microemulsion, which may lead to phase separation, flocculation and potentially, drug precipitation. In an attempt to regulate the drug release profile, Fan *et al*. included 1% Carbopol® 980 to the internal phase of a w/o microemulsion, to stabilise the microemulsion droplets that formed on dilution and reduce protein leakage for the inverted microemulsions (126). The impact of dilution *in vivo* needs to be adequately addressed.

Compared to other peptide delivery technologies, microemulsion-based technologies, in particular self-emulsifying systems, may offer distinct advantages, as the avoidance of using organic solvents and/or high shear conditions during formulation, which can adversely influence protein stability, is eliminated. While considerable commercial success has been achieved with SMEDDS for oral administration of hydrophobic peptides and peptide-like drugs, most notably the lipophilic peptide cyclosporine (Neoral®, a SMEDDS formulation with ~30% bioavailability (127), research involving hydrophilic peptides remains largely pre-clinical.

3.1.4. *Nanocapsules (NC)*

Nanocapsules (NC) are core-shell nanostructures consisting of a liquid core, normally an oil, surrounded by one or more polymer coating layers. The core is considered to acts as a drug reservoir, whereas the surrounding shell is intended to control peptide release as well as to help overcoming biological barriers, such as stability and mucodiffusion. The methods to prepare the NC involve two steps: emulsification and polymer formation or deposition around the oily nanodroplets. The emulsification can be achieved using high-pressure or high-energy sources, the solvent displacement technique (68-70) or temperature cycling treatment (128, 129). The formation of the polymer shell has been so far attained with two main approaches (i) polymerization at the interface of the nanoemulsion (130-132); (ii) polymer precipitation around oily nanodroplets, a process that has been reported using a variety of terminologies: *emulsion-diffusion* (133), *interfacial deposition* (134), *solvent displacement* (135, 136) and *emulsion coacervation* (137). In addition to these variety of techniques, our lab and others have made possible the formation of a multi-layer coating based on the layer-by-layer deposition technique (138).

From our knowledge, Couvreur and co-workers were the first to report on the encapsulation of proteins within NC (130). These authors showed the possibility to entrap insulin with poly (alkylcyanoacrylate) (PACA) NC using the emulsion-polymerization technique. Following on from these studies significant advances in terms of the capacity of NC to load peptides have been achieved (Table 5) (81, 135, 139-144). For example, proteins such as OVA, BSA, insulin and D-Lys6-GnRH have been entrapped within PACA NC with a high efficiency (> 90%). In this case, the association efficiency was found to be influence by the density of the polymer shell (79-82) and also by the chemical reactivity of monomers (145).

Chitosan NC, originally developed by our group (146), have also been proposed as

carriers for the oral administration of sCT (81, 135, 139). The encapsulation of this cationic peptide was favoured by its interaction with the surfactant lecithin and probably by the acidic nature of the oily core.

The NC technology offers the advantage and the versatility of both, the inner core and the polymer shell. The nature of these components, and also that of the surfactant/s used to stabilize the NC, may have an effect on the encapsulation and release properties. For example, in the case of insulin-loaded PLA NC, it was found that an increase in the amount of surfactant (Span® 60 or Span® 80) was beneficial for the incorporation of insulin into polylactide NC (142). Similar to the case of both SLN and microemulsions, employment of w/o/w emulsion core also effectively increased the peptide loading in this formulation (147).

In common with other lipid based nanocarriers, despite the high entrapment values, the final loading of the NC are not uniformly reported and the values disclosed are generally modest (~2%) (140, 148). This may be attributed to the limited capacity for interaction/solubilisation/entrapment of hydrophilic peptides within lipidic nanocarriers. In this specific sense, attention should be paid to the way the final loading is calculated (taking into account the weight of the individual components used to prepare the NC, or the final weight of the NC).

With regard to the mechanism of release of peptides from NC, most of the information available applies to the NC prepared by interfacial polymerization. For example, it has been reported that the release of insulin from PACA NC was pH-dependent, being very low in acidic medium and more pronounced in a neutral medium. In addition, based on the decrease of the NC size over the time, the authors assumed that the NC's surface was altered due to erosion leading to the sustained release of the encapsulated peptide (149-152). On the other hand, the MW of the peptide was found to significantly affect the release rate, according to the following

ranking: insulin (5.8k) > OVA (45k) > BSA (65k) > urease (483k) (148). The explanation for this behaviour was related to the diffusion capacity of these macromolecules across the polymer shell. However, these data should not lead to the conclusion that the release of the encapsulated peptides is solely driven by the passive diffusion across the pores of the polymer shell. Indeed, the potential interaction between the peptides and the different constituents of the NC should also be taken into consideration in the interpretation of the release mechanisms.

Beyond the work performed with PACA NC, in general, it is assumed that the polymer coating around the NC may contribute to the controlled release of the encapsulated peptide and there are some illustrative examples of such behaviour. For example, the release of insulin from NC with an alginate/chitosan shell was dramatically reduced as compared to that observed for the control nanoemulsion (141). Similarly, our group observed that the release of sCT from chitosan NC was slower than from the control emulsions, although the emulsion also showed a capacity to hold the drug (139). This was attributed to the interaction between the cationic peptide and lecithin. Other studies have also described the role of the surfactants in the prevention of the drug release (142).

As compared to other lipidic systems, peptide encapsulation and release from the NC does not only depend on the lipid composition of the oily cores and their potential interaction with the peptide molecules, but also on the affinity of the peptide for the polymer shell and the erosion of the shell in biologically relevant conditions. Therefore, overall the release profile is expected to be affected by a combination of different processes, which may involve lipid and polymer degradation - followed possibly by the destabilization of the nanostructure - , diffusion across the oily medium and the polymer shell, and disassociation of the peptide molecules form the counter-interacting parts, these being either lipids, polymers or surfactants.

Peptide	Lipids	Coating	Size & Z-pot	AE (%)	*In vitro* release profile	Ref.
Insulin	C8/C10 TG	PBCA	220-300nm	55-98	Sustained release as PBCA degrades	(130, 145, 153-156)
	C8/C10 TG, C8/C10 mono-/diglycerides	PECA/PBCA	151-210nm	Up to 97	pH, oil-water ratio and mass of monomers used alters the release	(149-151)
	Isopropyl myristate (w/o or o/w)	PECA/ PBCA	326-353nm -9.7 to -33mV	11.5-52.3	Higher amount of monomer slowed down release	(152, 157)
	Propylene glycol dicaprylate/dicaprate, PEG-HCO	Chitosan-alginate	488nm -1 to -70mV	47.3	pH dependent leakage & *in vitro* drug release	(141)
	Span® and Tween® (no oil)	PLA	159-322nm -32 to -41mV	34-66	Span®60-Tween®60 NC slower released than Span®80-Tween®80 NC; higher amount of Span® caused increase in AE and decrease in drug release	(142)
sCT	C8/C10 TG	PBCA	39nm - micron	Up to 23	No delayed release	(158, 159)
	C8/C10 TG	CS	251-331nm, +31 to +34mV	38-39	Biphasic release in pH 4 acetate buffer, nearly 30% burst	(81, 83)
	C8/C10 TG	CS-PEG	247nm, +34.8mV	44	Biphasic release in pH 4 acetate buffer, 20% burst	(135)
	C8/C10 TG	Carbopol	110nm	-	-	(143)
OVA	Ethyl oleate	PECA	211-309nm -13 to -21mV	8 to 95	-	(160)
BSA	Light mineral oil	TDI	~50nm -20 to -25mV	92	Potentially slowed drug release	(144)

BSA	Glycerol trioleate	PBCA	160nm	DL(%): 1-4	Higher pH / loading and lower drug MW promote burst release; surface erosion induced drug release; lower MW protein diffuse through PBCA wall easily	(148)
D-Lys6-GnRH	Ethyl oleate	PECA	190nm, +8.8mV	98.6	<5% release at 6h in GIT media; 60% release in plasma	(161)

Carbopol: Carbopol®940, cross-linked polyacrylate polymer; C8/C10 TG: caprylic/capric triglycerides; DL: drug loading, PBCA: poly(butyl-cyanoacrylate); PECA: poly(ethyl-cyanoacrylate); TDI: tolylene 2,4-diisocyanate.

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Table 5. Nanocapsules-based oral peptide delivery formulation indicating lipids cores and polymer shells composition, physicochemical properties, drug entrapment and *in vitro* release profile.

3.1.5. Liposomes

Liposomes are vesicles with a diameter from 10nm to more than micron, comprising a well-defined aqueous core and one or more amphiphilic bilayers made of phospholipids and cholesterol. In addition, some polymer compositions incorporate in their structure different surfactants and polymers (162, 163). These vesicles are by far the most extensively studied vesicular system since its discovery in 1961 (164, 165) and, so far, this research activity has been translated into 13 FDA approved liposomes-based products for human use, containing low molecular weight drugs and intended for injection. This prior knowledge has been fundamental for the development of liposomal formulations intended for oral peptide delivery. Other liposome-like nanostructures, such as niosomes and archeosomes, have also been proposed for the oral administration of peptides and proteins (166).

In most cases, peptide-loaded liposomes have been prepared by the *film hydration* (167-183), and *reverse-phase evaporation* methods (184-192), followed by particle size reduction treatments including sonication, extrusion or high pressure homogenization. To date numerous peptides and proteins such as insulin, sCT, albumin, adamantyltripeptides, globulin, leuprolide and others have been entrapped into liposomes, with the final goal of enhancing oral bioavailability (193). Table 6 shows representative examples of compositions reported in the last years for oral peptide delivery.

Peptides can be entrapped into the aqueous core of the liposomes. However, as described for other lipid-based nanostructures, the association efficiency of peptides within liposomes is also greatly influenced by its ionic/hydrophobic interaction with the liposomal components and also by the rigidity of the by-layer formed (179, 180).

Up to now, over 90% association efficiency of insulin and sCT has been successfully achieved, making use of a variety of lipids including, diplamitoyl-phosphatidylcholine (DPPC), distearoyl-phosphatidylcholine (DSPC), stearylamine, dicetyl phosphate, or blends of these lipids with cholesterol. For example, when DPPC was mixed with higher phase transition temperature lipids like dipalmitoyl-glycero-phosphoethanolamine (DPPE) to form liposome, the AE of insulin was significantly enhanced (175). It has also been shown that an increase in the concentration of lipids in liposomes commonly leads to a higher entrapment of peptides, while the absolute drug payload is inevitably decreased simultaneously. Finally, the association other biomaterials, i.e. lectins, to the surface of the liposomes, has sometimes resulted in an increase in the entrapment efficiency (167).

As described for other nanocarriers, the peptide loading capacity of peptides into liposomes is limited by the capacity of the aqueous core and the lipid bi-layer to hold the peptide molecules. In some cases, it has been found that an increase in the amount of peptide to be loaded may lead to a destabilization of the nanostructure (173, 175, 182). Therefore, it is important to carefully design the composition and structure of the liposomes in order to reach the maximum loading, while maintaining the stability and controlled release properties of the system.

The peptide release rate from liposomes is known to be highly influenced by liposomal fluidity, which depends on the phase transition temperature of phospholipids involved. In particular the use of long and hydrophobic phospholipid chains, as well as the presence of cholesterol are known to enhance the rigidity of the liposomes wall and, hence, their colloidal stability and capacity to control the release (194). Additionally, the use of special lipids, such as diether or tetraether lipids – the resulting liposomes were named as *archeosomes*- were reported to maintain the structural integrity against extreme pH, to prevent the degradation by bile salts and lipases and to help the control of the release (170). Similarly, the use of

polyoxyethylene alkyl ethers - the resulting nanostructures were named as *niosomes*- have led to a significantly delayed insulin release (171).

More drastic procedures for enhancing the stability and controlling the release of peptides from liposomes have relied on their association with other biomaterials and nanostructures. For instance, a formulation of thiolated chitosan-coated niosomes exhibited a 12% insulin release in SGF in 5h (189), a result that was attributed to the protection of the vesicle structure in the GI environment. On the other hand, liposomes coated by silica nanoparticles exhibited an improved stability and a sustained release profile for the insulin (169). Our group also reported that the complexation of liposomes with insulin-loaded CS nanoparticle resulted in an enhancement of the colloidal stability of CS nanoparticles with the possibility to tailor the insulin release profile (174, 195). Finally, the encapsulation of the insulin-loaded liposomes into larger liposomes (196), or the surface PEGylation of liposomes were adequate strategies towards this goal (214).

Overall, efficient peptide incorporation in the inner aqueous core of liposomes has been achieved, although the loading capacity of these nanocarriers clearly depends on the interaction of the peptide molecules with the lipids, surfactants and potential polymers involved in the process. In general, it is accepted that a substantial amount of peptide is associated to the by-layer, rather than encapsulated into the core, and this often leads to the unpredicted leakage of the peptide. However, an increasing number of approaches are being disclosed with the final goal of a tailor-made design of liposomes for oral peptide delivery. Currently, some liposomal drugs for oral peptides delivery are in clinical trials, like clinical phase II drug HDV-I (Diasome pharmaceuticals, USA) for oral insulin delivery.

Peptide	System	Size & Z-pot	AE (%)	*In vitro* release profile	Ref.
Insulin	Biotin-modified liposomes (SPC)	85-309nm	22-65	-	(184, 185)
	Bile salts added Liposome (SPC)	150nm (SGC)	30	In 6h, 57.7% release in SGF; 63.8% release in FaSSGF	(186-188)
	Trimethyl CS coated niosome (Span®60)	100-180nm (non-coated)	75	12% release in SIF by 5h	(189)
	Folic acid added multi-coat liposome (PC, SAM)	266nm +25mV	93	< 20% release in SGF, SIF & pH 7.4 PBS in 2h; up to 72% release in PBS in 24h	(168)
	Silica nanoparticle coated liposome (DPGPC)	255-297nm ~-15mV	~ 70	Negligible release 45min in SGF; slowly and continuous release for up to 8 h; slower release for coated liposomes	(169)
	Archaeosome (tetraether lipids)	210nm -36mV	18.6	70% and < 50% release in 4h in artificial intestinal and gastric condition, respectively	(170)
	Niosome (Brij™,DCP)	4.2-13.4µm	8.7-41.5	26.3% insulin released during 24h in SIF	(171)
	Fusogenic liposome (EPC, PG)	-	3.3	-	(197)
	Lectin modified liposome (SPC)	190-194nm +3.4 to +8.7mV	40-83	-	(190)
	PEG-2000 or mucin coated liposome (DPPC, DSPE-PEG)	453-479nm	35-38	Surface coating resulted in resistance to bile salt digestion and slow release in GI tract	(172)
	Aprotinin added double liposomes (SPC, SA/PS)	8-9µm	7.6-23	Double liposome show slightly slower release	(196)
	Complex of liposome & CS nanoparticle (DSPC, DPPS)	1.5-3µm -17.7mV	-	Lipid coat induced delayed drug release	(174)
	Liposome (PEtOH)	50-250nm	> 80	-	(175)
	WGA-carbopol modified liposome (DSPC, SAM)	187-201nm -44 to -57mV	91	-	(176)
sCT	Thiolated CS coated liposome (DPGPC, DPPE-MCC)	605-709nm +28 to +44mV	69	No evident release in SGF; sustained release in SIF with less than 20% burst	(198, 199)
	Pectin-liposome nanoxomplexes (DSPC, SAM)	Below 1µm -40 to -60mV	49.7	-	(177)

	CS coated liposomes (DSPC, DCP)	305nm-1.8µm	100 (20% EE)	-	(178)
	CS coated liposome (DSPC, DCP) with different size	473nm-4.1µm +30 to +35mV	> 90	Sustained release at pH 6.8	(179, 180)
	Double liposomes (DMPC, DPPC, SAM, DPPG)	2.1µm	36.2-62.9	-	(196, 200)
Albumin	CS coated liposome (SPC or EPC)	128nm +5.4mV	50% Drug : lipid ratio 2.5%	-	(182)
Octreotide	Archeosome (tetraether lipids)	130-207 nm	Drug : lipid ratio 1.2 to 13%	-	(183)
Leuprolide	PSCG coated liposome (PC)	112-168nm -8.8 to -20mV	29.4-37.1	Drug release due to vesicle disruption	(167)
Epidermal growth factor	Liposome (DMPG, DOPC, triolein)	1.4µm	Up to 60	Sustained release; 47% release in SGF and 35% release in SIF in 6h	(191)

Brij™: polyoxyethylene alkyl ethers; DCP: dicetyl phosphate; DMPC: dimyristoyl-phosphatidylcholine; DMPG: dimyristoyl-phophatidyl-glycerol; DOPC: dioleoyl-phosphatidyl-choline; DPPC: dipalmitoyl-phosphatidyl-choline; DPPCG: dipalmitoyl-glycero-phosphocholine; DPPE-MCC: dipalmitoyl-glycero-phosphoethanolamine-maleimidomethyl-cyclohexane-carboxamide; DPPG: dipalmitoyl-phosphatidyl-glycerol; DPPS: dipalmitoyl-phosphatidyl-serine; DSPC: distearoly-phosphatidyl-choline; DSPE-PEG: distearoylphosphatidylethanolamine-poly-(ethylene glycol) 2000; FaSSGF: fasted state simulating gastric fluid, PC: phosphatidylcholine; PEtOH: phosphatidylethanol; PG: L-dimyristoyl phosphatidylglycerol; PS: phosphatidylserine; PSCG: O-palmitoylscleroglucan; SAM: stearylamine.

Table 6. List of vesicular systems for oral delivery of p/p drugs: lipids involved, physicochemical properties, drug encapsulation and release profile.

