



**UNIVERSIDAD DE SANTIAGO DE COMPOSTELA**

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DEPARTMENT OF PHARMACY AND PHARMACEUTICAL TECHNOLOGY

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Doctoral Thesis

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Lungile Nomcebo Thwala

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**Santiago de Compostela, 2016**

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**Report:**

That the experimental dissertation entitled: "**Protamine nanocapsules as carriers for oral peptide delivery**" presented by **Lungile Nomcebo Thwala** was conducted under their supervision in the Department of Pharmacy and Pharmaceutical Technology at the University of Santiago de Compostela and in the Department of Pharmacy at the Université Catholique de Louvain, Belgium. 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 Brussels, March 29th and Santiago de Compostela, March 31th 2016.

| | | |
|---|---|---|
| Prof. María José Alonso Fernández | Dr. Noemi Stefania Csaba | Prof. Véronique Préat |

***For my angels…..my parents***

***“Simakadze ngumelusi wami, ngeke ngeswele lutfo”***

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# Abstract

The number of peptide drugs in the industry pipelines has significantly increased in the last decades due to their high therapeutic efficacy and excellent selectivity. However, their utility has been limited due to their difficulties for crossing mucosal barriers, making it virtually impossible to deliver them by the oral route. The aim of this work was to design and develop protamine-based nanocapsules (NCs), capable of encapsulating insulin, protecting it from the harsh intestinal environment and promoting its absorption across the epithelial wall. The NCs consisted of an oily core, containing Miglyol® and sodium glycocholate (SGC), surrounded by a polymer layer made of protamine and PEG-stearate. The NCs were also provided with a double protamine/polysialic acid (PSA) layer. These polymer layers were intended to enhance the stability against intestinal degrading enzymes and to facilitate the interaction of the NCs with the epithelial mucosa.

Protamine and PSA/protamine NCs exhibited a size between 300-400 nm, with a spherical shape and the capacity to load insulin. The provision of the NCs with a PSA layer reinforced their colloidal stability in different simulated intestinal media and preserved the loaded insulin from enzymatic degradation when incubated in fed-state simulated intestinal fluid. In addition, both formulations released insulin in a controlled manner. These NCs could also be lyophilized and stored as a thermostable powder formulation up to 6 months at 25 ℃, while preserving insulin stability.

The interaction of the protamine and PSA-protamine NCs with the Caco-2 cells was found to occur at the transcellular and paracellular level. The NCs were able to cross the monolayer and, simultaneously open the tight junctions between cells. Moreover, both systems interacted with the epithelium of human intestinal tissue without any cytotoxic effects. Finally, *in vivo* efficacy studies showed a moderate decrease (20% reduction) of blood glucose levels in non-diabetic rats. Overall, these results highlight the potential for protamine-based NCs to interact with intestinal mucosa and promote the absorption of the peptide across the intestinal epithelium.

# Resumen

El número de biofármacos presentes en las “pipelines” de las industrias farmacéuticas se han incrementado significativamente en las últimas décadas debido a su alta eficacia terapéutica y a su excelente selectividad. Sin embargo, su utilidad se ha visto limitada debido a las dificultades inherentes a dichas moléculas para cruzar barreras mucosas, imposibilitando de esta forma su administración por vía oral. El objetivo de este trabajo consiste en el desarrollo de nanocápsulas basadas en protamina (NCs), capaces de encapsular insulina, protegerla de la degradación en el desfavorable ambiente del tracto gastrointestinal y promover su absorción a través de la barrera epitelial. Las NCs están constituidas por un núcleo oleoso, formado por Miglyol® y sodio glicocolato (SGC), y rodeadas por una cubierta polimérica a base de protamina y PEG-estearato. Una doble cubierta con protamina/ácido polisiálico (PSA) ha sido explorada asimismo con el objetivo de aumentar la estabilidad frente a enzimas digestivas y facilitar la interacción de las NCs con la mucosa epitelial.

Ambas NCs, recubiertas con protamina o PSA-protamina, muestran un tamaño comprendido entre 300-400nm, una forma esférica y capacidad de incorporación de insulina. La presencia de la cubierta de PSA en las NCs refuerza su estabilidad coloidal en diferentes medios intestinales simulados y preserva la insulina incorporada en las nanoestructuras frente a la degradación enzimática cuando estas son incubadas con FeSSIF-V2. Adicionalmente, ambas formulaciones demuestran una liberación controlada de insulina y pueden ser liofilizadas y almacenadas como polvo seco durante al menos 6 meses a 25 ºC preservando la estabilidad de la insulina durante todo este periodo.

La evaluación de las NCs de protamina y PSA-protamina en células Caco-2 ha demostrado que la interacción de los nanosistemas con las células se produce a nivel transcelular y paracelular. Las NCs son capacers de cruzar la monocapa de células y simultáneamente abrir las uniones estrechas entre las células. Ambos sistemas interaccionan con el epitelio intestinal humano sin presentar efectos citotóxicos. Finalmente, estudios de eficacia in vivo han demostrado una reducción moderada (20% de reducción) de los niveles de glucosa en sangra en ratas no diabéticas. Estos resultados remarcan el potencial de estos nanositemas para interaccionar con la mucosa intestinal y promover la absorción de péptidos a través del epitelio intestinal.

# Resumen *in extenso*

## 1. Introducción

Según la Federación Internacional de Diabetes, 415 millones de personas en todo el mundo padecen diabetes y se espera que este número aumente hasta 642 millones en el año 2040 [1]. Se distinguen dos tipos principales de diabetes, denominadas tipo I y tipo II en función del origen y características de la enfermedad. Por un lado, la diabetes tipo I tiene su origen en una reacción autoinmune que provoca que el sistema de defensa del organismo ataque a las células beta pancreáticas productoras de insulina y como consecuencia, el organismo no produce la insulina necesaria. En el caso de la diabetes tipo II, el organismo es capaz de producir insulina, pero con el tiempo va desarrollando resistencia a esta hormona. A la larga, esta situación puede acabar desembocando en unos niveles de insulina insuficientes. Ambas causas, tanto la deficiencia de insulina como la resistencia a la misma, generan niveles elevados de glucosa en sangre, que generalmente son controlados mediante terapia de sustitución con insulina [2].

La insulina humana es una hormona (peso molecular de 5808 Daltons) compuesta por 51 aminoácidos organizados en dos cadenas polipeptídicas (A y B), unidas entre sí por puentes disulfuro. La estructura primaria de la insulina humana, con 21 aminoácidos en la cadena A y 30 en la cadena B, se representa en la **Figura 1A** [3]. Esta hormona juega un papel fundamental en la regulación de los niveles de glucosa en sangre. La elevación de la glucemia se produce como consecuencia de la absorción intestinal de glucosa a partir de la degradación de hidratos de carbono de la dieta, como el almidón o la sacarosa. Dicho aumento estimula la secreción de insulina del páncreas hacia el torrente sanguíneo y la actividad biológica de la misma se inicia cuando ésta se une a sus receptores celulares de membrana, desencadenando así distintas cascadas de activación proteica (**Figura 1B**). A través de ellas, esta hormona promueve la absorción, la utilización, el almacenamiento y el transporte de glucosa desde la sangre hacia las células de los tejidos-diana, como hígado, músculo esquelético o tejido adiposo (**Figura 1B**) [4].

La terapia actual, a base de insulina, es eficaz en cuanto a que consigue una reducción de la glucemia en pacientes diabéticos, pero su utilización se ve restringida debido a la necesidad de ser administrada mediante inyección. Además, la administración

parenteral de insulina evita su paso por el hígado introduciéndola directamente en el torrente sanguíneo, lo que a la larga puede provocar un estado de hiperinsulinemia asociada a hipertensión periférica, que finalmente puede traducirse en el desarrollo de aterosclerosis, hipoglucemia y otros efectos metabólicos adversos [5]. Asimismo, los pacientes diabéticos tienen que soportar múltiples inyecciones diarias, con los consiguientes efectos indeseados tales como dolor, invasión de tejidos, posibles infecciones y daños nerviosos, lo que conduce a una baja adherencia al tratamiento. Como consecuencia, el uso de la insulina inyectable es claramente inferior al deseable y se cree queel desarrollo de terapias alternativas, menos invasivas, ha de ser un objetivo prioritario en la innovación farmacéutica [6].

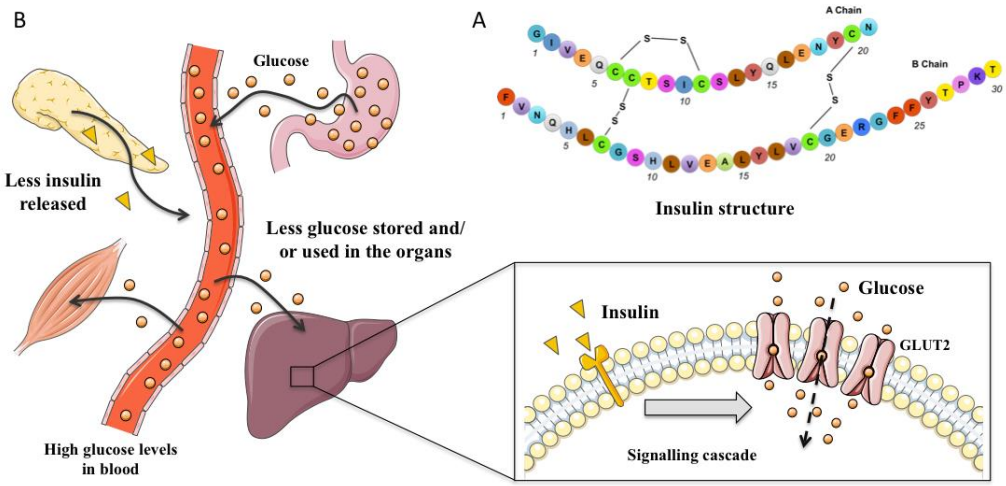


**Figura 1:** A) Secuencia de aminoácidos de la insulina humana, B) Ilustración esquemática de los efectos de la insuficiente producción de insulina en la diabetes tipo I; ilustración de la función fisiológica de la insulina en la absorción y metabolismo de la glucosa (la insulina se une a su receptor, iniciando diversas cascadas de activación proteica incluyendo la translocación de GLUT-2 hacia la membrana plasmática, influjo de glucosa y síntesis de glucógeno).

### *1.2 El reto de administrar insulina por vía oral*

A pesar de los grandes avances en la tecnología asociada aldesarrollo de formulaciones inyectables, se estima que más del 5% de la población mundial continúa teniendo fobia a estos dispositivos. Asimismo, de entre todas las vías de administración no invasivas (oral, nasal, pulmonar, bucal y transdérmica), la oral es la preferida. Por ello, el desarrollo de formulaciones de péptidos que permitan la administración oral de los mismos es muy importante ya que cuando se trata de una terapia crónica, la administración parenteral desemboca en una baja adherencia al tratamiento, reduciendo así su eficacia [7]. La administración oral de insulina permitiría, además, el acceso de este fármaco a la circulación portal, alcanzando el hígado antes de llegar a circulación sistémica, de forma similar a lo que ocurre con la insulina secretada fisiológicamente por el cuerpo humano [8].

Sin embargo, la administración de insulina por vía oral sigue siendo un gran reto debido a las dificultades inherentes al desarrollo de una formulación destinada a este propósito. Los fármacos administrados por vía oral deben soportar las condiciones agresivas del estómago, estando expuestos a la degradación por parte de ácidos y de enzimas. En el entorno gastrointestinal el fármaco queda expuesto a la degradación por enzimas como las proteasas pancreáticas, entre las que se incluyen la tripsina, α-quimotripsina, elastasa, exopeptidasas, carboxipeptidasas A y B. Los fármacos que resisten estas condiciones deben posteriormente atravesar la capa de mucus que recubre la superficie de absorción del tracto gastrointestinal (TGI) y pasar a través de las células epiteliales para lograr acceder al torrente sanguíneo. Como la insulina es una molécula peptídica, puede ser fácilmente degradada a su paso por el TGI debido a las condiciones hostiles del mismo, lo que conlleva que su biodisponibilidad oral se vea ampliamente disminuida. Además, su elevado peso molecular (sobre 6 kDa), su carga y su hidrofilicidad limitan su absorción por las vías paracelular y transcelular [9].