3.2. Interaction of lipid-based nanocarriers with the biological environment

Given that lipids are biodegradable, and in particular susceptible to intestinal lipase mediated degradation, a comprehensive understanding of the behaviour of the lipidic nanocarriers upon contact with the gastro-intestinal milieu is needed in order to reliably predict *in vivo* performance. This degradation of the lipid materials may also be followed by the degradation of the associated peptides. Finally, the transport of these nanocarriers across the mucus barrier and subsequent interaction/transport across the intestinal epithelium are expected to have important consequences on the overall performance of the nanocarriers. In the following sections, we will report the knowledge accumulated so far with regard to the ability of lipid-based nanocarriers to deal with these biological barriers.

3.2.1. Stability in simulated biological fluids

As indicated in section 2.1 (table 1), selected lipid excipients, namely long chain glycerides and fatty acids, may confer lipid-based nanocarriers with a resistance against enzymatic degradation (5, 6). In the case of SLN, the solid physical state of the lipids is expected to help protect the encapsulated peptide (48, 77, 78, 85, 87). Similarly for NC, the polymeric shell may offer protection from exposure to low gastric pH and enzymatic degradation (81, 83, 130, 135, 145, 152-157). PEG surfactants have also been employed as a general strategy to protect microemulsion systems from aggregation and degradation (8, 104, 107). Finally, the colloidal stability of liposomes and their capacity to protect the encapsulated drug has been achieved through adequate lipid selection (170, 183, 197), the inclusion of enzyme inhibitors (186-188, 196), or the attachment of a protective polymer around the phospholipids bilayer (168, 178).

In terms of selecting lipid excipients to protect from enzymatic degradation, it appears that long chain fatty acids are favoured. For example, in a study it was shown that the stability of SLN and their subsequent capacity to protect the encapsulated

molecules followed the ranking: tripalmitin (C16) > trimyristin (C14) > trilaurin (C12) (72). In a separate study involving liposomes, it was found that the use of phospholipids with a high phase transition temperature leads to the formation of a gel membrane, which helps in preventing their colloidal stability as well as the stability of the encapsulated peptide

Apart from the mentioned triglycerides and phospholipids, there are other lipid materials, which may significantly contribute to the colloidal and molecular stability of lipid-based nanocarriers. Bile salts, in addition to their penetration promoting capacity, have been reported as efficient stabilizers. As an example, insulin loaded-liposomes containing sodium glycocholate (SGC), sodium taurocholate (STC), or sodium deoxycholate (SDC) were reported to have an improved stability in simulated gastrointestinal fluids (186-188). SGC-containing liposomes displayed the highest drug protection and longest residence time. More importantly, this improved stability profile was translated into an enhanced *in vivo* efficacy in terms of more pronounced glucose level decrease and sustained drug action.

As indicated above, the presence of a polymer coating around the liposomes, SLN or NC, can positively influence the stability of these nanocarriers. A common approach involved the use of PEG derivatives and, in particular PEG stearate, which has been used to improve the stability of SLN and the entrapped peptides in gastric-intestinal fluids (75, 80). Similarly, the PEGylation of liposomes with PEG-2000 was reported to enhance the resistance to digestion (172). An alternative approach has made use of the polysaccharide chitosan and PEG-Chitosan in combination with lipids. Different type of nanocarriers, including liposomes and NC, combined with chitosan were found to exhibit an improved stability and overall in vivo performance (81, 135, 139, 174, 195). Chitosan coated tripalmitin SLN maintained particle size upon contact with simulated gastric-intestinal media (71).

A drastically different strategy oriented to increase the stability of the encapsulated peptide has consisted in involved the formation of conjugates of peptide/protein with lipids. In particular, fatty acids have been linked to peptidic drugs (sCT, insulin, enkephalin, INF-α, octreotide, tetragastin, demopressin, etc.), the result of which was an improvement of the peptide stability against enzymatic degradation (201-204). Although not systematically investigated, the increased in the fatty acids carbon number (from 2 to 5) in fatty acids was found to positively impact the stability of the resulting peptide-lipid conjugate (205). This could be, in some cases, related to the capacity of the amphiphilic conjugates to form micelles (206, 207). Palmitic acid (C16) is the most studied molecule for this peptide lipidization approach.

Overall, it can be concluded that the appropriate selection of lipids with different properties and/or the addition of auxiliary excipients, such as surfactants and polymers, to lipid-based nanocarriers has been successfully employed to overcome the harsh conditions present in the GI environment. However, given that these studies have been generally performed using simulated gastric-intestinal fluids in the presence of enzymes, the extrapolation of this in vitro data to the *in vivo* conditions remains unclear.

3.2.2. Interaction with the mucus layer

In theory, due to their innate hydrophobicity, lipid-based nanocarriers are not supposed to optimally diffuse across the water-rich mucus layer. However, the combination of hydrophobic lipids with amphiphilic lipids, surfactants and polymers offers the possibility to modify the muco-diffusion of lipid-based nanocarriers.

Traditionally, the use of bioadhesive polymers and, in particular chitosan and its thiolated version (71, 139, 208), alginates (141), pectin (177), and acrylic polymers (143, 176, 180), have been proposed as a strategy to favour the interaction of the nanocarriers with the mucus, with a view to prolonging residence time in the intestinal

tract. However, more recently, it has been recognised that increasing the muco-interaction may not be an optimal strategy for nanocarriers to penetrate to the underlying epithelium. In fact, the nanocarriers may be retained in the mucus layer and be eliminated via mucus turnover. On the contrary, the emphasis is now oriented towards achieving an adequate mucodiffusion. It is generally considered that a small nanocarrier size, a neutral or negative surface charge, and enhanced surface hydrophilicity are favourable properties for achieving adequate muco-permeation (209, 210). This postulate has been confirmed for a number of lipid-based nanocarriers, including SLN (80), SNEDDS (211, 212), NC (129) and liposomes (179, 186-188).

Clearly, the surface characteristics of the nanocarrier can be tailored to optimise muco-diffusion. The potential of protecting the nanocarriers with a hydrophilic layer made of PEG, is particularly noteworthy given the ability to enhance diffusion through the hydrophilic mucous layer and also prevents interaction with biological enzymes and mucin. The first evidence of this positive role of PEG was reported by our group in the late 90's (213) and has been extensively validated thanks to the work by Hanes *and co-workers* (210, 214-216). Mechanistic studies demonstrating the benefit of PEG in the mucodiffusion of lipid-based nanocarriers has been shown for NC containing PEG stearate (129), SNEDDS involving PEG oleate (211) and liposomes modified by poloxamer 407 (217). Finally, it is important to keep in mind that the concentration of nanocarriers interacting with the mucus layer may significantly alter their mucodiffusion behaviour (216, 218).

While our understanding of nanocarrier interaction with the mucous layer has been greatly improved as a result of these studies, there remains a need for a more systematic study of the properties influencing muco-adhesion, muco-diffusion and stability of nanocarriers in the intestinal environment. Additional efforts should focus on identifying experimental techniques illustrating the intestinal mucus environment

and allowing for a systematic and comparative analysis of different nanocarriers (219). Multiple particle tracking (MPT) and fluorescence recovery after photobleaching (FRAP) techniques appear to be among the most promising techniques and the advances in this area are well illustrated in the paper entitled *"How to design the surface of peptide loaded-nanoparticles for an efficient oral bioavailability?"* in ADDR journal (Malhaire *et al.*).

3.2.3. Interaction with the intestinal epithelium

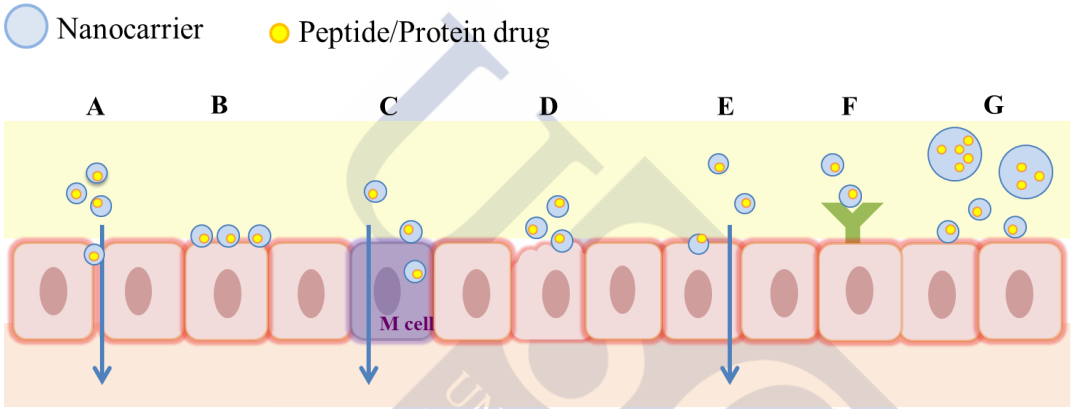
Lipid-based delivery nanocarriers are made of natural biodegradable and biocompatible lipids and, therefore, they are generally regarded as safe (GRAS) (220). However, a concentration dependent toxicity might be observed for specific nanocarriers containing additional ingredients (83, 182), i.e. surfactants, bile salts, polymers and any other synthetic material, which may be part of the nanocarriers.

The majority of the studies on lipid-based nanocarriers for oral peptide delivery, justify the use of lipids based on their functionality as penetration enhancers. This is particularly the case of medium chain fatty acids and bile salts, as well as medium chain caprylic/capric (C8/C10) glycerides, which are known to digest into fatty acids, thereby contributing to the enhanced intestinal permeability (21, 32, 221). The mechanism of penetration enhancement of these lipids has been mainly associated to the alteration of the intercellular junctions (34, 222, 223).

On the other hand, long chain fatty acids, e.g. stearic acid, oleic acid, linoleic acid, linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are classified as penetration enhancers or membrane fluidizers, due to their membrane-associated effects at cellular level (224-226). The presence of double C bonds has been found to contribute to the cell membrane disrupting ability. Despite this interesting permeability enhancement effect, it has been suggested that this membrane perturbation could have consequences in terms of long term toxicity (222).

Therefore, a composition balance in terms of medium chain and long chain glycerides might be option to achieve an acceptable penetration enhancement effect.

In addition to the inherent properties of lipids with regard to their penetration enhancement capacity, the physical and physicochemical properties of the nanoparticles may influence how the lipid-based nanocarriers interact with the intestinal epithelium. Figure 5 shows the different mechanisms whereby the lipid-based nanocarriers interact with the intestinal epithelium.



A. Paracellular pathway with opening of tight junction
B. Enhanced cell affinity
C. M cell mediated transport
D. Cell membrane perturbation
E. Enhanced endocytosis (clathrin-/caveolae- mediated)
F. Cell targeting (ligand modified nanocarrier)
G. Enhanced uptake by size regulation (advantage of nanometric size)

Figure 5. Approaches how the lipid based nanocarriers enhance peptide permeability

Dating back to the first trials of insulin loaded NC, Damgé *et al.* observed that these 220nm PBCA NC could promote the insulin absorption, crossing through ileal epithelium rapidly via M cells (154). In the case of SLN, they have been found to be transported by a clathrin- and caveolae-dependent endocytosis (72) and the affinity of the fatty acids towards the cell membrane has been associated with the melting point, the length and saturation degree of the of fatty acid chain. In addition, particle size has been shown to play an important role in the mechanism of uptake of SLN (227) and

liposomes (187) with the epithelial cells. Nanometric liposomes (80-400 nm) are more easily absorbed than those over micron; SLN around 247 nm showed enhanced cell uptake than other size (165 nm and 1 μm).

The modification of the surface of the nanocarriers has also been a frequently used strategy to promote uptake. For instance, based on SLN template, the addition of cell penetrating peptide octaarginine to the constituent lipid (SA) was reported to increase 18.44 folds insulin internalization on Caco-2 cells (78). Similarly, the incorporation of targeting ligands, i.e the goblet cells CSK ligand (80), lectins (48), or chitosan (82, 208) have all led to an improved interaction of lipid-based nanocarriers with the intestinal epithelium. All these have been discussed the paper entitled "*Mechanisms of transport of polymeric and lipidic nanoparticles across the intestinal barrier*" in ADDR journal (Beloqui *et al*), and consequently are not detailed here.

4. *In vivo* performance: PK/PD analysis of lipid-based nanocarriers

Lipid NC consisting of an oily core and a PBCA coating was the first lipid-based nanocarrier proposed for oral administration of insulin (130). Insulin-loaded PBCA NC induced significant long term (6 or 20 days) hypoglycaemic effect when injected orally to fasted diabetic rats (12.5 or 50 IU/kg), however, no significant drug efficacy was evidenced in normal rats (130). The authors attributed the enhanced efficacy to the drug protection against enzymatic degradation and also to the internalization of the NC into the intestinal epithelium. The long term effect was believed to be related to an accumulation of NC in the intestinal epithelium and/or a sustained release of insulin from NC's. Subsequent studies led the authors to conclude that the NC were taken up by M cells (Peyer's patches) and that only a small portion could cross the intestinal layer via the paracellular pathway (153, 154, 156, 228). The same group also reported that the ileum was the most significant intestinal region for the insulin absorption (153, 154) and that there were significant variations among rats in the pKa profiles (155). This could be the reason for the discontinuation of the preclinical

evaluation of these originally promising prototypes. Irrespective of this, other authors have explored other polyacrylic polymers (Carbopol®940, cross-linked polyacrylate polymer) NC for the oral administration of sCT and reported high bioavailability values as high as 14.7%, following intracolonic administration (143, 229).

Our group was the first to report the potential of chitosan NC (81, 135, 139) for the oral absorption of sCT. Other authors have also reported the efficacy of NC with a shell made of chitosan and alginate for the oral delivery of insulin, showing pharmacological bioavailability (relative to s.c) of 8.42% (25 IU/kg) and 5.72% (50 IU/kg) in normal rats and 8.19% (25 IU/kg) and 7.84% (50 IU/kg) in diabetic rats following oral gavage. The high bioavailability was attributed to an efficient drug protection, an interaction of the NC with the intestinal mucosa and an enhanced paracellular transport of the associated peptide (141). Overall, the control studies with non-coated nanoemulsion formulation made evident the role of the polymer shell on the *in vivo* behaviour of these lipid based nanocarriers (81, 135, 141).

Liposomes have received the greatest attention for oral peptide delivery. A common strategy intended to adapt the liposome's features to the requirements of the GI environment has relied on the modification their surface. For example, using lectin (WGA) as a targeting ligand, some authors reported a 9.12% insulin bioavailability (50 IU/kg) following oral gavage in diabetic mice compared to subcutaneous injection (190). Other authors studied the efficacy of folic acid-PEG-poly(allyl amine) coated liposomes for oral insulin administration (50 IU/kg). The very long-lasting hipo-glycaemic effect (18h) was thought to be a consequence of folic acid receptor mediated endocytosis (168). A more complex composition was the so-called fusogenic liposomes, consisting of conventional liposomes loaded with inactivated *sendai* virus and the enzyme inhibitor p-chloromercuribenzoate. Following intra-colonic administration of these liposomes loaded with insulin, the authors reported a high bioavailability of 8.4% (10 IU/kg) (197). Other simpler formulation

options have also resulted in promising *in vivo* data. For instance, the coating of sCT-loaded liposomes with muco-adhesive pectins led to a significant reduction of the calcium levels for up to 48h when administrated intra-gastrically (500 IU/kg) (177). On the other hand, the complexation of liposomes and insulin-loaded CS nanoparticle led to a response that varied depending on the lipids composition. For the best formulation, a 50% glucose reduction was observed over 24h, following intra-gastric administration (10 IU/kg) to rats (174, 195, 230). Promising *in vivo* results has been also reported for other peptides, such as octreotide and epidermal growth factor using different liposomal compositions (183, 191). In summary, the formation of a functional coating around the liposomes, or their combination with mucoadhesive materials has been the overall tendency for their use as oral peptide delivery carriers. In addition to this, it has been generally accepted that a small size (around 150 nm), is preferable in order to facilitate the diffusion of liposomes across the mucus and epithelium barrier (179, 186-188).

Conventional composition of SLN, based on a blend of stearic acid and tripalmitin have been reported to increase the intestinal absorption of sCT (72). However, similar to the case of liposomes, the most promising *in vivo* results attained for both insulin and sCT loaded SLN have made use of combinations of these lipid nanocarriers with penetration enhancers, e.g. the cell penetrating peptide r8 (78) or targeting ligand, e.g. the CSK targeting ligand (80). SLN based on r8 modified SA achieved 13.86% insulin pharmacological availability when intra-duodenally administrated to diabetic rats (25 IU/kg) (78); while up to 12.41% absolute bioavailability was observed for the sCT loaded SLN formulation after intra-duodenal administration (250IU/kg) to rats (80).

Microemulsions have also been reported to enhance the oral administration of peptide drugs. For instance, the bioavailability of a glycoprotein IIb/IIIa antagonist, was as high as 27% upon its incorporation into a caprylic/capric mono/di/triglyceride microemulsion (116). Similarly, the BA of a fibrinolytic enzyme (EFE-d) has been

reported to be 17.55% following formulation as a microemulsion involving caprylic/capric triglycerides, caprylocaproyl macrogol-8 glycerides and polyglyceryl-3 dioleate (115). Some promising data have also been reported for insulin loaded microemulsions mainly composed by fatty acids and their glycerides. A sustained hypoglycaemic activity and a 37.5% decrease in the plasma glucose levels were observed upon oral administration of a dose of 20 IU/kg to healthy rats (103). Finally, it is worthwhile to mention that the combination of microemulsion systems with other nanocarriers has also led to interesting *in vivo* results. For example, the incorporation of the insulin-chitosan nanocomplex into oleic acid based w/o emulsion, containing polyglyceryl-3 dioleate and caprylocaproyl macrogol-8 glycerides as surfactants, has resulted in a 7% pharmacological bioavailability in diabetic rats following oral administration (50 IU/kg) (104).