Se han explorado distintas estrategias para mejorar la biodisponibilidad oral de la insulina, entre las que se incluyen su modificación química, su co-administración con promotores de la absorción y/o inhibidores enzimáticos y su incorporación en sistemas de liberación [10]. En esta línea, la nanotecnología ha mostrado resultados prometedores en el diseño de nanotransportadores con potencial para la administración oral de péptidos, por lo que se espera que la investigación en este área

consiga mejorar sustancialmente el diagnóstico y tratamiento de la diabetes en el futuro [11].

### *1.3. Nanotransportadores para la administración oral de insulina*

La principal estrategia en el área de la nanotecnología para lograr la administración oral de péptidos y proteínas va dirigida a conseguir la encapsulación de los mismos en vehículos de tamaño nanométrico (10-1000 nm) que los protejan frente a la degradación, controlen su liberación en los tejidos diana y faciliten su transporte transepitelial. Los nanosistemas utilizados para el transporte de insulina incluyen nanopartículas, nanocápsulas, micelas y liposomas (**Figura 2**) [13], entre otros. Algunos de estos nanotransportadores han sido ampliamente estudiados para la administración oral de insulina, llegando incluso a ensayos clínicos, tema analizado en detalle en el **capítulo 1**.

Características como el tamaño, la carga superficial y la hidrofobicidad/hidrofilicidad de las partículas han sido identificadas como factores determinantes para lograr su internalización en las células del organismo [13, 14]. La modificación de estas propiedades de superficie puede conseguirse, bien a través de un recubrimiento de los nanosistemas con polímeros biodegradables hidrofílicos, o bien a través de la inclusión de surfactantes adicionales en la formulación. Por ejemplo, debido a sus propiedades estabilizantes, se ha utilizado ampliamente el polietilenglicol (PEG) como material de recubrimiento o como componente de la matriz en nanosistemas cargados con insulina. Esto se debe a que las cadenas de PEG forman una barrera estérica en la superficie de las nanopartículas que incrementa su estabilidad frente a proteasas, previene su opsonización y reduce su inmunogenicidad [15]. También ha sido demostrado que en algunos casos la combinación de ambas estrategias (el uso de polímeros y de surfactantes en el mismo sistema) podría llevar a una potenciación de sus propiedades y, consecuentemente, a un aumento de la biodisponibilidad oral del péptido encapsulado [7].

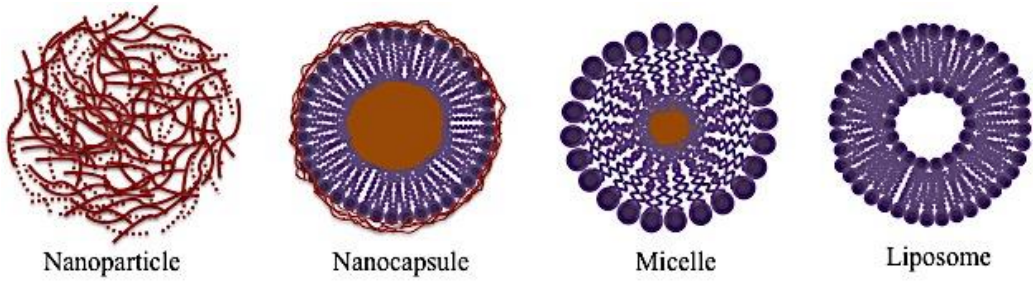


**Figura 2:** Diferentes tipos de nanotransportadores para liberación de insulina.

### *1.4 La protamina como biomaterial en el desarrollo de nanotransportadores de fármacos*

De entre los diferentes biomateriales explorados, los **poliaminoácidos** con capacidad de penetrar en las células constituyen una estrategia prometedora para la administración oral de insulina [16].Las principales razones son su capacidad para interactuar con estas moléculas, reducir su degradación enzimática y promover su absorción oral [17], propiedades que comparten con los péptidos de penetración celular (CPPs). Morishita *et al.* fueron pioneros a la hora de mostrar el potencial de las oligoargininas como CPPs, para mejorar la absorción de insulina a través de la pared intestinal [18] través de membranas mucosas, logrando así alcanzar niveles muy efectivos de biodisponibilidad [19]. La adsorción electrostática de la octarginina a los proteoglicanos presentes en la superficie celular y su subsecuente internalización han sido asociadas con su efecto promotor en la absorción de insulina en mucosas.

Por otro lado, la protamina es una proteína nuclear de pequeño tamaño rica en arginina, que juega un papel esencial en la condensación nuclear de los espermatozoides y en la estabilización del ADN [20]. Una vez combinada con la insulina, la protamina retrasa el inicio la actividad biológica de esta hormona, además de aumentar la duración de su actividad (ver insulina NPH). Debido a su capacidad de natural para condensar ácidos nucleicos, la protamina también es un biomaterial atractivo y ampliamente utilizado en aplicaciones de terapia génica [22], [23].

Nuestro grupo de investigación tiene experiencia en el empleo de biomateriales ricos en arginina, como poliarginina [24] y protamina [25] en el diseño de sistemas de liberación de fármacos con capacidad para transportar distintas moléculas terapéuticas. En particular, la protamina ha sido inicialmente utilizada en la preparación de

nanopartículas y nanocápsulas, demostrando gran potencial para interactuar con células y promover la internalización de antígenos [25]. Asimismo, nanopartículas multicapa (técnica "*layer-by-layer")* han sido también desarrolladas para la co-encapsulación y liberación de agentes inmunoestimulantes y antígenos, utilizando una combinación de poliarginina y protamina con polisacáridos como el sulfato de dextrano o el alginato [26]. Nuestro grupo también ha diseñado recientemente nanocápsulas de protamina con estructura lipídica de tipo núcleo-cubierta, con núcleo oleoso de vitamina E, para la encapsulación de curcumina como molécula anti-inflamatoria. Con este sistema se logró aumentar de forma significativa la permeabilidad de este fármaco en células Caco-2 [27]. En todos los casos, los efectos positivos observados con la utilización de protamina se atribuyen a la presencia de una secuencia repetitiva de arginina en su estructura (sobre el 70%) (**Figura 3**), la cual otorga a este polipéptido una eficiente capacidad de translocación a través de membranas biológicas, incluyendo las células epiteliales intestinales [28]. Se sabe que esta capacidad de translocación se produce principalmente por endocitosis, como consecuencia de la interacción entre las unidades de arginina cargadas positivamente y las cargas negativas presentes en la superficie de las membranas [29].