The emergence of numerous commercial successfully SMEDDS formulations, including the cyclosporine SMEDDS mentioned previously, clearly demonstrates the increased regulatory acceptance of such lipid based systems, coupled with the knowledge that SMEDDS have been used safely in patients after chronic administration. However, in the case of hydrophilic peptides, the number of studies relating to clinical trials remains scarce, despite promising reports from pre-clinical studies. For example, while Human Parathyroid Hormone (rhPTH1-34) has negligible oral bioavailability, a SMEDDS formulation resulted in an absolute bioavailability to 5.4% in rats (231). Similarly, oral bioavailability of leuprolide acetate in a SMEDDS increased 6.5 fold in rats, relative to a drug solution. Interestingly, the bioavailability was increased even further by using a hydrophobic ion pair of leuprolide with sodium oleate (increased 17.2 fold relative to a solution) (232).

An alternative strategy to enhance oral bioavailability of peptides is based on site specific targeting of peptides to the colon. Relative to the upper small intestine, the colon has a lower brush-border membrane peptidase and pancreatic enzyme activity

and is also believed to be sensitive to absorption enhancers (12, 233). The merits of lipid excipients, in terms of enhancing permeability via the colonic mucosa have been well established (234, 235). Table 7 presents a summary of studies involving colon targeted local delivery of oral peptides, where lipid excipients are involved as a component in these multifunctional drug delivery systems, as mentioned in section 2.1. However, despite significant efforts to develop site-specific targeting of peptides, most notably insulin, developing drug delivery system with demonstrate reliable and consistent systemic absorption from the large intestine remains problematic, as recently reviewed (236, 237).

In summary, though the technologies described here suggest the potential of lipid-based nanocarriers for oral peptide delivery, further mechanistic and bio-relevant studies are required in order to get a comprehensive knowledge on the complex interactions that occur *in vivo* between peptides, lipid excipients and the intestinal milieu. This mechanistic knowledge will help understanding the *in vivo* performance of the nanocarriers.

Peptide	Lipid excipient (PE)	Delivery system	Additional functional materials	PK/PD observations	Ref.
Insulin	SGC	Enteric coated capsules	HPMC phthalate (coat), chitosan (capsule shell)	5.73% BA (rats) Inclusion of SGC increased PA to 3.5%	(238)
	Sodium oleate	Enteric coated microspheres	Bacitracin (PI), hydroxypropylethyl cellulose acetate succinate (coat)	Hypoglycaemic effect observed between 2-7h post dosing (rats)	(239)
	SGC	Coated tablets (CODES®)	Lactulose (core), Eudragit E, HPMC, Eudragit (coat), Camostat mesilate (PI), EDTA (PE)	Absolute BA of 0.34% by inclusion of SGC from 0.13% (not significant). Significantly reduced glucose levels between 6-8h post dosing (dogs)	(240)

	Oleic acid decyl ester, PEG-8 C8l/C10 glycerides, polyglycerol oleate	Enteric and time-controlled release coated capsules	Eudragit S, NE30D and cellulose acetate phthalate (coat), Carbosil (gelling agent) aprotinin (PI), silicium dioxide (gelling agent)	PA increased to 6.2% from 2.1% (dogs)	(50)
Calcitoinin	SGC	Enteric coated capsules	HPMC phthalate (Coat) chitosan (capsule shell), bacitracin, aprotinin (PI), S-Nitro-N-acetyl-penicillamine (PE)	PA increased to 6.3% from 0.04% for control (rats)	(241)
	Sodium Caprate	Minispheres (Smpill®)	Caprylic/capric/linoleic TG, Cremophor EL (microemulsion), gelatin (core)	Absolute BA of 22.3% after intracolonic administration from 7.0% (rats)	(242)
Ciclosporin (CsA)	Caprylic/Capric Triglyceride, Cremophor EL	Enteric and time controlled release coated minispheres (Smpill®)	Ethyl cellulose/pectin (coat), gelatin (core)	Increased colonic delivery of CsA Increased CsA levels in colonic tissue	(243)
Erythropietin (EPO)	PEG-8 capryl/caprylic acid glycerides	Drug/lipids adsorbed on solid adsorbent (carbon nanotubes)	Casein, lactoferrin, Explotab	Absolute BA of 11.5% after intra-jejunal administration (rats). >6 fold BA compare to non-lipid control	(244)
Heparin (LMWH)	Capric acid (C10)	Enteric coated capsules (GIPET I®)	Eudragit (coat)	Absolute BA of 3.9-7.6% (humans)	(245)
Desmopressin	Microemulsions	Enteric coated capsules (GIPET II®)	Eudragit (coat) mono- and di-glycerides of caprylic an capric acid	Absolute BA of 2.4% from 0.2% for control in humans	(245)
Octreotide	Sodium caprylate	Enteric coated capsules (Octreolin®)	Oily suspension including polyvinyl pyrrolidone, polysorbate 80, glyceryl monocaprylate, glyceryl tricaprylate, and magnesium chloride	**Relative oral BA of was similar to SC injection in humans.** Achieved efficacy in controlling IGF-1 and GH up to 13 months	(13, 14)

PI: protease inhibitor; PE: permeation enhancer.

Table 7. Colonic local peptide/protein delivery using lipid excipients

5. Conclusion and future perspectives

In summary, an appropriate design and development of optimal lipid-based nanocarriers requires consideration of the distinct merits of various formulations and the peptide or protein characteristics. Using a range of lipid based nanocarriers systems, adequate peptide entrapment has been accomplished and certain formulations have shown ability to control the drug release. It is also increasingly recognised, that in order to overcome physiological, biochemical and biopharmaceutical barriers to delivery, a multifunctional drug delivery system employing protease inhibitors to enhance stability, permeation enhancers to enhance uptake and modified release technology for site specific delivery. Critically, the roles of lipid excipients will therefore hold significant promise. To date, good entrapment, adequate protection (especially against enzymatic degradation), prolonged retention in GIT and enhanced permeation have been demonstrated employing lipid based nanocarriers. However, more mechanistic studies are needed to shed new insights on the understanding of the interplay between nanocarriers, peptides and physiological conditions in the intestine. It will also be helpful to create databases to summarise the existing data as well as to have uniformed model for both *in vitro* and *in vivo* tests. Meanwhile, the *in vitro - in vivo* bio-relevance must receive more attention and the adequate correlation could be crucial for the robust development of commercially viable products. From the formulation point of view, the combination of drug and vehicle oriented approaches, i.e. lipidization of peptide prior to encapsulation into the nanocarriers, maybe crucial. As new techniques develop, the behaviour of LBDDS in GIT is also subject to be triggered by external elements like force, heat, light, ultrasound and electro-magnetic field. Novel ideas in within the area of nanotechnology offer a bright future of LBDDS for oral peptide and protein delivery.

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Antecedentes, Hipótesis y Objetivos

Background, Hypothesis and Objectives

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Antecedentes

1. En los últimos años se han diseñado diferentes nanovehículos destinados a mejorar la absorción intestinal de los péptidos administrados por vía oral (1-3). Entre ellos, nuestro grupo fue pionero en el desarrollo de nanocápsulas (NCs) formuladas con polímeros cargados positivamente, tales como el quitosano, poniendo además de manifiesto la utilidad de las mismas para mejorar la absorción intestinal de péptidos (4-7). Por otro lado, nuestro grupo fue también el primero en dar a conocer el papel del PEG en la mejora de la estabilidad de las nanopartículas de ácido polilíctico-PEG (PLA-PEG) en las superficies mucosas y su transcendencia en la absorción oral o nasal de proteínas (8, 9).

2. La poliarginina (PARG) es un polímero catiónico que posee la capacidad de abrir las uniones intimas (TJ) intercelulares (10, 11), facilitando así el paso de macromoléculas hidrófilas a través de mucosas, tales como la nasal, ocular y pulmonar (12-17). Además, se ha reportado el hecho de que la inclusión de PARG en nanoparticulas permite mejorar la absorción pulmonar de fármacos (15). Asimismo, nuestro grupo de investigación, demostró por la primera vez la capacidad de las nanocápsulas de PARG para la liberación intracelular de moléculas bioactivas (18).

Hipótesis

Sería posible incrementar en mayor medida la ya conocida capacidad promotora de la absorción de la PARG al combinarla con otros promotores de carácter lipídico, por ejemplo, aceites y sales biliales. Además, partiendo de un diseño adecuado, será posible obtener nanocápsulas dotadas de una buena estabilidad en los fluidos intestinales y con la capacidad de interaccionar con el epitelio intestinal.

Una vez allí, los promotores de la absorción deben ayudar a la absorción del sistema o del péptido encapsulado, i.e. insulina.

Objetivos

Teniendo en cuenta los antecedentes y la hipótesis descrita anteriormente, esta tesis ha tenido como objetivo el diseño y desarrollo, en base a criterios racionales, dos nanotransportadores distintos para la administración oral de péptidos. Estos dos nanotransportadores presentan CPP como componente común. No obstante el resto de componentes así como su organización estructural y propiedades físicas son drásticamente diferentes. Por otro lado, se espera que tanto la caracterización sistemática de estos dos nanotransportadores, así como los estudios mecanísticos *in vitro* e *in vivo* contribuyan a comprender mejor los fenómenos de interacción de nanotransportadores con las barreras biológicas presentes en la vía oral.

Más detalladamente, desde un punto de vista experimental, se han llevado a cabo los siguientes estudios:

Nanocápsulas de PARG:

1. Diseño y desarrollo de nuevas formulaciones de PARG con un tamaño nanométrico, una distribución de tamaños estrecha, así como una adecuada estabilidad en fluidos biológicos simulados.

2. Determinación de la capacidad de las nanocápsulas para encapsular insulina y liberarla de manera controlada. Con este objetivo se estudiaron una serie de parámetros de formulation que resultaron clave lograr la encapsulación y liberación controlada del péptido.

3. Estudio de la toxicidad *in vitro* y mecanismo de interacción de estos nanosistemas con el epitelio intestinal (cultivos celulares de Caco-2 y epitelio intestinal humano). Evaluación de la capacidad de las nanocápsulas para promover la absorción y el transporte de la insulina. La capacidad como promotor de la adsorción del sistema se comparó con el de la PARG en solución.

4. Estudios *in vivo* orientados a comprender el mecanismo de interacción entre las NCs de PARG marcadas con fluorescencia y el epitelio intestinal después de su administración oral, así como a determinar la bioactividad de las nanocapsulas con insulina cargada mediante la inyección subcutánea y intra-intestinal a ratas sanas.

Nanocomplejos :

1. Diseño y desarrollo de nano-complejos de insulina y polimero A/polímero B con un tamaño nanométrico, una distribución de tamaños estrecha y una alta eficiencia de encapsulación de insulina.

2. Estudio de la estabilidad de los nano-complejos en fluidos intestinales simulados y en condiciones de almacenamiento..

3. Estudio de la toxicidad de los nanocomplejos desarrollado y de su mecanismo de interacción con el epitelio intestinal (cultivos celulares de Caco-2 y epitelio intestinal humano).

4. Evaluación de la capacidad de los nanocomplejos para promover la absorción y el transporte de la insulina en el cultivo celular Caco-2. La capacidad como promotor de la adsorción del sistema se comparó con respecto a la mezcla de polymero A y insulina.

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Background

1. A variety of nanocarriers have been explored for enhancing the intestinal absorption of orally administered peptides (1-3). Among them, our group pioneered the development of nanocapsules (NCs) made of positively charged polymers, i.e. chitosan, which were shown to enhance the intestinal absorption of peptides (4-7). On the other hand, our group was the first reporting the critical role of a polyethylene glycol (PEG) corona around polylactic acid (PLA) nanoparticles for enhancing their stability following either, oral or nasal administration (8, 9).

2. Polyarginine (PARG) is a polycationic polymer, which has been described for its capacity to open the intercellular tight junction (TJ) (10, 11), thereby facilitating the transmucosal delivery of macromolecules across the nasal, ocular and pulmonary epithelium in animals (12-17). Proteins like exendin-4 and rhG-CSF were delivered nasally with success (12, 16). In addition, nanoparticles containing PARG were reported to enhance pulmonary drug absorption (15). Finally, our group, reported for the first time the potential of PARG nanocapsules for the intracellular delivery of pharmaceuticals (18).

Hypothesis

It would be possible to further promote the known penetration enhancement capacity of PARG by combining it with other enhancers of lipidic character, i.e. oils and bile salts. Moreover, if adequately engineered, PARG NCs will have a good stability in the intestinal fluids and an ability to interact with the intestinal epithelium. Once there, the penetration enhancers may help the internalization of the nanocarrier and/or that of the associated peptide, i.e. insulin.

Objectives

Bearing in mind the background information and hypothesis described above, the objective of this thesis has been to rationally design and develop two different nanocarriers for the oral administration of peptides. The systematic characterization of these two different nanocarriers and the evaluation of their mechanistic behavior both, *in vitro* and *in vivo*, has been expected to contribute to the understanding of the interaction of nanocarriers with the biological barriers associated to the oral modality of administration.

More precisely, from an experimental viewpoint, the following activities have been undertaken:

*Polyarginine nanocapsules***:**

1. Development of new compositions of PARG with a nanometric size, a narrow size distribution, and an adequate stability in simulated biological fluids.

2. Determination of the capacity of the nanocapsules to load and control insulin release. For this, a study of the key factors that influence the entrapment of the peptide has been performed.

3. Study of the *in vitro* toxicity and mechanism of interaction of the nanocapsules with the intestinal epithelium (Caco-2 model cell line and human intestinal epithelium). For this, the capacity of the nanocapsules to enhance the permeability and the transport of insulin has been evaluated. This capacity was compared to that of the free PARG.

4. *In vivo* studies aimed at understanding the interaction of fluorescence-labelled PARG NCs with the intestinal epithelium after oral administration, and also at determining the bioactivity of insulin-loaded nanocapsules following subcutaneous and intra-intestinal injection to healthy rats.

Nanocomplexes:

1. Development of complexes of insulin and polymer A and polymer B with a nanometric size, a narrow size distribution, and high insulin association efficiency.

2. Study of the stability of the r colloidal stability of the nanocomplexes in simulated intestinal fluids and under storage condition, enhance their capacity to protect the entrapped peptide drug from enzymatic degradation, and to optimize their muco-interaction profile.

3. Study of the *in vitro* toxicity and mechanism of interaction of the nanocomplexes with the intestinal epithelium (Caco-2 model cell line and human intestinal epithelium).

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Chapter 2

Polyarginine Nanocapsules: a Potential Carrier for Oral Peptide Delivery

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Abstract

The aim of this work has been to rationally design and characterize a new type of nanocapsules composed of an oily core and a polyarginine (PARG) shell intended for oral peptide delivery. PARG, a cationic polyaminoacid, was selected based on its known penetration enhancing properties. After the screening of a number of formulation conditions, a composition containing oleic acid and sodium deoxycholate (SDC) as additional penetrating enhancers, was defined and characterized. The NCs, prepared by a mild solvent displacement technique, exhibited an average size of 180 nm, a low polydispersity (0.1) and high insulin association efficacy (AE). Another relevant feature of these NCs was their stability upon incubation in simulated intestinal fluids (SIF, FaSSIF-V2, FeSSIF-V2), in the presence of lipase and pancreatic enzymes. They were also stable during long-term storage (over 45 days). Moreover, studies on Caco-2 cells indicated that the NCs exhibit a concentration-dependent cytotoxicity, whereas no evident toxicity was found on human intestinal tissue. With regard to their mechanism of action, the results showed that PARG NCs led to a reversible reduction of the TEER of Caco-2 monolayers, which might be responsible for the observed facilitated transport of the associated insulin (3.54% as compared to 2.73%, observed for insulin mixed with PARG in solution). Finally, *in vivo* studies performed in rats showed that insulin bio-activity is not depraved during the NC preparation. Overall, this work shows that PARG NCs fulfill specific requirements that make them an attractive vehicle for oral peptide delivery.

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1. Introduction

Diabetes is a chronic disease that threatens the life of human beings. There are 382 million of diabetes sufferers worldwide today and an alarming number of 471 million is supposed to be reached by 2035 (1). Insulin has been widely used to treat this disease via subcutaneous injection. However, this route of administration is not the best option due to the pain and stress caused by repetitive injections. The oral route of administration is generally considered as the best alternative thanks to the good patient compliance and the physiological pathway followed by the peptide after intestinal absorption. Despite these clear advantages, the delivery of peptide molecules by the oral route represents a huge challenge due to the important biological barriers that the peptide molecules need to overcome. Firstly, the labile peptide should be able to resist the harsh environment of the GIT. Secondly, the peptide should cross the mucus layer and the underlying epithelium (2). As a consequence of these critical barriers the oral bioavailability of current peptides administered orally is below 1%.

The approaches investigated so far to improve the absorption of peptides administered orally, include the chemical modification of the peptide molecule, the co-administration of protectors or permeation enhancers and the formulation of adequate nanocarriers (3, 4). Nanocarriers such as polymeric nanoparticles, liposomes, micelles, solid lipid nanoparticles, microemulsions, self-emulsifying drug delivery systems, nanoemulsions and NCs have attracted much attention over the past years (5-14). Within this frame, our laboratory has contributed with the design of NCs consisting of an oily core surrounded a shell made of chitosan (7, 15). These NCs allowed us to encapsulate the peptide salmon calcitonin (sCT) and the resulting nanocomposition was found to facilitate and prolong sCT absorption following oral administration.

Taking into account this background information, the objective of this work was to engineer a NC-type of nanocarrier including the ingredients, and having the structural organization that would endow them with the adequate properties for overcoming the biological barriers associated to the oral modality of administration. Hence, the main criteria for this design was: (i) to be able to have an efficient encapsulation of the selected peptide, i.e. insulin; (ii) to be stable in the intestinal fluids, in the presence of enzymes and bile salts; (iii) to diffuse across the mucus layer and, (iv) to interact with the intestinal epithelium and to facility the transport of the associate peptide across it. Based on these criteria, we performed a performed a thoughtful analysis of the potential ingredients to form the lipid core, and selected oleic acid and SDC. These core components, are known as permeation enhancers that can promote intestinal absorption of insulin (16-20). Besides, SDC can form complexes with insulin during the formulation process, which can help improving its encapsulation of insulin (21, 22).On the other hand, we chose polyarginine (PARG) as a material to form the polymer shell, because of the known capacity of arginine to facilitate the insulin transport across the intestinal epithelium (23, 24). In addition, the incorporation of PARG in a variety of nanosystems, such as NCs and nanoparticles, has been recently reported to enhance the intracellular as well as transmucosal (pulmonary) delivery of drugs (25-27). Despite of this, no work has been reported so far on the use of PARG-based nanocarriers for oral peptide delivery. Finally, with the objective to facilitate the dispersion of the oily droplets and to improve the stability of the NCs in the intestinal fluids, we selected surfactants with different HLB values, i.e. poloxamer 188 and Span®80 (28). Once formulated, the physicochemical properties of these NCs and their capacity to promote the absorption of insulin in different *in vitro* and *in vivo* intestinal models have been evaluated.

2. Materials and methodology

2.1. Materials:

Recombinant human insulin hexamer Insuman® (Mw 5808 Da) was kindly provided by Sanofi (Paris, France). Poly-L-Arginine (Mw 26-37 kDa) was purchased from Polypeptide Therapeutic Solutions (PTS, Valencia, Spain). Analytical grade poloxamer 188, oleic acid, Span® 80, SDC and Triton™ X-100 were purchased from Sigma Aldrich (St. Louis, USA). Pharmaceutical poloxamer 188 was purchased from BASF (Ludwigshafen, Germany); pharmaceutical oleic acid and Span® 80 were purchased from Croda (Snaith, UK); pharmaceutical SDC was purchased from New Zealand Pharmaceuticals (Palmerston North, New Zealand). The pancreatin (8xUSP) was purchased from Biozym (Hamburg, Germany). The Sephadex G-50 was purchased from GE healthcare (Little Chalfont, UK). The 1,10-dioctadecyl-3,3,30,30-tetramethylindodicarbocyanine perchlorate fluorescent dye (DiD oil, Em 644nm; Ex 663nm) was obtained from Life Technologies (Eugene, USA). Human colorectal adenocarcinoma Caco-2 cells (ATCC® HTB37™) were purchased from American Type Culture Collection (Manassas, VA, USA). High glucose Dulbecco's modified eagle medium (DMEM) and non-essential amino acid (NEAA) solution were purchased from Sigma Aldrich (St. Louis, USA), while heat inactivated fetal bovine serum (FBS), penicillin-streptomycin solution, L-glutamine, phosphate-buffered saline (PBS), Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS) were purchased from Lonza (Basel, Switzerland). Reagents for cytotoxicity assays were MTS based CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, USA), LDH cytotoxicity detection kit plus (Roche, Mannheim, Germany) and Neutral Red based In Vitro Toxicology Assay Kit, (Sigma Aldrich, St. Louis, USA). Ultrapurified water was obtained from Millipore Milli-Q Plus water purification system (Darmstadt, Germany). The acids and bases are purchased from Scharlab (Barcelona, Spain). All other chemicals were of analytical grade.

2.2. Preparation of the PARG NCs

Polyarginine based NCs were prepared by modified solvent displacement technique previously utilized in our laboratory (29, 30). Different oils (oleic acid, Miglyol® 812N) and surfactants (Span®80, Tween®80, Labrasol®) at different concentrations were explored for the formation of the NC's core. Finally, as the optimum condition, insulin was dissolved in 0.01N HCl (pH~2.1) at concentration 15 mg/mL, and 0.1 mL of this solution were transferred to an organic phase composed by 62.5 µL oleic acid, 20 mg surfactant Span® 80, 4.1 mL acetone and 0.8 mL ethanol with 2.5mg SDC. This phase was mixed using a vortex agitator (VELP Scientifica, Usmate, Italy) and immediately poured onto 10 mL of ultrapure water or pH 5.5 acetate buffer. In both cases, the aqueous phase solution contained 0.05% (w/v) PARG and 0.25% (w/v) of poloxamer 188. When buffer is not used, the pH of the external aqueous phase was adjusted by 0.1N NaOH. Alternatively, 10mM, 20mM, 30mM and 50mM pH 5.5 acetate or citrate buffer was used to prepare the aqueous to ensure the desired final formulation pH (pH 5.5). After 10 mins under magnetic stirring, the solvents were evaporated under vacuum from 15 mL to 5 mL by rotavapor (Heidolph Hei-VAP Advantage, Schwabach, Germany). Oleic acid NEs, used as controls for some experiments, were prepared by the same method but with an aqueous phase without PARG. In case of the NCs used for fluorescent studies, 50 µg DiD was dissolved in the ethanol of the organic phase.

2.3. Physicochemical and morphological characterization of PARG NCs

Particle size distribution and PDI were determined by dynamic light scattering (DLS) and zeta potential was calculated from the electrophoretic mobility values determined by laser doppler anemometry (LDA). Both were obtained with Malvern Zeta-sizer device (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) equipped with a red laser light beam (λ=632.8 nm). To measure the particle size and PDI, a volume of 50 µL of the formulations was diluted with 950 µL of ultrapure water. For

the Z-potential measurements, the sample was diluted with 1mM KCl solution. The analysis was performed at 25 ℃ in at least, three different batches and each batch was analyzed in triplicate. The morphological analysis of the NCs was carried out in a transmission electron microscope (TEM, CM12, Philips, Netherlands). The samples were stained with phosphotungstic acid (2%, w/v) solution and placed on cupper grids with Formvard® for TEM observation.

2.4. Association of insulin to PARG NCs

The AE of insulin to PARG NCs was determined upon separation of the NCs from the suspending aqueous medium. The analysis was done by both indirect and direct method. Briefly, in the indirect method, 2mL of NC formulation was ultracentrifuged (Beckman Coulter, Optima L-90K, Brea, USA) at 82,656g for 1h at 15 ℃, and the amount of free insulin in the undernatant was determined using reverse phase HPLC (Agilent, 1100 Series, Santa Clara, USA) method. The phosphoric acid/ sodium perchlorate buffer was mixed with acetonitrile at different volume phase ratios, in order to produce two different mobile phases (93:7 as phase A and 43:57 as phase B), and C18 column (Superspher® RP-18 endcapped) was used as stationary phase. The AE of insulin in NCs was calculated according to the equation:

$$AE(\%) = \frac{Total\ insulin - Free\ insulin}{Total\ insulin} X100$$

Where *Total insulin* is the theoretical total insulin concentration in the formulation, and *Free insulin* is the insulin concentration determined by HPLC. Analysis was done in triplicate.

When using the direct method, 500 μL of NCs were isolated from the suspension medium by size exclusion chromatography using Sephadex-G50 column. The cream fractions and the transparent aqueous fractions were collected in microtubes. Insulin was extracted from the NC cream following the next steps: 1). 100 μL of the cream

was vortexed with 100 µL of acetonitrile for 2mins 2). 100 µL of Triton™ X-100 were added to the previous mixture and vortexed for 2mins; 3). 700 µL of 0.1% (v/v) of a trifluoroacetic acid (TFA) solution were added to the mixture and all the components were vortexed. Finally, both the broken NCs and the transparent aqueous fractions were analyzed by HPLC in order to determine the amount of insulin associated to the NCs and also in the suspending medium formulation. Simultaneously, 500µL of non-isolated NCs were degraded following the same procedure and the total insulin concentration was determined by HPLC, by which the gross mass of insulin in 500µL NC formulation was obtained. The AE of insulin in NCs was calculated dividing the amount determined in the isolated NC cream by the amount determined in the non-isolated NCs. Analysis was done in triplicate.

The final insulin loading (w/w) was calculated by dividing the amount of insulin associated (AE x total insulin in the formulation) by the total weight of the NCs. For the calculation of the total weight of the NCs, 500 µL of formulation was isolated by ultrafiltration using 100k Amicon® filters (Merck Millipore, Carrigtwohill, Ireland). The NC cream was re-suspended to 500 µL and lyophilized solely in microtube, which was weighed before adding NC sample and after freeze drying.

2.5. Stability of PARG NCs in simulated intestinal media

The colloidal stability of NCs in simulated intestinal fluids was evaluated analyzing the particle size and PDI by DLS. Additionally, particle concentration of the sample was monitored by the analysis of the light intensity count rate. To mimic the intestinal environment following oral delivery, SIF, fasted and fed state simulated intestinal fluids, FaSSIF-V2 (pH 6.5) and FeSSIF-V2, (pH 5.8) respectively, were prepared according to the composition shown in Table 1 (31). The study was carried out using 50 µL of NCs that were diluted in 950 µL simulated intestinal media, and then placed in an incubator at 37 ℃ (Heidolph Instruments GmbH & Co. KG, Schwabach,

Germany) with a horizontal shaking at speed 300rpm. At different time points (0 h, 0.5 h, 1 h, 2 h and 4 h), 50 µL samples were withdraw and the particle size, PDI and count rate were determined at the different time points using Malvern Zeta-sizer (Attenuator 6). Each analysis was performed in three different batches in triplicate. To quantify the effect of the pancreatin in the FeSSIF-V2 medium, a blank control of FeSSIF-V2 medium was used to exclude the interference of the pancreatin particles in suspension.

Composition	SIF	FaSSIF-V2	FeSSIF-V2
Sodium hydroxide	15.4mM	34.8mM	81.65mM
Monobasic potassium	50mM		
Sodium taurocholate		3mM	10mM
Lecithin		0.2mM	2mM
Maleic acid		19.12mM	55.02mM
Glyceryl monooleate			5mM
Sodium oleate			0.8mM
Sodium chloride		68.62mM	125.5mM
Pancreatin (8xUSP)			100Unit/mL
Calcium chloride			5mM
pH	6.8	6.5	5.8

Table 1. Composition of SIF, FaSSIF-V2 and FeSSIF-V2 media

Colloidal stability of the NCs under storage condition was analyzed as follows: different aliquots of the formulation were conserved at room temperature (about 20^{o} C). The particle size, PDI and count rate were monitored up to 45 days to check the potential destabilization of the formulation. The insulin remaining in the formulation was also determined.

2.6. *In vitro* release profile of insulin from PARG NCs

The in vitro release profile of insulin from PARG NCs was evaluated in both, SIF and FaSSIF-V2 media in three replicates. Specifically, 5 aliquots of 0.25mL of insulin-loaded NCs from the same batch were diluted with 1.25 mL of the desired intestinal media and placed in an incubator at 37ºC under horizontal shaking (300 rpm). After 0, 2, 3, 3.5 and 4 hours the different samples were ultracentrifuged (82,656g; 4 ℃; 1h) and the free insulin present in the undernatant was quantified by HPLC. Additionally, the insulin present in the NCs cream was evaluated by HPLC through the degradation of the cream with Triton™ X-100, acetonitrile and 0.1% TFA (same method as section 2.4), in this case, the released insulin was calculated by

2.7. Caco-2 cells culture

Caco-2 cells were grown in DMEM high glucose with L-glutamine supplemented with 10% heat inactivated fetal bovine serum, 1% Penicillin (100 U/mL), streptomycin (100 µg/mL), and 1% NEAA solution. Cells were maintained at 37 °C in a humidified incubator supplied with 5% CO_2.

2.8. Toxicity studies on Caco-2 cells

For cytotoxicity evaluation, Caco-2 cells were seeded in 96-well plates at the density of 10,000 viable cells/well, and incubated 24h to allow cell attachment. Cells were then incubated with increasing concentrations of the tested samples. The cytotoxicity of both blank and insulin loaded PARG NCs was determined by measuring metabolic activity with MTS assay and neutral red uptake (NRU). Lactate dehydrogenase (LDH)-based cytotoxicity assay was also used to measure LDH released into media from damaged cells as a biomarker for cellular cytotoxicity and cytolysis. In the study, the cell culture medium was replaced by the blank or insulin loaded PARG NC suspension in cell culture medium at concentration 0.1855, 0.371, 0.742, 1.484, 2.968,

4.452 and 5.936 mg/mL. The plate of Caco-2 monolayers were transferred to a humid incubator at 37 ℃ with 5% CO_2. After 2h incubation, the tested samples were removed. The cells were rinsed with PBS and incubated at 37 ℃ for 3h with 120 µL of fresh culture medium containing 20% MTS solution. Cellular supernatants were then transferred into a new 96-well plate and the amount of soluble formazan produced by cellular reduction of MTS was determined recording absorbance at 490 nm with Synergy 4 microplate reader (BioTek Instruments, Inc., Winooski, USA). Before performing MTS assay, 50 µL of cell culture media were transferred into a new 96-well plate, mixed with 50 µL of working reagent for LDH detection and incubated for 20 min at room temperature in the dark. Reaction was blocked by adding 25 µL of stop solution, and the amount of produced formazan was measured recording absorbance at 500 nm with Synergy 4 microplate reader. Cytotoxicity of PARG NCs was also evaluated by neutral red uptake (NRU) assay. Treated Caco-2 cells were rinsed with PBS and incubated for 3h at 37 ℃ with 100 µL of cell culture medium containing 10% Neutral Red solution. After incubation, medium was removed and cells rinsed twice with Dulbecco's PBS before adding Neutral Red Assay solution. Plate was shaken 45 minutes at room temperature, and absorbance recorded at 540 nm with Synergy 4 microplate reader.

The cytotoxicity of a control NE (without PARG shell) and also that of the PARG polymer solution was evaluated by NRU assay. In the study, the NE was tested at same concentration as the PARG NCs, while the PARG polymer solution was tested at concentration 0.009, 0.017, 0.034, 0.068, 0.137, 0.205 and 0.274 mg/mL, which are the amounts of PARG polymer involved in the tested PARG NCs concentration successively. The assays were repeated three times independently, each run as three independent technical replicates. Results are reported as a percentage of control and expressed as mean ± standard deviation. Data from *in vitro* testing were analyzed with dose–response sigmoidal fit function to estimate minimum effective concentration and EC50 values.

2.9. Interaction of PARG NCs with the Caco-2 cells monolayer

Entry of nanoparticles into Caco-2 cells was studied quantitatively by flow cytometry and qualitatively by confocal laser scanning microscopy (CLSM), for which DiD (λ_{em}= 644 nm) loaded nanoparticles were employed. For the flow cytometry study, Caco-2 cells were seeded in 24-well cell culture plates at a density of $5x10^5$ cells per well and allowed to adhere for 48h until confluency. Cells were co-incubated with 400 µL of a DiD loaded nanoparticles suspension in HBSS (0.371 mg/mL NCs). After 2h of incubation with fluorescent NCs, cells were washed three times with PBS and detached from the plates by trypsinization. Cells were then centrifuged at 1500g, the supernatant was discarded, the cells were resuspended in PBS and fluorescence was measured using a BD FACSVerse™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA, US). Cell fluorescence was quantified by measuring the fluorescence of DiD. For cell viability measurements, the propidium iodide reagent was employed. The reagent was added to each sample at a final concentration of 10 µg/mL, and, after 10min of incubation, the fluorescence corresponding to dead cells was measured at 620nm (FL2). For each sample, 10,000 events were collected. The data were subsequently analyzed using the FlowJo data analysis software package (TreeStar, USA). For the CLSM study, the Transwell® inserts fixed in PFA 4% were gently washed in HBSS. Actin was stained with 200 µL of alexa-phalloidine (1:50) in buffered HBSS+0.2% (v/v) Triton X-100 for 10 min in the dark to reveal cell borders, as described by des Rieux et al (32). Subsequently, inserts were washed in HBSS, cut and mounted on glass slides. Images were captured using a Zeiss™ confocal microscope (LSM 150). Data were analyzed by the Axio Vision software (vs 4.8) to obtain *y-z*, *x-z* and *x-y* views of the cells monolayers.

2.10. Toxicity and permeability on human intestinal tissues

Concerning the toxicity on human intestinal tissue, jejunal tissue samples were collected from patients undergoing laparoscopic Roux-en-Y gastric bypass. Patients

had given full informed consent. The study has been reviewed and approved by the regional ethical review board. Tissue samples were immediately transferred into a vessel containing cold, oxygenated Krebs-Ringer buffer and quickly transported to the laboratory. Arriving the epithelium was dissected away from sub-epithelial tissues and mounted in horizontal as well as vertically oriented Ussing chambers with 9 mm openings between the two chambers. The chambers were kept at 37 ℃ and bubbled with 95% O_2/5% CO_2 for the duration of the experiment. Electrophysiology of the tissue was monitored throughout the experiment to assure continued tissue viability. After mounting, the tissues were allowed to equilibrate for 40 minutes with two medium exchanges (35, 36). NCs (1mg/mL) were then added to the chambers. At the end of the experiment, continued viability of the tissues was tested by addition of the cAMP-agonist forskolin. Viable tissue with oxidative metabolism will form cAMP in response to forskolin leading to an opening of CFTR Cl channels, the response was monitored as changes in potential difference and short-circuit current over the epithelium. In respect of the NC transport on intestinal tissue, studies has been performed on human jejunal tissues using Ussing chamber models as described above. DiD labeled PARG NCs up to 3.5mg/mL were added to the chambers, and samples from donor and acceptor side chambers were taken at regular intervals for the 120 min duration of the experiment. Permeability of fluorescently labeled NCs were analyzed in a plate reader (33).