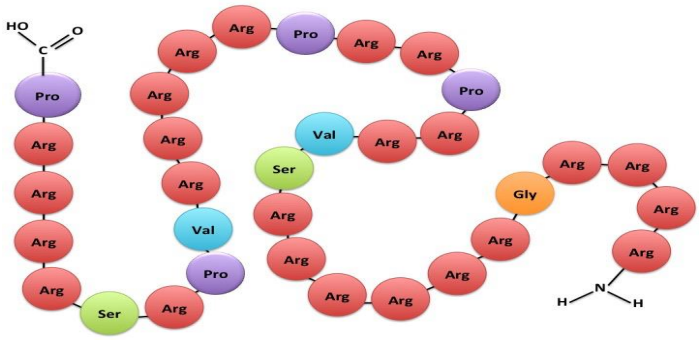


**Figura 3:** Representación esquemática de la estructura convencional de la protamina, conteniendo los aminoácidos prolina, arginina, serina, valina y glicina.

## 2. Hipótesis

En base a las evidencias aportadas por estudios anteriores, se han formulado las siguientes hipótesis:

1. La protamina es un biomaterial prometedor a la hora de desarrollar

nanocápsulas para una eficaz administración oral de macromoléculas como la insulina. La capacidad de este polipéptido para translocarse a través de membranas celulares podría verse potenciada al combinarlo con otros promotores de la penetración celular (lípidos y surfactantes), aumentando así la absorción intestinal de la insulina.

2. La combinación racional de protamina con polímeros estabilizadores y mucopenetrantes, como el poliácido siálico (PSA) y derivados del PEG, podría conducir al desarrollo de un nanosistema optimizado capaz de asociar el péptido, protegerlo de la degradación enzimática y transportarlo a través de la barrera epitelial.

## 3. Objetivos

El principal objetivo de este trabajo es el diseño racional y desarrollo de un nuevo nanosistema basado en la combinación de protamina, con otros biomateriales seleccionados para incrementar sus propiedades promotoras de la absorción y mejorar su estabilidad. Tomando la insulina como péptido modelo, el objetivo último de este nanosistema es conseguir una eficaz administración oral de péptidos. Con este fin, el objetivo global se divide en los siguientes objetivos específicos:

1. **Desarrollo y caracterización *in vitro* de nanocápsulas de protamina cargadas con insulina.**
2. **Evaluación de los mecanismos de acción *in vitro* y de la toxicidad de las nanocápsulas de protamina en células epiteliales.**
3. **Evaluación de la interacción de las nanocápsulas de protamina con la barrera epitelial, incluyendo su biodistribución y eficacia *in vivo*.**

## 4. Resultados y discusión

Las nanocápsulas de protamina fueron preparadas mediante la técnica de desplazamiento de disolvente [8]. Se incorporó ácido polisiálico (PSA) como cubierta externa a través de interacciones iónicas con la protamina, confirmando su presencia con la inversión de la carga superficial de los nanosistemas (**Tabla 1, Figura 4**). Las nanocápsulas de protamina, recubiertas o no con PSA, presentaron un tamaño de 300-400 nm y una buena capacidad para asociar insulina.

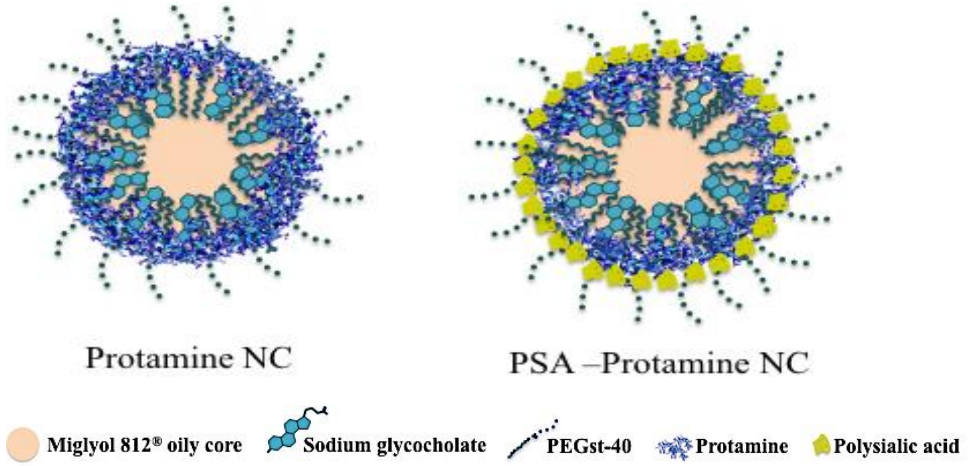


**Figura 4:** Representación esquemática de las nanocápsulas de protamina con y sin recubrimiento de ácido polisiálico (PSA).

**Tabla 1:** Propiedades fisicoquímicas, eficacia de asociación de insulina (AE%) y capacidad de carga (LC%) de las nanocápsulas de protamina en comparación con la nanoemulsion control (NE) (media ± SD, n=3).

| Formulación | Tamaño (nm) | PdI | ζ-pot (mV) | AE % | LC % (w/w) |
|---|---|---|---|---|---|
| NE | 345 ± 13 | 0.3 | -21 ± 1 | 35 ± 11 | 0.67 ± 0.03 |
| NCs Protamina | 382 ± 59 | 0.2 | +6 ± 3 | 62 ± 16 | 1.0 ± 0.06 |
| NCs PSA-Protamina | 301 ± 34 | 0.2 | -4 ± 1 | 51 ± 9 | 0.82 ± 0.01 |

Las nanocápsulas demostraron ser estables en medios intestinales simulados, especialmente las recubiertas con PSA. Además, para ambas formulaciones se observó un patrón de liberación de insulina bifásico en FaSSIF, con una liberación gradual durante la primera hora, seguida de una liberación constante hasta liberar aproximadamente un 70% de la insulina encapsulada tras las 6 h de estudio. La presencia de la cubierta externa de PSA reforzó la estabilidad de las nanocápsulas, protegiendo así a la insulina encapsulada de la degradación enzimática (**Figura 5**). Además, la inclusión de SGC, sal biliar conocida por inhibir la actividad proteasa, también mejoró la estabilidad de las formulaciones y contribuyó significativamente a la protección de la insulina frente a la degradación enzimática [11].