2.11. Measurement of the trans-epithelial resistance (TEER) and insulin transport across the Caco-2 monolayer

Caco-2 cell monolayers were cultured on tissue-cultured-treated PET filters (1 μm diameter, 1.1 cm^2, Millipore Transwell® 12 well/plate) and were used for experiments 21 days after seeding. The evaluation of PARG NCs was performed in Caco-2 cell monolayers with two NC concentrations: 0.5 mg/mL and 1 mg/mL. The variation in the TEER values for the cell monolayer integrity assessment was measured with a

Millicell-Electrical Resistance System (Endohm-12, Millipore Corp). Monolayers with a TEER values in the range of 800-1500 Ω cm^2 were used. Simultaneously, samples were collected (500 µL) from the receiver compartment and the apical compartment 2h after NC cell monolayer exposure and the insulin concentrations were measured using a LC/MS. Cell monolayers were gently washed with NaCl 0.9% and frozen at -80 ℃ for insulin quantification within the cells. Liquid chromatography (Shimadzu HPLC system LC 20AD) with a 150 x 2.1 mm - 5µm - 300Å HPLC C8 column (Interchim) was used for elution of insulin. The mobile phase A/B, where solvent A was H_2O containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid; the flow rate was 0.6mL/min to avoid pressure rise. 100 µL of tested sample was treated with 200 µL of chloroform / methanol / water at 1/ 1/ 0.3 and 100 µL of 0.1M NaOH, and then 40 µL of analyte was injected onto the column placed in an oven at 60 ℃. The total run time was 13min. Detection was done by tandem mass spectrometry (Quantum ultra) in positive electrospray mode. System control and data processing were carried out using MassLynx software version 4.1. Spray voltage was 3.0kV, and sheath and auxiliary gas pressures were 50 and 15 (arbitrary units), respectively. The in-source CID energy was fixed at 12V, and capillary temperature was 350 ℃. Tube lens and collision energy values were optimized for insulin. Multiple reaction monitoring was used for the detection of the ion transitions. The multiple reaction monitoring transitions for analytes were as follows: m/z insulin/hexameric 709.805 > 731.76, m/z bovine insulin 1284.73 > 1104.60. Analytes were quantified by means of calibration curves using bovine insulin as internal standard. The standard curves showed linearity for creatine over a range of 0.025 - 10 $\mu g \cdot mL^{-1}$ for insulin. The methodology for this assay involves reduction with dithiolthreitol 45mM and alkylation with 100mM of iodoacetamide 100mM of intact insulin for measurement of the free B chain.

2.12. *In vivo* fluorescence imaging of DiD-loaded PARG NCs

Two BALB/c mice were placed on a low manganese diet to reduce autofluorescence from normal mouse chow, and abdominal fur was removed by depilation where requested. One week later, 200µL of the DiD loaded PARG NCs (NC 20 mg/mL, DiD 10 µg/mL) were administered to mice by oral gavage. *In vivo* biodistribution was performed by total body scanning at different time points (0, 1, 3, 6, 24 hours) on isoflurane/oxygen anesthetized animals, using the MX2 scanner (ART, Montreal, Canada).

2.13. Bioactivity study of encapsulated insulin

All animal experiments were reviewed and approved by the ethics committee of the University of Santiago de Compostela (procedures Prof. Carlos di éguez, 1500AE / 12 / FUN01 / FIS02 / CDG3) of according to the European and Spanish regulations for the use of animals in animal studies; performed therefore in compliance with the Directive 2010/63 / EU of the European Parliament and Council of 22nd September 2010 on the protection of animals used for scientific purposes; Spain Royal Decree 1201/2005, of October 10th, on the protection of animals used for experimental and other scientific purposes and under the Royal Decree 296/2008 of Spain 30th December on the protection of animals used for experimental and other scientific purposes, including teaching. Male Sprague-Dawley rats (247-272g) were obtained from from the Central Animals House of the University of Santiago de Compostela (Spain). The animals were fasted for 4h prior to experiments, with free access to water, and kept conscious during the whole experiment. A dose of insulin loaded PARG NCs (1 IU/kg) in a volume–weight ratio of 250µL:250g was administered subcutaneously (n=8). As control, plain insulin solution was administered to the animals following the same procedure at the same dosage (n=8). Blood samples were collected from the tail vein 30min prior to the subcutaneous administration to establish the baseline blood glucose level. At time point of 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8 hours after

administration the blood samples were collected to monitor the glucose level change following the PARG NC or insulin administration. The glucose level was measured using a glucometer (Glucocard^{TM} G+ meter, Arkray Factory, Japan).

2.14. Intra-duodenal / jejunal administration

Male Sprague-Dawley rats (240-290g) were obtained from the Central Animals House of the University of Santiago de Compostela (Spain). The animals were fasted for 4h prior to experiments, with free access to water, and kept conscious during the whole experiment. PARG NCs were administered intra-duodenally or intro-jejunally to the rats at insulin dosage 50IU/kg body weight in a volume of 0.3mL through an intra-duodenal cannula operated 1 week before the experiment (n=8). As control, blank PARG NCs without insulin loading was administered to the animals following the same procedure (n=4). Additionally, an insulin solution in saline was subcutaneously injected at dose of 1 IU/kg body weight to a different group (n=8). Blood samples were collected from the tail vein 30min prior to the oral administration to establish the baseline blood glucose level. At time point of 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8h after dosing, the blood samples were collected to monitor the glucose level change following the PARG NC or insulin administration. The glucose level was measured by glucometer.

3. Results and discussion

As presented in the introduction, the main objective of this work was to design, develop and characterize a peptide nanocarrier with a potential to confront all the barriers associated to the oral modality of administration. Because of our previous experience and positive results obtained with chitosan NCs (7, 8), we decided to adopt this NCs technology and to engineer it in order to obtain a NC prototype fulfilling the requirements associated to the oral administration. The tailored properties were i) a

capacity to load insulin, ii) a nanometric size and a monodispersed population, iii) a neutral / negative zeta potential and a capacity to interact with the intestinal epithelium and facilitate the transport of the associated peptide.

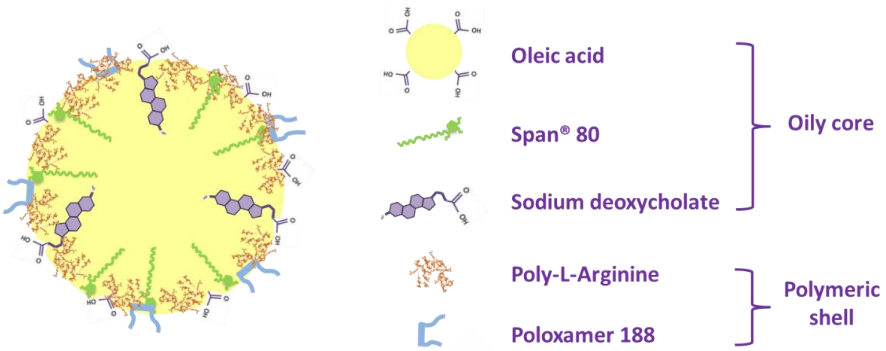


Figure 1. Structural illustration of PARG NC with the compositions.

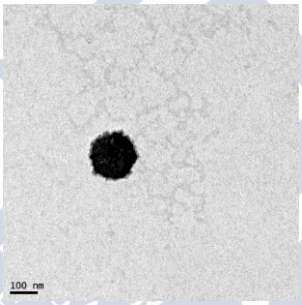


Figure 2. Transmission electron micrographs of the PARG NCs

3.1. Physicochemical characteristics of NCs and association efficiency of insulin

The schematic representation highlighting the components and the organization within the NCs is shown in Figure 1. The selection of these components and well as the appropriate concentrations of them were selected upon a thoughtful screening. Among the oils explored, oleic acid and Miglyol® 812N, the most commonly used one to formulate NCs (8, 9, 34-37), we selected oleic acid, because of the penetration enhancing effects of these long fatty acid chains as compared to the medium chains of Miglyol® 812N (38, 39). In addition, the important negative charge of oleic acid was supposed to facilitate the subsequent envelopment with cationic PARG. On the other hand, Span®80 was selected as surfactant among Span®80, Tween®80 and Labrasol®,

because it facilitate the attachment of PARG onto the NCs (confirmed by increased surface charge). The explanation can be that the PEG moieties in the other two emulsifiers saturated the NC surface area, which tremendously reduced the space for the interaction of PARG. Finally, SDC was selected as a co-surfactant because in addition to help reducing the NC particle size, it is known to form hydrophobic ionic pairs with insulin (21, 40), thereby facilitating its encapsulation.

The selection of the prototypes resulting from the above-indicated screening process was based on the evaluation of specific properties. With regard to the particle size distribution and zeta potential, it was found that using specific amounts of the ingredients illustrated in Figure 1, it was possible to obtain PARG NCs with a hydrodynamic mean size of 178 ± 20 nm, low PDI (0.11) and a negative Z-Potential of -23 ± 2 mV. The low negative zeta potential was attributed to a compensation of the positive charge of PARG with the negative charges associated to other ingredients, such as SDC and oleic acid, as well as to the shielding effect of polyethylene oxide-polypropylene oxide (poloxamer 188). This barrier was considered to be a positive feature because, firstly, it is expected to increase the stability of the NCs when interacting with intestinal lipids and enzymes (41), and secondly, it may also facilitate the diffusion of the NCs across the mucus. On the other hand, using TEM, it was found that the NCs have a size smaller than the one observed by DLS. This could be due to the shrinking of the NCs during the drying process. The images also showed that the NCs have a spherical shape (Figure 2).

In theory, the incorporation of insulin into the NCs is expected to depend on its electrostatic and hydrophobic interactions with various components of the NCs. Such interactions are obviously dependent on the solubility and ionization of insulin, both related to its isoelectric point (IP). In order to expose insulin molecules to different pH values, we adjusted the pH of insulin aqueous phase to different values and this

had an impact on the final pH of the formulations. As shown in Figure 3, when the final pH of the formulation was close to the insulin IP (IP=5.49), it was possible to reach an AE close to 80%. However, at pH values far away from this IP (either higher or lower), the insulin AE was clearly reduced (see Figure 3). The increased insulin entrapment at the pH close to its IP could be attributed to the predominance of hydrophobic interaction between the hydrophobic domains of this peptide with the components of the NC core.

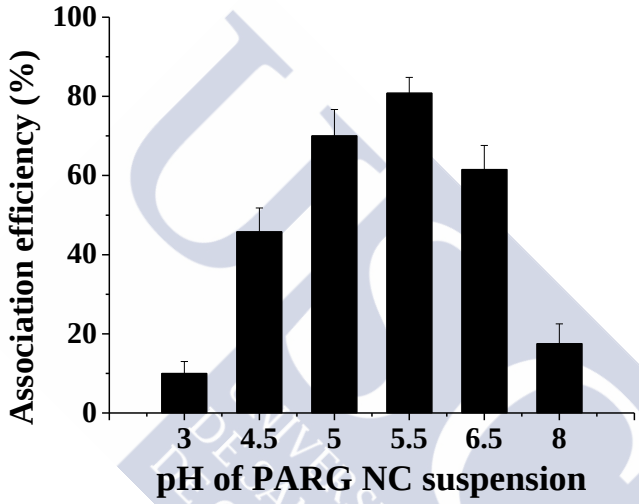


Figure 3. Influence of pH of the PARG NC suspension on insulin association efficiency. Mean ± S.D., n = 3.

Given the importance of pH on the insulin encapsulation process, we explored the inclusion of a buffer as the aqueous phase in order to ensure a final formulation pH close to 5.4. Among the different buffer systems investigated, we found that the use of 20 mM acetate buffer led to the formation of NCs with a size of 185 ± 6 nm, a low PDI and an AE of 88 ± 5% (Table 2, determined by both direct and indirect method described in methodology section). Taking into account that the production yield of the NCs is 74.21 ± 0.26% (w/w), the final insulin loading was 1.49% (w/w).

NC Formulation	pH (PARG solution)	pH (NCs suspension)	Size (nm)	PDI	Z-pot (mV)	AE (%)
Insulin-NCs	4.9	3-3.5	213±27	0.1	-3±4	<10
Insulin-NCs	10.8	5-6	178±20	0.1	-30±2	81±6
Insulin-NCs	5.5 (buffer)	5.2-5.4	185±6	0.2	-24±3	88±5
Blank NCs	10.8	5-6	189±5	0.1	-33±3	-
Blank NCs	5.5 (buffer)	5.2-5.4	177±5	0.1	-23±2	-

Table 2. Physicochemical properties of insulin-loaded PARG NCs and blank PARG NCs, showing influence of pH / salt content (acetate buffer) of external aqueous phase (PARG solution) on insulin association efficiency. Mean±S.D., $n \geq 3$.

3.2. Stability of PARG NCs in simulated biological media

Among other factors, the success of the formulation depends on its capacity to maintain intact its physicochemical properties under physiological conditions. Figure 4 shows the particle size and count rate evolution of insulin loaded-PARG NCs in SIF, FaSSIF-V2 and FeSSIF-V2 media during 4h. FaSSIF-V2 and FeSSIF-V2 are updated versions of the FaSSIF and FeSSIF media in the pharmacopeia to better mimic the in vivo fasted and fed state intestinal conditions (31, 42). Independent of the composition of the simulated intestinal media, PARG NCs displayed a quite stable size during the incubation time. In line with these results, the count rate, providing an indication of the number of particles in suspension, was hardly altered during the experiment (43). These results indicate that PARG NCs were stable in biologically relevant media containing enzymes and bile salts. These positive results have been attributed to the adequate protection of both the lipid components and the shell provided by the combination of Span®80 and poloxamer 188 at the oil/water interphase (41).

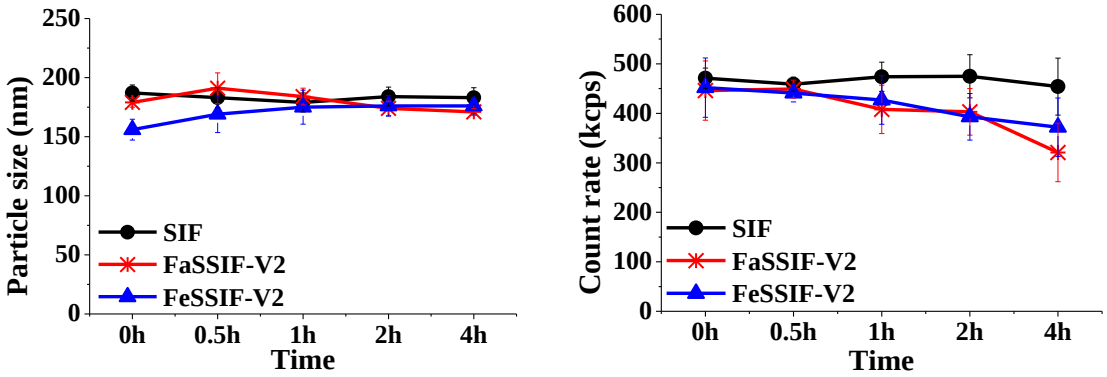


Figure 4. Evaluation of the particle size and count rate of insulin-loaded PARG NCs upon incubation in SIF, FaSSIF-V2 and FeSSIF-V2 media at 37 ℃. Attenuator of DLS fixed to 6. Mean±S.D., n = 3.

3.3. Colloidal stability of insulin-loaded PARG NCs during storage

Insulin-loaded PARG NCs were stored, as a suspension, at room temperature (~20 ℃). The particle size and count rate were monitored up to 45 days, and these PARG NCs remained stable over this period of time. In addition, insulin content in these NCs was determined at 0, 1, 3 and 15 days, and the insulin remaining was 98.5±1.8%, 93.3±3.9% and 82.2±0.9% of the initially detected amount at 1, 3 and 15 day respectively.

3.3. *In vitro* release of insulin from PARG NCs

The release of insulin from PARG NCs was studied in different simulated intestinal media, i.e. in SIF and FaSSIF-V2, which mimic the fasted state of the animals in the *in vivo* assay. The results obtained in SIF medium showed a negligible peptide release (data not shown). Similarly, the amount of insulin detected in FaSSIF-V2 medium, was very low and variable (between 10-20%) (Figure 5, dash line). In contrast, when the amount of insulin remained encapsulated was quantified, and from this, the amount of insulin released deduced (Figure 5, plain line), a continuous release profile was obtained. The differences observed in the release profile depending on whether insulin was determined directly in the release medium or indirectly, from the amount that remains encapsulated, could be attributed to the aggregation followed by

precipitation on the insulin released. The mechanism behind this release profile could be understood as follows. The pH (6.5) and the presence of bile salts and surfactants in the FaSSIF-V2 release medium were supposed to trigger the release of insulin, and this release process is supposed to be determined by the necessary insulin diffusion through and/or disassociation from the PARG/surfactants shell (10, 44, 45). As a consequence, a slow release profile, compatible with the targeted profile was attained.

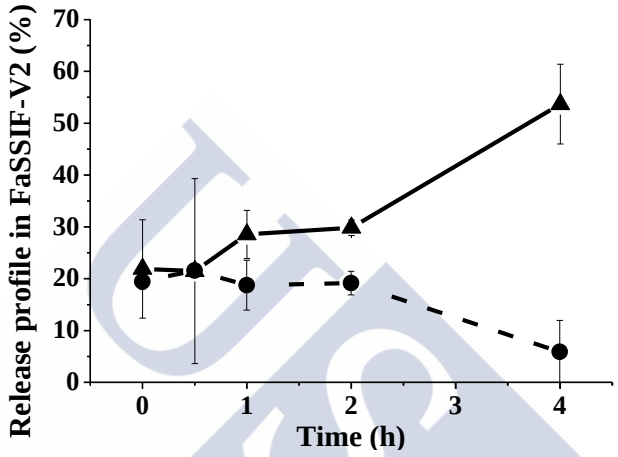
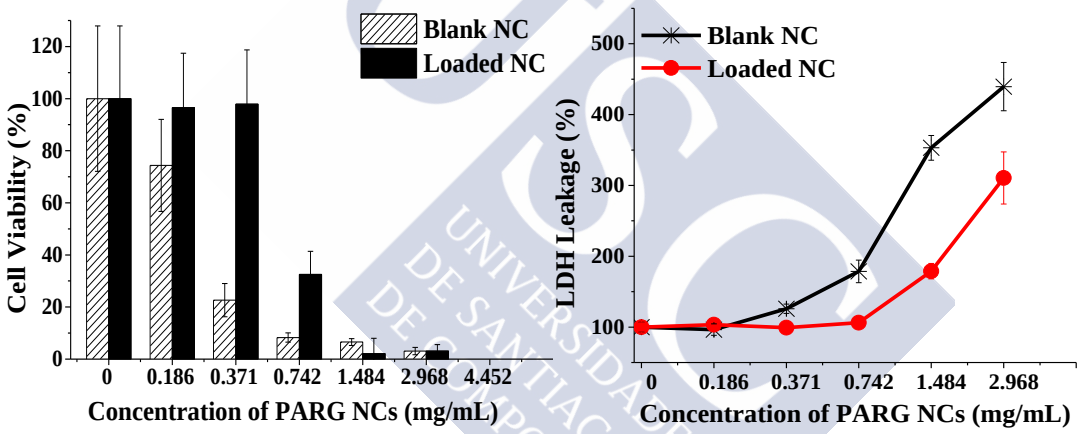


Figure 5. The *in vitro* insulin release profile of PARG NCs in FaSSIF-V2 medium: the insulin amount released to FaSSIF-V2 medium (straight line) and the insulin amount determined in FaSSIF-V2 medium (dash line).

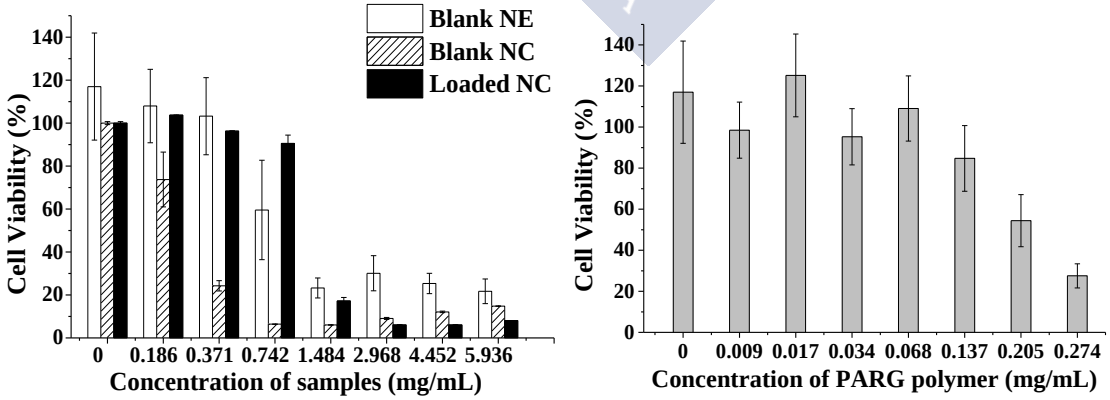
3.4. Toxicity of PARG NCs on Caco-2 cells and human intestinal tissue

The cytotoxicity of both, blank and insulin loaded PARG NCs was evaluated in the Caco-2 model cell line by MTS, LDH and NRU assay. Both blank and insulin loaded PARG NCs induced a dose-dependent toxicity after 2h incubation in MTS test (Figure 6A left). Interestingly, the toxicity of the loaded NCs was lower than that of the non-loaded ones. These results were confirmed by measuring the amount of LDH released from the damaged cells. (Figure 6A right). Finally, cellular viability of the loaded and un-loaded NCs was evaluated by NRU assay. The results showed that, as observed with the MTT and LDH tests, the toxicity of the loaded NCs was reduced as

compared to that observed for the blank ones. On the other hand, the toxicity of the control NE (having the same constituents as the NCs except PARG) was lower than that of the PARG NCs (Figure 6B left), corroborated by measuring directly the cytotoxicity of the PARG polymer alone. The results in Figure 6B right make evident that the toxicity of the polymer was superior to that of the nanoemulsion, however, considering the limited amount of PARG polymer in the NCs (0.045 mg/mL in 1 mg/mL NC), this led to the conclusion that the NE core was the main responsible for the cytotoxicity of the NCs. The toxicity of NE, free PARG and PARG NCs could be attributed to their interaction with the enterocytes and their penetration enhancing effect (39, 46). The reduced toxicity of the insulin-loaded PARG NCs, might be related to a different organization of the NCs shell due to the entrapment of insulin.



A



B

Figure 6. Cytotoxicity of the blank and insulin loaded PARG NCs and the control groups (Non PARG coated NE and PARG polymers) in Caco-2 cell line after 2h incubation. (A) Cytotoxicity of blank and insulin loaded PARG NCs as determined by MTS and LDH assays. (B) Cytotoxicity of blank, insulin loaded PARG NCs and control NE determined by NRU assay (left); cytotoxicity of PARG polymers determined by NRU assay (right) (Mean ± S.D., n=3).

The effects of the PARG NCs on the human jejunal tissue was also evaluated by both electrophysiology parameters and LDH release. Forskolin response was used to estimate the tissue physiological function (47). The results showed that no evident change in forskolin response was seen in the tissue incubated with PARG NCs (Figure 7). On the other hand, no significant LDH release occurred during 3h incubation in the Ussing chambers (data not shown). It can be concluded that up to 3.5 mg/mL of PARG NCs displayed no toxicity effect on the human jejunal tissue. The discrepancy in the toxicity values observed in Caco-2 cells and in human intestinal tissue could be related to the presence of mucus, which might reduce the accessibility of the NCs to the epithelium.

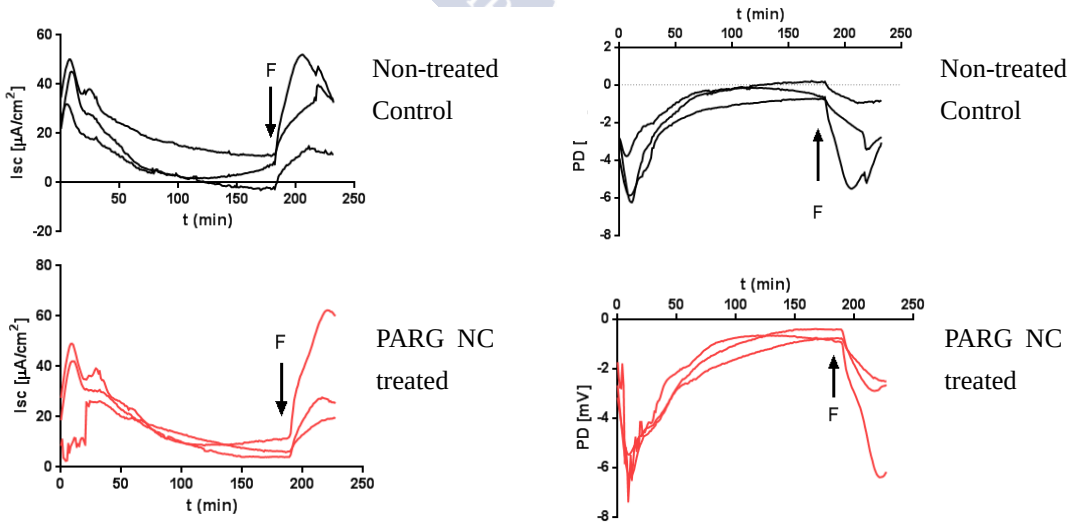
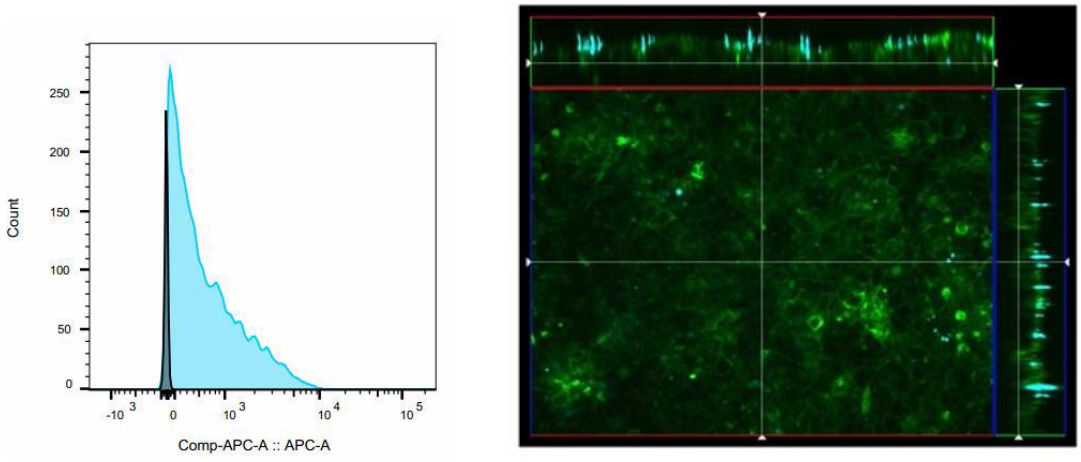


Figure 7. Forskolin response of human jejunal tissue after 3h incubation with PARG NCs at 3.5 mg/mL. F: addition of forskolin. n=3.

3.5. Interaction of PARG NCs with Caco-2 cells and human intestinal tissue

It has been reported that nanocarriers may interact with enterocytes according to different mechanisms: (i) they may adhere to the cells and offer a localized drug delivery, (ii) they may enter the cell membrane and deliver their cargo at the intracellular level and, (iii) they may get across the whole barrier carrying the cargo along (48). In theory, these three mechanisms may happen at once at a lower or higher extent. Due to the presence of the PARG shell, the NCs were expected to interact with intestinal epithelium. To confirm this hypothesis, PARG NCs labeled with DiD fluorescent dye (43) were added to the Caco-2 cells monolayer at a non-toxic concentration (0.371 mg/mL). Cell viability was assessed by staining dead cells with propidium iodide and alive cells were more than 90% in all cases, comparable to untreated control group. The results of the flow cytometry analysis (Figure 8A) show that the peak of the fluorescence-internalized cells (light blue peak) was not shifted from the non-fluorescent control cells (dark blue peak on the left) after 2 hours incubation with fluorescent PARG NCs, which means the fluorescence corresponding to DiD-labeled PARG NCs were mainly allocated on the surface of the cells (Figure 8A). These results were confirmed by confocal laser scanning microscopy (CLSM). The images presented in Figure 8B, indicate that DiD-labeled PARG NCs are mainly adhered to the cell surface rather than being internalized.

In a parallel study, the uptake of the PARG NCs was tested on human jejunal tissue mounted in Ussing chamber. Similar to the cell studies, no evident translocation of these NCs across the mucosa was observed at the tested NC concentration (up to 3.5 mg/mL, data not shown).



A B

Figure 8. Visualization of the interaction of DiD-labeled PARG NCs (0.371 mg/mL) with the Caco-2 cells monolayer after a 2h incubation time (n=3). (A) Flow cytometry profile showing the peak of DiD internalized cells (light blue) as compared to the non-fluorescent control cells (dark blue). (B) CLSM images (*y-z*, *x-y* and *x-z* sections) of the cell membranes upon staining with Alexa-phalloidine (green); DiD-labelled NCs are shown in blue color.

3.6.Effect on the transepithelial electrical resistance (TEER)

Some cationic polymers, among them chitosan (7, 15), are well-known for their capacity to open the TJs, thereby altering the trans-epithelial resistance and facilitating paracellular transport of drugs. A recent report has also claim this functionality for PARG (46). In this study, we evaluated the effect of PARG NCs on the TEER of the Caco-2 monolayer and used a PARG solution as a control. The results indicated that the TEER value of Caco-2 cells was not affected at PARG NC concentration of 0.5 mg/mL (data not shown). However, when the concentration was increased up to 1mg/mL, both, PARG NCs and the free PARG, induced a significant TEER decrease (15% and 38%, respectively), after a 2-hour incubation time (Figure 9). Interestingly, after removing PARG NCs, a significant TEER recovery was observed at 24 hour, thus evidencing the transient opening of the TJs. In contrast, this recovery of the TEER value was not observed upon exposure to the free polymer.

Overall, this study confirmed the capacity of PARG polymer to open the intercellular TJ (46), and showed that by the incorporation of this polymer to the shell of the PARG NCs it is possible to modulate the permeation enhancing capacity of the polymer. This might also suggest that the toxicity observed on the Caco-2 cells maybe transitory.

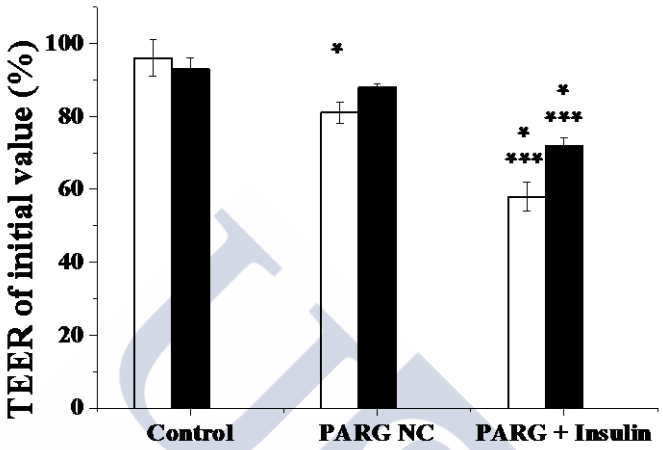


Figure 9. TEER assay on Caco-2 cell monolayers exposed to PARG NCs (1 mg/mL, containing 0.045mg/mL PARG and 0.014mg/mL insulin) or to PARG polymer (0.045 mg/mL) + insulin (0.014 mg/mL). ☐ 2h after exposure; ■ 24h after removal of the prototypes from the cells. Data expressed as mean ± SD, n=3. Changes were considered statistically significant at $P < 0.05$: *$p < 0.05$ compared to the control group; ***$p < 0.05$ compared to PARG NC group.

3.7. Capacity of PARG NCs to enhance the transport of insulin

As shown in previous sections, PARG NCs are able to adhere to the Caco-2 cell monolayer and alter in a transient manner the TEER. In a subsequent study, we studied whether or not these mechanistic details were translated into an enhanced insulin transport. The results showed that at upon a 2-hour incubation time, 3.54 ± 0.27% of insulin associated to the NCs was transferred to the basolateral compartment. This transport was significantly higher than the one observed for the physical mixture of PARG and insulin (2.73±0.32%) (Figure 10A). In addition, the amount of insulin detected inside the enterocytes was higher (1.29±0.53%) when the peptide was

administered associated to the NCs, with respect to the administration of the physical PARG-insulin mixture (0.67±0.08%, Figure 10B). Based on the observed adherence of the NCs to the monolayer and the transient changes in the TEER, it could be speculated that the enhanced insulin transport occurs by the paracellular pathway. Although the small amount (1.29 %) of insulin internalized in the monolayer also suggests the possibility of an enhanced intracellular uptake, as previously reported for octaarginine (49), it could be concluded that the dominant mechanism of transport is the one taking advantage of the paracellular route. In addition, the higher insulin transport achieved with PARG NCs, as compared to that achieved with the free PARG, could be explained by the co-localization of the peptide in association with PARG and other permeation enhancers present in the formulation. In fact, the oleic acid and bile salts present in the NCs core are known to increase the fluidity of cell membrane, and enhance the membrane permeability (16, 18, 39, 50-52).

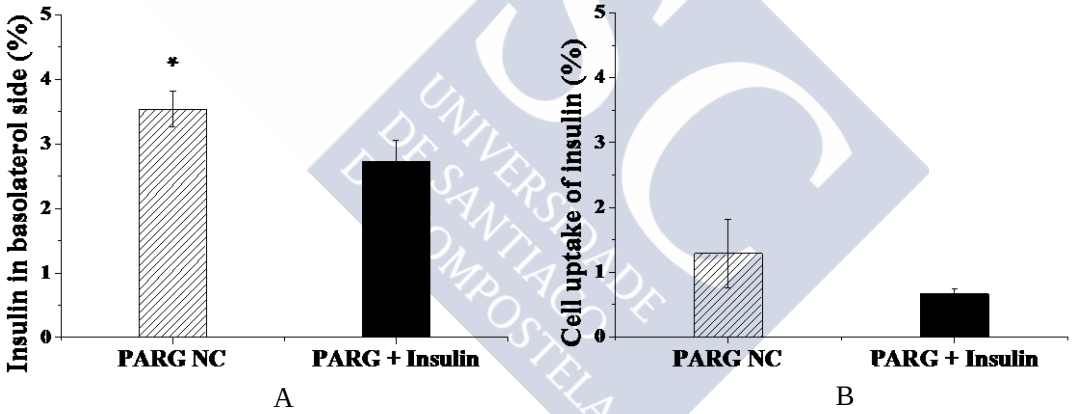


Figure 10. Apical to basolateral transport of insulin across the Caco-2 cell monolayer under 37 ℃, after 2h incubation with insulin loaded PARG NCs (1mg/mL, containing 0.045mg/mL PARG and 0.014mg/mL insulin) or PARG polymer (0.045 mg/mL) + insulin (0.014 mg/mL). Data expressed as mean ± SD, n=3. Changes are considered statistically significant at $p < 0.05$, evaluated by ANOVA following Tuke's multiple comparison post hoc test (SigmaPlot SyStat Software Inc, San Joes, CA).