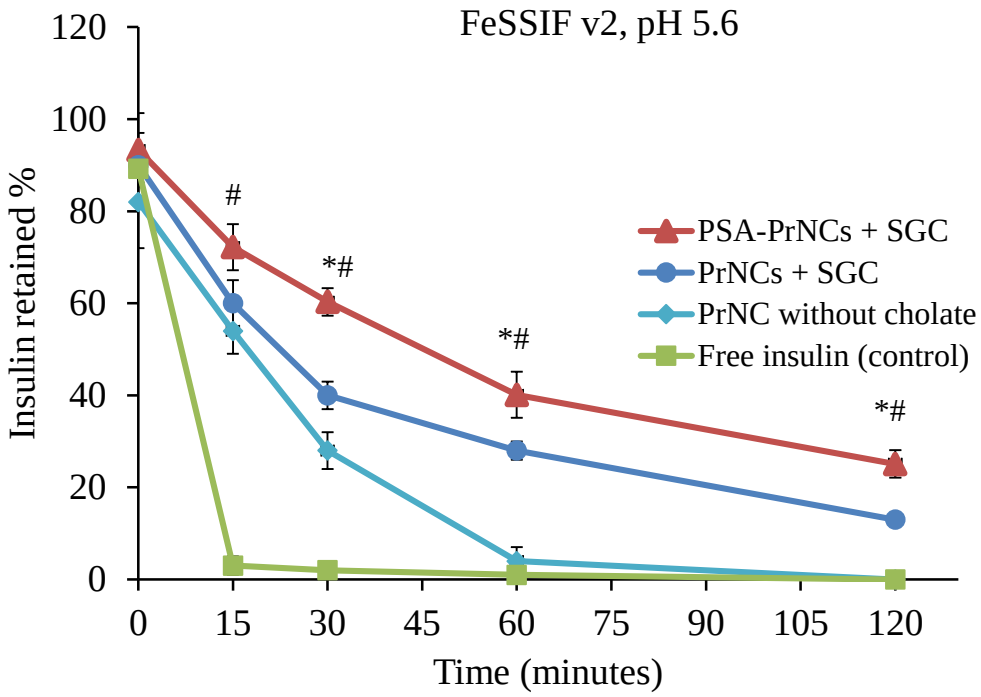


**Figura 5:** Perfil de estabilidad de la insulina encapsulada en los distintos nanosistemas tras la incubación en FeSSIF-V2, conteniendo enzimas pancreáticas (media ± SD, n=3 diferencia significativa ( $p < 0,05$ ) para PSA-PrNCs + SGC en comparación con *PSA-PrNCs + SC y #PrNC sin colato / insulina libre.

Ambas formulaciones demostraron no ser toxicas en células Caco-2 y en tejido intestinal humano a las concentraciones de 2 y 5 mg/mL respectivamente, confirmando la seguridad de estos nanosistemas para administración oral. Las células Caco-2:HT29-MTX sembradas en una relación 3:1 exhibieron una capa homogénea de mucus que no afectó a las propiedades promotoras de la absorción de estos nanosistemas (**Figura 6**).

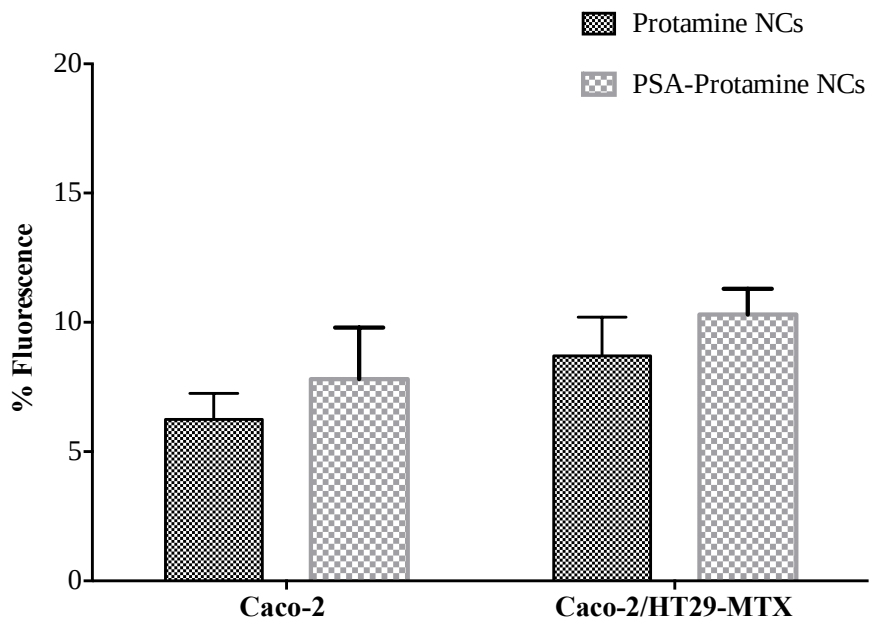


**Figura 6:** Transporte de nanocápsulas de protamina con y sin cubierta de PSA en monocapas de células Caco-2 y Caco-2/HT29-MTX tras 2 horas de incubación (media ± SD, n=3).

Finalmente, la eficacia *in vivo* de las formulaciones desarrolladas fue evaluada mediante la administración intraduodenal (50 IU/kg) a ratas sanas, tras 4 horas de ayuno [18], [30], [31]. Los resultados relativos a los niveles de glucosa en sangre mostraron un descenso moderado pero significativo de los mismos (≈20 %), el cual se mantuvo durante el todo estudio (**Figura 7**). Esta respuesta moderada si la comparamos con otros estudios publicados en la literatura, se atribuyó al modelo animal utilizado (ratas no diabéticas), al corto periodo de ayuno (4 h) y a la elevada viscosidad de la formulación administrada que puede dificultar la miscibilidad de las nanocápsulas con los fluidos intestinales impidiendo, por lo tanto, su absorción.