3.8. *In vivo* fluorescence imaging of DiD-loaded PARG NCs

In order to have an *in vivo* preliminary estimation of the interaction of PARG NCs with the intestinal tract, we traced fluorescent DiD- labelled PARG NCs after oral administration to mice (Figure 11). The bioluminescent image on the left (acquired by the Optix Optiview^{TM}, ART, Montreal, Canada) shows the biodistribution of PARG NCs or free DiD dye following oral gavage to mice, and the histogram on the right shows the mean of total photons emitted from the regions of interest (ROI, the whole rat body). The images suggest that both, the PARG NCs and the free DiD dye, remain associated to the gastro-intestinal tract for up to 24 hours (Figure 11). The association of the free amphiphilic dye to the mucosa could be explained by its affinity for the cell's membrane. However, the analysis of the florescence intensity gave some preliminary evidence of the greater retention of the NCs as compared to the free dye. More detailed *in vivo* studies are needed in order to confirm this interaction of the NCs with the intestinal mucosa.

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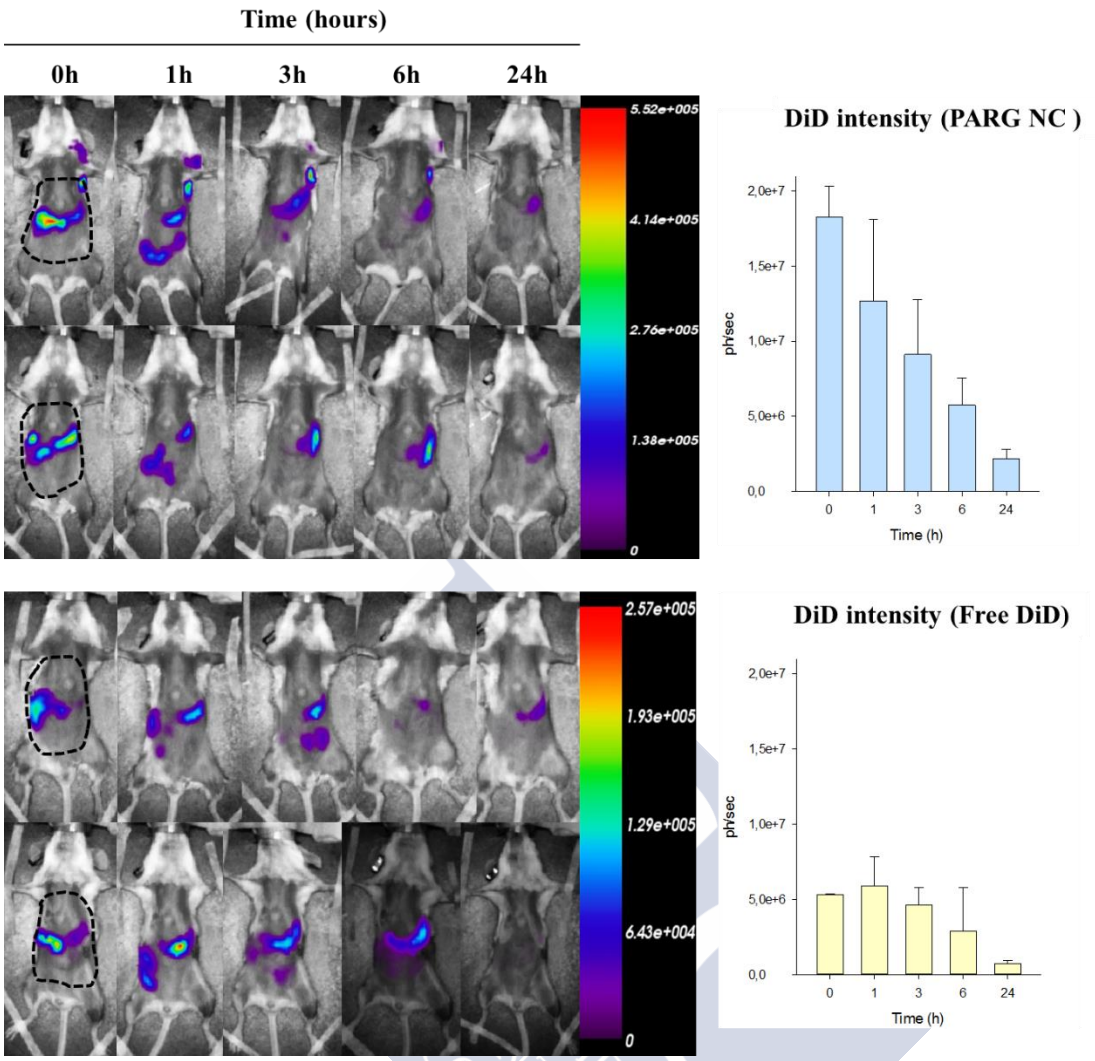

Figure 11: Fluorescence images (on the left) of representative mice at 0h, 1h, 3h, 6h and 24h following oral administration of (A) DiD labelled PARG NCs, (B) free DiD dye; and the mean of total photons ± SD emitted from regions of interest (ROI) around the GIT (on the right).

3.9. Bioactivity of encapsulated insulin and *in vivo* efficacy of PARG NCs

It is well known in the biotechnology field that the formulation process may result in the inactivation of labile macromolecules, such as peptides (53). Within this context, and before studying the *in vivo* efficacy of the oral formulation, we analyzed the bioactivity of the peptide following subcutaneous (s.c.) administration. Thus, insulin-loaded PARG NCs were administered subcutaneously to fasted (4h) healthy rats, using an insulin saline solution as control. The blood glucose level was

normalized taking the 0h mean glucose baseline value as 100%. As shown in Figure 12, following the s.c. injection, both, the insulin solution and insulin-loaded NCs, exhibited a very similar profile, where a drastic decrease in the glucose level was observed in 0.5h and the normal levels were recovered in 3h. From these results, it can be concluded that the mild conditions formulation process adopted for this system has not affected to the bioactivity of insulin.

Finally, the *in vivo* efficacy study was performed following either intra-duodenal or intra-jejunal administration (50IU/kg) to conscious healthy rats after 4h fasting (Figure 12). In both cases, the administration of insulin-loaded PARG NCs led to a slight blood glucose level decrease along the first 3 hours of the assay in comparison to blank PARG NCs controls. However, there were no significant differences in the responses observed for insulin-loaded NCs and the blank NCs. Overall, it could be concluded that the amount of insulin that reached blood circulation, after intra-duodenal or intra-jejunal administration (50 IU/kg), was much lower than the one achieved after sc. administration of 1 IU/kg insulin.

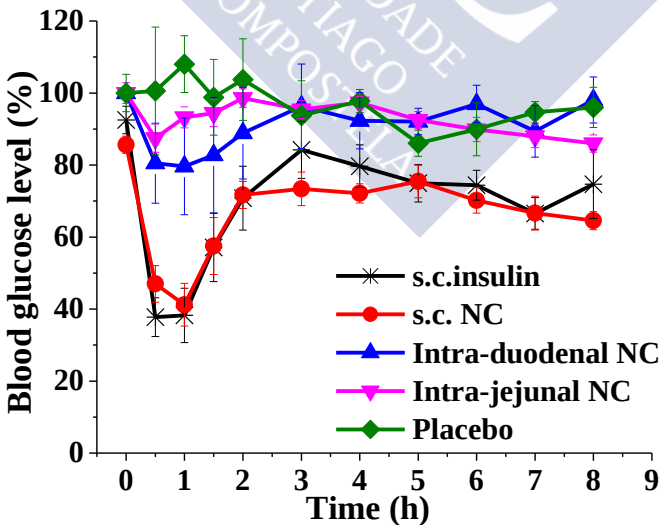

Figure 12. Standardized hypoglycemic effect in healthy rats following subcutaneous administration of insulin-loaded PARG NCs and insulin saline solution at 1 IU/kg, intra-duodenal administration of insulin-loaded PARG NCs at 50 IU/kg, intra-jejunal

administration of insulin-loaded PARG NCs at 50 IU/kg, and intra-jejunal administrated blank PARG NC as placebo. Data represents the mean ± S.E., n=8 for all the groups except for placebo (n=4).

This poor in vivo performance is somehow contradictory with the rational design of PARG NCs. In fact, the observed preservation of the insulin activity during the formulation process, the adequate stability in the intestinal media and the remarkable capacity of this formulation to promote the transport of insulin across a Caco-2 cells monolayer (Figure 9) would rather suggest a potential for this formulation to facilitate insulin oral absorption. A number of hypothesis have been formulated to explain the limited performance of the nanocarriers. First, the viscosity of the NC formulation, which might prevent the adequate mixing with the mucus fluids; second, despite of the controlled release properties of the formulation, there is the possibility that some enzyme (pancreatin) molecules may interact with the NCs and promote insulin degradation; third, although NCs may interact with the intestinal epithelium, it is possible that the interaction is insufficient in the in vivo situation; finally, even if the NCs interact with the epithelium, the associated insulin maybe retained and even degraded at the intracellular level. Finally, it is also possible that the in vitro assays and/ or the in vivo experimental conditions used in the reported studies have a limited predictive value of the performance of these formulations. Specific studies, i.e. mucodiffusion and quantification of insulin absorption are underway to validate these hypotheses.

From the studies performed so far we could conclude that the rational development of oral peptide delivery formulations and the translation of the *in vitro* data into the *in vivo* situation possess significant difficulties. However, the information reported here is supposed to help identifying the critical steps to be considered in the design of such formulations. Further work of PARG formulations aimed at enhancing the peptide

loading, as well as the incorporation of the NCs in a final dosage form (beads or capsules) might additionally help increase the performance of the formulation.

4. Conclusion

In this work we report the rational design of NCs consisting of an oily core made of penetration enhancers (oleic acid and SDC) and a polymer shell made of PARG, a polymer that is known for its ability to interact and increase the permeability of the cell membranes. The hypothesis for this design was that the combination of lipids and penetration enhancers in the form of nanostructures would reinforce the capacity of the penetration enhancers to increase insulin transport. Although this hypothesis was clearly validated *in vitro* using simulated intestinal fluids and the Caco-2 model cell line, the limited *in vivo* performance of the NCs suggest that further attention may have to be given to the final form of administration of the NCs. In the meanwhile, the validity of the tools used in the *in vitro* and *in vivo* screening, and in particular the predictive value of such experiments remain under question.

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Chapter 3

Polymer Nanocomplexes for Oral Insulin Delivery

This work is done in collaboration with: Aloïse Mabondzo [1], Josep Garcia [2], Patrik Lundquist [3], Meritxell Teixidó [2], Ernest Giralt [2], Per Artursson [3]

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Abstract

Polymer A similar structures have been explored as a way to enhance the transport of insulin across the intestinal epithelium. In this study, our goal has been to design and engineer a polymer A-based peptide nanocarrier endowed with the capacity to prevent insulin from degradation and facilitate its transport across the intestinal epithelium. For this, we used polymer A and polymer B to form nanocomplexes with insulin. The nanocomplexes exhibited an average particle size of 200 nm, a narrow size distribution (PDI 0.1), a negative (-44 ± 1mV) or neutral (+2 ± 2mV) zeta potential and a 100% insulin association efficiency (AE). As expected from our design, the nanocomplexes showed good colloidal stability and the capacity to effectively protect insulin from proteolysis in simulated intestinal fluids (SIF) with pancreatin. In addition, studies performed in Caco-2 cells indicated that the nanocomplexes led to 47.59% insulin cell uptake and 2.11% insulin transport to the basolateral side of the cell monolayer, whereas the physical mixture of a polymer analogue with insulin led to a negligible insulin transport. Finally, permeability studies across human intestine also showed that the nanocomplexes were capable of penetrating the mucus layer that cover the intestinal cells. Overall, these data show that the combination of polymer A with polymer B may represent a useful strategy for making feasible the oral administration of peptides.

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Confidential chapter

The content of this chapter was removed due to patent application.

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Overall discussion

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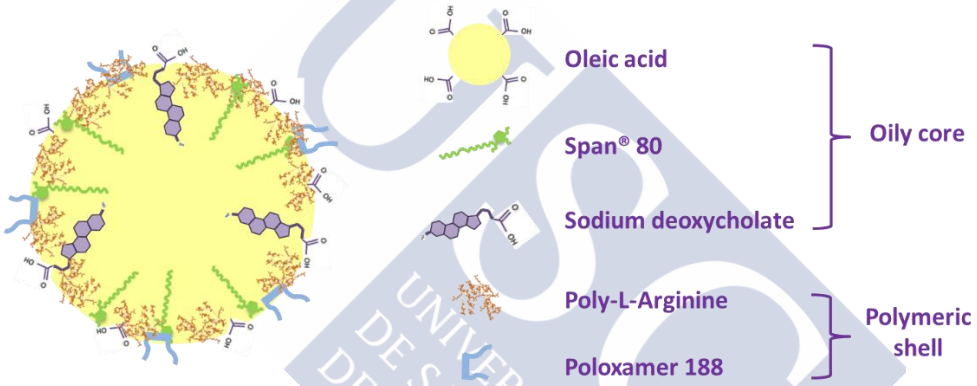
Overall discussion

The advances in the biopharmaceutical field along the past decades have allowed the production of therapeutic peptides on a large scale. The administration of these peptides should face several challenges such as *in vivo* instability, poor absorption and short half-life. As a result, most of these therapeutic peptides need to be administered by parenteral routes, a fact that has raised important concerns (1). The search for alternative routes of administration, and in particular, the exploration of the oral route has been an appealing option. The harsh GIT environment as well as the low permeability of most of the peptides have persuaded pharmaceutical technologist to design new platforms that allow the oral administration of peptides. As indicated in the introduction and review chapter 1, a significant number of nanocarriers including polymeric nanoparticles, liposomes, solid lipid nanoparticles, micelles, nano / microemulsions, nanocapsules (NCs), self - nano / micro - emulsifying drug delivery systems (SNEDDS / SMEDDS) and nano-conjugates are being explored as key enabling nanotechnologies of oral peptide delivery. Although none of these prototypes have reached the market yet, many of them have exhibited promising properties with regard to their utility as peptide delivery systems and some candidates are under clinical trials, i.e. the hepatic-directed vesicle insulin HDV-I (clinical phase II) and the silica-based nanoparticle Oshadi Icp (clinical phase I) for oral insulin delivery (2, 3).

In this study we have focus our efforts to the design and the *in vitro*/*in vivo* evaluation of two different nanostructures that might enable the oral administration of insulin: i) polyarginine (PARG) NCs and ii) polymer B/polymer A - insulin nanocomplexes. The rationale behind these two formulations is to promote the peptide transport over the intestinal membrane exploiting the peptides of these polymers. Additionally, it was assumed that the incorporation of this peptide into the nanostructure might offer the possibility to protect the encapsulated peptide and to facilitate the transport across the intestinal mucosa. It is important to highlight that the limited number of success in

clinical trials underlines the necessity to perform systematic studies in order to have a clear understanding on the influence of the nanosystem's properties on their interaction with the biological barriers. Bearing this in mind, we performed a comprehensive *in vitro* and *in vivo* characterization of the two designed family nanocarriers, aiming not only to improve insulin bioavailability after oral administration, but also to shed light on the relation-ship between the properties of these nanosystems and their in vivo performance.

1.1 Physicochemical / pharmaceutical characterization



A

PARG NCs (Figure 1) prepared by modified solvent displacement technique (4) exhibited particle size around 200 nm, narrow size distribution and negative superficial charge. Despite to the negative superficial charge of the system, the reduction of this parameter as compared with the one corresponding non-PARG coated nanoemulsion is justified by the incorporation of PARG onto the oily droplets. On the other hand, the entrapment of insulin in the formulation was dependent on the medium pH (Figure 2), reaching an optimal association when the pH of this final suspension medium was close to the insulin isoelectric point (IP = 5.4). At this pH the peptide shows a high hydrophobic character, and, thus could interact more easily with the inner lipid core. The highest insulin association efficiency 88% was obtained when using an aqueous phase at 20 mM pH 5.5 (20 mM acetate buffer), . The final

drug loading of this formulation was 1.48% (w/w) (Table 1).

Prototype	Size(nm)	PDI	ζ-pot (mV)	AE %	pH	Final loading (%)
PARG NC	185±6	0.2	-24±3	88 ±5	5.3-5.5	1.48
COMPLEX	236±27	0.1	+2 ±2	100	7.0-7.2	25.7

Table 1. Physicochemical properties of insulin-loaded into specific nanocarrier prototypes, which fulfilled the design requirements: Polyarginine nanocapsules (PARG NCs) and nanocomplexes. Mean±S.D., n > 3.