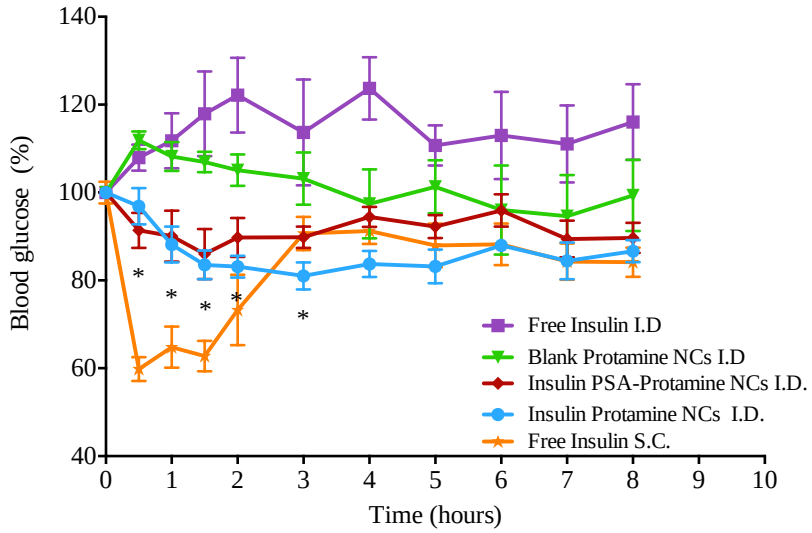


**Figura 7:** Efecto hipoglucémico estandarizado tras la administración intraduodenal de nanocápsulas de protamina cargadas con insulina (50IU/kg, n=11), nanocápsulas de protamina recubiertas de PSA y cargadas con insulina (50IU/kg, n=15), nanocápsulas de protamina vacías (placebo) (n=12) a la misma concentración y solución salina de insulina (50IU/kg, n=16) a ratas no diabéticas. (Media ± SEM, *p < 0.05 nivel de significación al comparar nanocápsulas de protamina y nanocápsulas de protamina recubiertas de PSA con el placebo).

## 5. Conclusiones

El presente trabajo de tesis se ha centrado en el diseño y desarrollo de un nanosistema a base de protamina, con estructura formada por un núcleo oleoso y una cubierta polimérica, para una eficiente liberación oral de insulina (y otras macromoléculas similares). Los resultados obtenidos del trabajo experimental han permitido extraer las siguientes conclusiones:

1. Nanocápsulas (NCs) con una sola cubierta de protamina o una doble de protamina y ácido polisiálico (PSA) fueron eficazmente preparadas usando el método de desplazamiento del solvente. Estas nanocápsulas, protamina y PSA/protamina, presentaron una buena capacidad para encapsular insulina (62 y 51 %) y un tamaño nanométrico (382 y 301 nm). Ambos sistemas resultaron en poblaciones homogéneas de forma esférica y con una carga superficial cercana a la neutralidad.

2. Las nanocápsulas de protamina y protamina/PSA demostraron ser estables en medio intestinal simulado protegiendo a la insulina encapsulada frente a la degradación enzimática. La cubierta extra de PSA junto con la presencia de la sal biliar glicocolato de sodio como surfactante (SGC), incrementaron la estabilidad del nanosistema y minimizaron la degradación enzimática de la insulina encapsulada.

3. Los estudios in vitro fueron llevados a cabo en diferentes modelos celulares que simulan el epitelio intestinal, poniendo de manifiesto que las nanocápsulas de protamina y protamina/PSA exhiben una baja toxicidad celular. Además, estos estudios indican que ambos tipos de NCs interaccionan con la monocapa de células principalmente mediante dos mecanismos, que posiblemente sean responsables tanto del transporte transcelular como paracelular del péptido asociado.

4. Estudios in vivo realizados en ratas no diabéticas confirman que las NCs son capaces de preservar la insulina encapsulada en la formulación, mantener su actividad biológica y minimizar su degradación enzimática. Ambas formulaciones han sido capaces de promover la absorción de insulina e inducir un descenso moderado (20%) de los niveles iniciales de glucosa en sangre después de una administración intra-intestinal.

5. Los nanosistemas desarrollados en este estudio fueron liofilizados a polvo seco, mostrando una estabilidad durante almacenamiento y a temperatura ambiente (25 ºC) así como preservando la insulina asociada durante al menos 6 meses.

En términos generales, el trabajo presentado en esta tesis plantea a las nanocápsulas a base de protamina como potenciales nanosistemas para la administración oral de péptidos. La extensiva caracterización in vitro de estas NCs ponen de manifiesto su estabilidad tanto en fluido intestinal como durante almacenamiento, propiedades ambas de mucha relevancia para el uso de estos nanosistemas en la administración oral. Por otra parte, es evidente que las NCs pueden modularse y adaptarse para mejorar la estabilidad y el perfil de liberación del péptido incluido. El perfil de toxicidad, tanto in vitro como en tejido intestinal humano, junto con el efecto de aumento de la penetración son propiedades igualmente interesantes de estos nanosistemas. Si bien los estudios de eficacia in vivo han mostrado una modesta variación en los niveles de glucosa en sangre, los datos presentados en este trabajo son alentadores, considerando que un mejor desarrollo de modelos animales y procedimientos experimentales realizados podrán proporcionar una respuesta más perceptible.

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# INTRODUCTION

## Oral nanomedicines for effective insulin delivery

## 1. Diabetes therapy

Diabetes mellitus (DM) is a chronic condition that occurs when the body cannot produce enough insulin or cannot use insulin. DM is considered a metabolic disorder since reduced or inactive insulin results in imbalanced food metabolism, characterized by the accumulation of glucose in the blood (known as hyperglycaemia). The resulting high levels of glucose in the blood causes damage to many tissues in the body, and with progression of the disease pathological changes like nephropathy, retinopathy and cardiovascular complications start occurring in the body [1, 2].