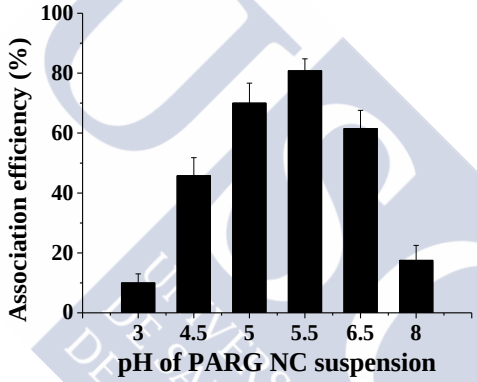


Figure 2. Major influential factors on insulin entrapment in the nanocapsules: pH of the formulation. Mean±S.D., n = 3

With regard to the stability of the designed prototypes in biological fluids, PARG NCs exhibited a good colloidal stability upon incubation in different intestinal simulated fluids (SIF, FaSSIF-V2 and FaSSIF-V2) at 37℃ for up to 4h. This is consistent with the fact that the selected components of the polymeric shell, are known to improve the colloidal stability of nanosystems (5, 6). Similarly, the nanocomplexes displayed a good colloidal stability in simulated intestinal conditions (SIF and FaSSIF-V2 media), thanks to the property of polymer B.

The nanocomplexes displayed good capacity to protect the entrapped insulin against

enzymatic degradation in 1% (w/v) pancreatin (8 USP) supplemented SIF media. Just after 15min of incubation, plain insulin solution suffered a tremendous degradation (7), whereas only 24.4% of the nanocomplexes insulin was degraded. After 2h of incubation in the proteolytic medium, there were still 25.3 ± 4.6% of insulin protected by the nanocomplexes. These interesting results showed the capability of the nanocomplexes to protect insulin from enzymatic attack in intestinal environment following oral administration.

The insulin release profile of PARG NCs in simulated fasted-state intestinal fluids (FaSSIF-V2) showed that this formulation presented an initial burst release of 22% of the encapsulated insulin, followed by a sustained release (up to 54%) during the following 4h (Figure 3). The mechanism behind could be understood as follows. The pH (6.5) and the presence of bile salts and surfactants in the FaSSIF-V2 release medium were supposed to trigger the release of insulin, and this release process is supposed to be determined by the necessary insulin diffusion through and/or disassociation from the PARG/surfactants shell. As a consequence, a slow release profile, compatible with the targeted profile was attained.

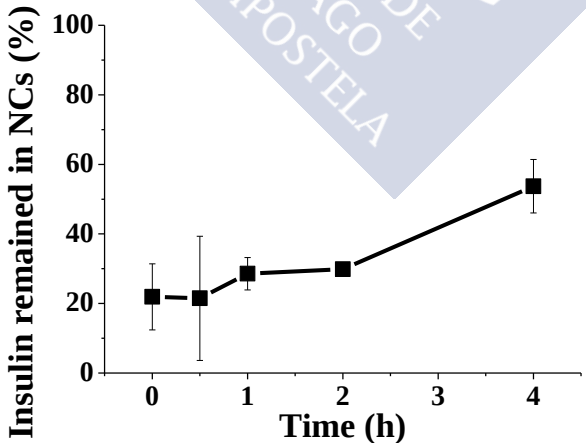


Figure 3. The insulin release profile from PARG NCs to FaSSIF-V2 medium up to 4h incubation.

On the other hand, both the nanocomplexes did not show any quantifiable insulin

release when incubated in SIF and FaSSIF-V2 media up to 6h. To understand the reason behind these results, another release study was performed in pH 4 and pH 5 acetate 100 mM buffers, where the ionic interaction between insulin and polymer A was minimized due to the protonation of insulin (pI~4.8). The absent insulin release in these buffers suggested that, apart from the electrostatic interaction, the hydrophobic interaction is also responsible for the restrained insulin release. More aggressive environment *in vivo* may trigger the insulin release from these nanostructure.

2. Interaction with the intestinal barriers

2.1 Mechanisms of cell transport

Both, PARG NCs and polymer nanocomplexes exhibited a concentration-dependent cytotoxicity profile on the Caco-2 cells monolayer. The insulin-loaded PARG NCs induced cytotoxicity from 0.742 mg/mL or 1.484 mg/mL, depending on different techniques (MTS or NRU/LDH). The nanocomplexes incurred cytotoxicity from 0.2 mg/mL. However, no toxicity was seen on intestinal tissues at the same nanocarrier concentrations or even higher (up to 3.5 mg/mL PARG NCs and 1 mg/mL nanocomplexes). These toxicity profiles were in line with the mechanism of action of these nanocarriers. The transport studies indicated that the nanocarriers were able to enhance the permeability of insulin on Caco-2 cells models (discussed later), however, the specific internalization routes promoted by these two nanocarriers are different.

A reduction in the TEER value of Caco-2 cells monolayer is normally related to the opening of the intercellular tight junctions (TJ). The PARG NCs and the PARG polymers induced respectively 15% and 38% significant TEER decrease 2h after exposure (Figure 4), suggesting that PARG NCs are able to open the TJ and hence to promote the insulin permeation by paracellular route. After removing PARG NCs, a significant TEER recovery was observed at 24 hour, while removing the free PARG did lead to a complete recovery of the TEER value. These results suggest that the toxicity observed for this prototype could be related to the increase in the permeability

of the cell monolayer. However, the fact that this effect is transitory, would highlight the potential value of this delivery carrier.

Opposite to what was observed for the nanocapsules, the TEER value of the Caco-2 cell monolayers was not modified after exposure to the nanocomplexes, this implying that the mechanism of action is other than that of the paracellular permeation. These results are in line with the results already published, where PARG can induce the transient internalization of TJ proteins to cells via clathrin-mediated endocytosis, leading to a transient increase of the paracellular permeability of the macromolecules on Caco-2 cell monolayer (8);

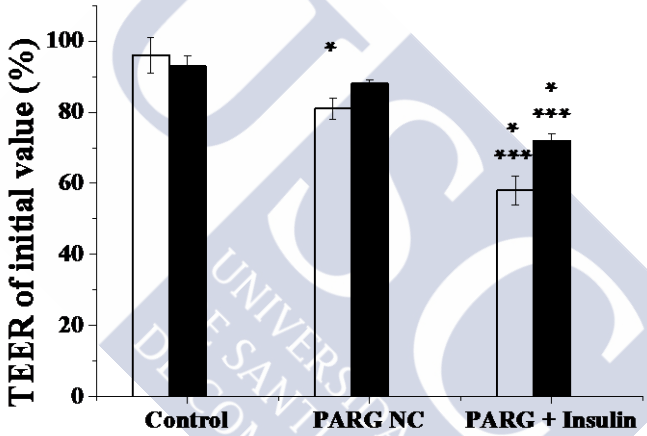


Figure 4. TEER assay on Caco-2 cell monolayers exposed to PARG NCs (1 mg/mL) or to PARG polymer (0.045 mg/mL) + insulin (0.014 mg/mL). ☐ 2h after exposure; ■ 24h after removal of the prototypes from the cells. Mean ± SD, n=3. Changes were considered statistically significant at $p < 0.05$: *$p < 0.05$ compared to control group; ***$p < 0.05$ compared to PARG NC group.

2.2. Quantitative cell uptake and transport of insulin

Following incubation of PARG NCs (1mg/mL of the NC formulation containing 0.014 mg/mL insulin, 0.045 mg/mL PARG) with the Caco-2 cells monolayer it was found that 1.29 % insulin was internalized into the cells (Figure 7A), a percentage that was higher than the one observed for the physical mixture of PARG and insulin at the

same concentration (0.67 %, data not shown). A much greater cellular internalization was observed when the monolayer was exposed to the nanocomplexes, this being 47.59 ± 5.79%. These results are in agreement with the expected properties of PARG and polymer A. The nanocomplexes showed much higher capacity to transport insulin via transcellular route than PARG NCs, the reason behind remains to be elucidated.

On the other hand, the analysis of the amount of insulin transported across the monolayer by the two different nanocarriers did not lead to drastic differences. Indeed, a in the case of PARG NCs, a 3.54 % of insulin was transported to the basolateral side of Caco-2 cells by a value higher than the amount of insulin internalized into the Caco-2 monolayer by PARG NCs. This results suggest that paracellular route is the dominant insulin transport way of PARG NCs. Moreover, the higher insulin transport achieved by PARG NCs as compared to that observed for the PARG-insulin physical mixture (2.73 %, data not shown) highlights the benefit of the combined use of permeation enhancers in the NCs.

In the case of the nanocomplexes, the results showed that a 2.11 ± 0.33% of insulin was ferried to the basolateral side, whereas the amount of insulin internalized by the cells was very high (47.59 %). In this study, it was also shown that both, particle size and surface charge of the drug carriers, played a relevant role in the peptide transport. Effect of the size on cell internalization was clearly shown by the null uptake (0 %) and the minimal concentration of insulin found in the basolateral compartment (0.6 %) when the physical mixture of polymer A analogue - insulin (micrometric clusters) was incubated with Caco-2 cell monolayers. This particle size dependent cell uptake is in agreement with the studies of other groups (11-13). On the other hand, the different surface charge of these nanocarriers made clear differences in insulin transport. Nanocomplexes formulated with polymer B internalized the 47.59 % of the insulin. Interestingly, independently on the cellular uptake, these nanocomplexes were the ones that led to the highest insulin transport across the monolayer, a result that could

be associated to their distinct intracellular fate

3. *In vivo* performance of the nanosystems

Prior to study the *in vivo* efficacy of the oral formulation, we assess the bioactivity of insulin following subcutaneous (s.c.) administration of the PARG NCs (1IU/kg) to 4h fasted healthy rats. As shown in Figure 6, the bioactivity of this labile peptide was well maintained along the mild conditions to formulate these PARG NCs.

Finally, the *in vivo* efficacy study was performed following either intra-duodenal or intra-jejunal administration (50IU/kg) to healthy rats after 4h fasting (Figure 5). In both cases, the administration of insulin-loaded PARG NCs led to a slight but non-significant blood glucose level decrease along the first 3 hours of the assay indicating a minimized absorption of insulin. Though this low absorption of insulin might be partially compensated by a reduced physiological insulin secretion (14, 15), a number of hypothesis have been formulated to explain the limited performance of the nanocarriers; *i)* the viscosity of the formulation may prevent the adequate mixing with the intestinal fluids and intestinal mucosa; *ii)* possibility that some enzymes may degrade the NCs or the sustained released insulin; *iii)* insufficient interaction of the nanocarriers and the absorption barriers in the *in vivo* situation; and finally *iv)* insulin retention or even degradation at the intracellular level.

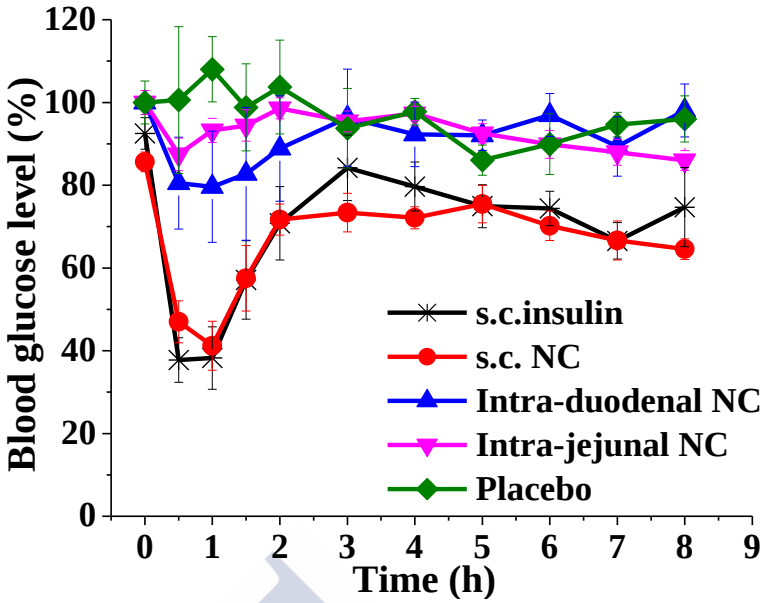


Figure 5. Standardized hypoglycemic effect in healthy rats following subcutaneous administration of insulin-loaded PARG NCs and insulin saline solution at 1 IU/kg, intra-duodenal administration of insulin-loaded PARG NCs at 50 IU/kg, intra-jejunal administration of insulin-loaded PARG NCs at 50 IU/kg, and intra-jejunal administrated blank PARG NC as placebo. Data represents the mean ± S.E., n=8 for all the groups except for placebo (n=4).

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Conclusiones - Conclusions

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Conclusiones

En esta tesis hemos diseñado dos tipos de nanosistemas: nanocápsulas de poliarginina (PARG NCs) constituidas por un núcleo oleoso rodeado por una capa de PARG, y nanocomplejos de polimero A/polímero B - insulina. Estos dos nanosistemas se han diseñado en base a criterios racionales, con el objetivo de valorar su adecuación para la administración oral de péptidos. Los resultados de este trabajo experimental han dado lugar a las siguientes conclusiones:

1. Las PARG NCs formuladas por la técnica de desplazamiento de disolvente presentaron un tamaño medio de 180 nm, una limitada polidispersion una carga superficial negativa y más de 80% de eficiencia de asociación de insulina. Aunque con una estructura muy diferente, los nanocomplejos envueltos mostraron características similares en términos de tamaño de partícula (200 nm), de distribución de tamaños (PDI 0,1), y de rendimiento de asociación de insulina (100%). Además de su distinta composición y estado físico (nanopartículas sólidas vs. nanocápsulas líquidas), y propiedades físicas (densidad, viscosidad) una diferencia importante entre los dos tipos de nanostructuras ha residido en su capacidad de carga de insulina (1,5% para las NCs y 25,7% para los nanocomplejos envueltos), y perfil de liberación de la insulina asociada.

2. Ambos tipos de nanovehículos, PARG NCs ynanocomplejos, presentaron una buena estabilidad coloidal tras la incubación en fluidos intestinales simulados que contenían enzimas. Además, las suspensiones de ambos nanovehículos fueron estables durante su almacenamiento a largo plazo (al menos 45 días para las NCs y 60 días para los nanocomplejos).

3. Los estudios realizados en cultivos celulares *in vitro* (modelo Caco-2) pusieron de manifiesto la capacidad de las nanocápsulas de PARG para reducir de forma

significativa, aunque transitoria, el valor de la TEER de las monocapas de Caco-2. Por el contrario, los nanocomplejosno alteraron significativamente la permeabilidad trans-epitelial. Independientemente de esto, ambos sistemas mostrado una gran capacidad para incrementar la internalización de insulina en los enterocitos (47,59% para nanocomplejos y 1,29% para PARG NC), así como el transporte de insulina a través de la monocapa celular (2,11% para nanocomplejos revueltos y 3,54% para PARG NC). Estos resultados sugieren que el mecanismo predominante para el transporte de insulina es transcelular o paracelular (PARG NCs) dependiendo del nanovehículos usado.

Aunque los datos preliminarios *in vivo* no nos permiten determinar la eficacia de las formulaciones desarrolladas, la conclusión general de esta tesis es que es posible diseñar nanotransportadores destinados a superar múltiples barreras biológicas, de una manera racional. Asimismo, de los resultados expuestos se puede concluir que el mecanismo de interacción entre los nanovehículos y el epitelio intestinal depende no sólo de sus propiedades fisicoquímicas, sino también e sus propiedades físicas (textura, viscosidad) y de la composición específica de los mismos. En este sentido, el diseño de nanoestructuras a partir de promotores de la absorción puede hacer factible la administración oral de péptidos.

Conclusions

In this thesis we have engineered two kinds of nanosystems, namely poly-arginine nanocapsules (PARG NCs) consisting of an oily core surrounded by a PARG shell, and polymer A/polymer B -insulin nanocomplexes. These two systems have been rationally designed to allow the oral administration of peptides and the results of the experimental work led to the following conclusions:

1. PARG NCs produced using solvent displacement technique had an average size of 180 nm, a narrow size distribution, negative surface charge and over 80% association efficiency of insulin. Although with a very different structure, the nanocomplexes showed similar properties in terms of particle size (200 nm,) particle size distribution (PDI 0.1), and insulin association efficiency (100%). In addition to their distinct composition, physical state (solid nanoparticles *vs.* liquid nanocapsules) and physical properties (density, viscosity), a major difference among the two delivery carriers relied on the final loading, this being 1.5% for the NCs and 25.7 % for the nanocomplexes, as well as their insulin release profile.

2. Both types of nanocarriers exhibited a good colloidal stability upon incubation in simulated intestinal fluids containing enzymes. Moreover, the suspensions were be stable upon storage for extended periods of time, at least 45 days for the NCs and 60 days for the nanocomplexes.

3. The *in vitro* cell culture studies indicated that PARG NCs induced a significant but transient reduction of the TEER of the Caco-2 monolayers, whereas the nanocomplexes did not significantly alter the trans-epithelial permeability. Irrespective of this, both systems exhibited the capacity to enhance the internalization of insulin into the enterocytes (47.59% for nanocomplexes and 1.29% for PARG NCs) and the insulin transport across the monolayer (2.11% for

nanocomplexes and 3.54% for PARG NCs). These results suggest a predominant transcellular (nanocomplexes) or paracellular (PARG NCs) mechanism of insulin transport for the two different nanocarriers.

Although the preliminary *in vivo* data do not allow us to establish the efficacy of the developed formulations, the overall conclusion of this thesis is that it is possible to rationally design nanocarriers intended to overcome multiple subsequent biological barriers, and that the mechanism of interaction of the nanocarriers with the intestinal epithelium will depend not only on their physicochemical properties, but also on their specific composition. In this respect, the design of nanostructured penetration enhancers may offer an array of possibilities towards making feasible the oral administration of peptides.

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