According to the International Diabetes Federation, 415 million people worldwide are diagnosed with diabetes and this number is expected to rise to 642 million by 2040 [1]. There are two main types of diabetes: type I and type II. Type 1 diabetes is caused by an autoimmune reaction, in which the body's defense system attacks the insulin-producing beta cells in the pancreas. As a result, the body does not produce the insulin it needs. In type II diabetes, the body is able to produce insulin but develops resistance over time so that insulin levels may subsequently become insufficient. Both the insulin resistance and deficiency lead to high blood glucose levels. The primary goal for the treatment of type I and type II diabetes is to cure the symptoms related to hyperglycemia. Type 1 is generally monitored through insulin replacement therapy [3].

## 2. Advances in insulin therapy

Human Insulin is a hormone (molecular weight of 5808 Daltons) composed of 51 amino acids arranged into two polypeptide chains (an A chain and a B chain), which are linked by disulfide bonds. The primary structure of human insulin, which contains 21 amino acid residues in chain A and 30 amino acid residues in chain B, is shown in (**Figure 1A**) [4]. Insulin plays a key role in the regulation of blood glucose levels. The intestinal absorption of glucose generated from the degradation of dietary carbohydrates including starch or sucrose, leads to elevated blood glucose levels, which stimulates the secretion of insulin from the pancreas into the blood stream. The biological actions of insulin are initiated when insulin binds to its cell surface receptor, which is followed by many protein activation cascades (**Figure 1B**) through which, insulin promotes the transport, intake, utilization and storage of glucose from the blood to the cells in the target tissues, such as liver, skeletal muscles and adipose tissue (**Figure 1B**) [5].

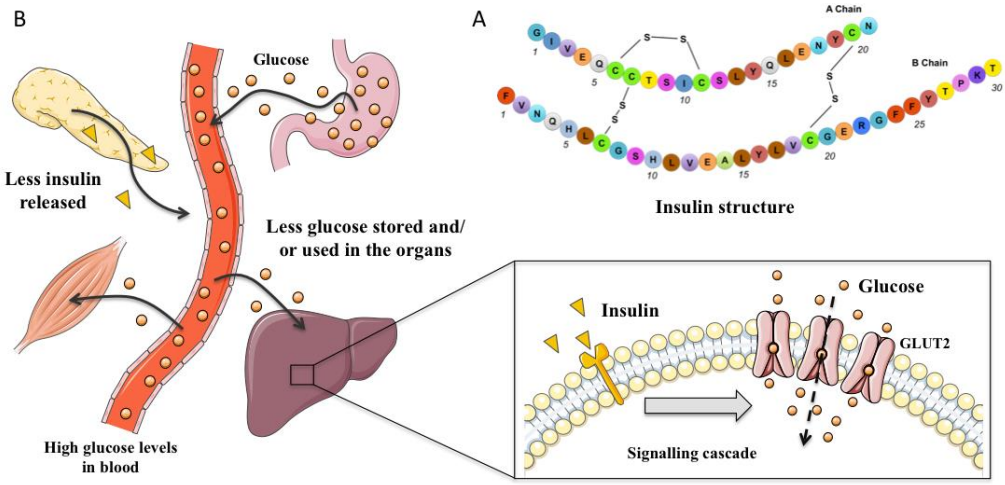


**Figure 1:** a) Amino acid sequence of human insulin, b) Schematic illustration of the physiological function of insulin on glucose uptake and metabolism. [Insulin binds to its receptor (1), which starts protein activation cascades (2) including translocation of GLUT-2 to the plasma membrane and influx of glucose (3), and glycogen synthesis (4)], c) Schematic illustration of the effects of insufficient insulin production in type 1 diabetes.

### 2.1. Injectable insulin

Current administration of insulin for diabetes treatment is almost entirely via subcutaneous injection and different insulin formulations have their unique pharmacokinetics. Human insulin preparations on the market can classified as (i) **rapid-acting insulin**: insulin lispro (Humalog®), insulin aspart (Novolog®), and insulin glulisine (Apidra®), (ii) **regular or short-acting insulin**: Humulin®R and Novolin®R (iii) **intermediate-acting insulin**: Neutral Protamine Hagedorn (NPH) insulin (Humulin N®), and Lente insulin, (iv) **long-acting insulin**: Ultralente, insulin glargine (Lantus®) and insulin detemir (Levemir®) (**Figure 2**) [4].

Rapid-acting insulins are inherited from the human insulin and characterised by a modification in the B chain e.g. for insulin lispro, the B28 (proline), B29 (lysine) amino acid sequence is reversed to be lysine-proline. In insulin aspart, the B28 amino acid proline is substituted with aspartic acid and finally for insulin glulisine (Apidra®) the amino acid aspargine at position B3 is replaced by lysine and the lysine in position B29 is replaced by glutamic acid. These modifications induce rapid dissociation of hexamers into dimers and monomers after injection. This rapid dissociation leads to rapid absorption after administration. Their onset peaks are sooner and they have

short durations compared to regular human insulin. Regular or short-acting insulin, on the other hand, is the basic human insulin without any modifications, (depicted in **Figure 1A**). It reaches the bloodstream within 30 minutes after injection, peaks at 2 - 3 hours after injection and is effective for approximately 3 to 6 hours [6].

The formulation Neutral Protamine Hagedorn (NPH) on the other hand, is a suspension of crystalline zinc-insulin combined at neutral pH with a positively charged, arginine-rich polypeptide, protamine. Its duration of action is intermediate due to delayed absorption of the insulin because of its conjugation with protamine, forming a less soluble complex. NPH blood absorption begins 1.5 h after subcutaneous injection; it has a peak plasma concentration at 4 to 12 h and disappears within 24 h (**Figure 2**) [4].

Long-acting insulin analogs attempt to replicate the body's basal insulin secretion. Insulin glargine consists of two modifications to human insulin. First, two arginines are added to the C-terminus of the B chain shifting the isoelectric point of the insulin from a pH or 5.4 to 6.7, making the insulin more soluble at an acidic pH. Secondly, the asparagine at position A21 is replaced by glycine. This substitution prevents deamidation and dimerisation that would occur with acid-sensitive asparagine. Insulin glargine is formulated at a pH of 4.0 and when it is injected into subcutaneous tissue (pH 6.8) the acidic solution is neutralized and microprecipitates of insulin glargine are formed, from which small amounts of insulin are released throughout a 24-hour period, resulting in a low level of insulin throughout the day (**Figure 2**) [3]. Insulin detemir, on the other hand, is a long acting insulin analog in which the B30 amino acid is omitted and a C14 fatty acid chain (myristic acid) is bound to the B29 lysine amino acid. It is slowly absorbed due to its strong association with albumin in the subcutaneous tissue and when it reaches the bloodstream it binds to albumin again, delaying its distribution to the peripheral tissues [7].

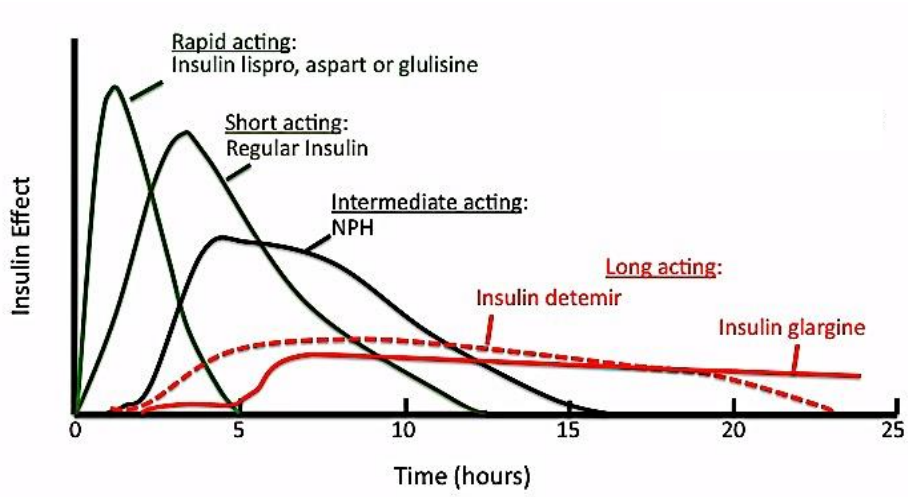


**Figure 2:** Idealized activity profiles of human insulin and analogues. Adapted with permission from [7].

The current therapy with insulin is effective at lowering blood glucose in patients with diabetes, but its use is constrained by the need to be injected subcutaneously and because of the concerns regarding interference with patients' lifestyle, risk of hypoglycemia. Parenteral administration bypasses the liver and goes directly into blood circulation leading over time to peripheral hyperinsulinemia, associated with peripheral hypertension, the development of atherosclerosis, cancer, hypoglycaemia and other adverse metabolic effects [2]. Moreover, diabetic patients have to endure multiple injections a day i.e. rapid acting insulin before meals to meet prandial insulin secretion and a supplemental injection of long acting insulin to mimic basal secretion levels. These repeated injections may bring about pain, tissue invasion, infections and nerve damage, which lead to poor patient compliance. Therefore, less invasive options for insulin therapy (and other similar peptides) are highly desirable and represent a priority objective in pharmaceutical innovation [8].

### 2.2. Oral insulin

Despite great improvements in needle technology, it is still estimated that >5% of the population are needle-phobic. Amongst all the alternative, noninvasive routes of administration (nasal, pulmonary, buccal and transdermal), oral insulin delivery remains the most preferred. Developing oral peptide formulations is important because parenteral administration by patients over a chronic period results in poor compliance thereby curtailing efficacy [9]. For patients, oral insulin would be pain

and stress free and this route would allow for the insulin to enter the portal circulation, reaching the liver before entering the systemic circulation, which resembles the pathway of physiologically secreted insulin [10].

Nevertheless, this ideal insulin delivery route has so far been elusive due to the difficulties faced in designing such a formulation. Orally administered drugs need to survive harsh gastric conditions in which they are exposed to acidic and enzymatic degradation. Subsequently, intestinal conditions further expose the drugs to a myriad of digestive enzymes; pancreatic proteases consisting of the serine endopeptidase (trypsin, α-chymotrypsin, elastase and exopeptidases, carboxypeptidases A and B), responsible for the degradation of proteins. The drugs that survive these conditions then need to penetrate the mucus layer overlying the absorptive surfaces of the gastrointestinal tract (GIT) and pass through the epithelial cells to enter the blood stream. Insulin, being a peptide molecule, is easily degraded by the conditions in the GIT, which significantly delimitates its oral bioavailability. Furthermore, the large molecular size (about 6 KDa), its charge and its hydrophilicity all preclude insulin absorption by paracellular and transcellular route [11].

To improve the bioavailability of insulin, different approaches have been explored, including chemical modification, co-administration with absorption enhancers and/or enzyme inhibitors and incorporation into carriers [12]. Nanotechnology has shown promising results in the design of potential oral peptide nanocarriers, and it is expected to lead to further improvement in the diagnosis and treatment of diabetes [13]. Nanoparticles have been engineered for the oral delivery of labile proteins and peptides for release into the systemic circulation [14].

## 3. Nanocarriers for oral insulin delivery

For insulin delivery the general idea is to encapsulate the peptide into carriers at a nanoscale range (10 – 1000 nm), allowing its protection from degradation, controlled release at target sites and transepithelial transport. Nanocarriers used for insulin delivery could broadly be categorized into: nanoparticles [15] (matrix-type particles), nanocapsules [16, 17], micelles [18] and liposomes [19] (**Figure 3**). Recently, a number of insulin nanocarriers for oral delivery have been investigated and a few have undergone clinical trials. This issue has been analyzed in detail in **chapter 1.**

Several properties have been identified as important for the uptake of nanoparticles, including the size, surface charge and surface hydrophobicity [20]. However, when it comes to increasing the bioavailability and pharmacological efficiency of the loaded peptide, the particle surface properties are of outmost importance for their interaction with intestinal mucosa and maintaining the integrity of the encapsulated peptide [21]. The modification of nanoparticle surface properties can be achieved either by a coating process with hydrophilic biodegradable polymers, incorporating stabilizing surfactants [22]. For example, PEG has been employed as a coating/matrix material in insulin nanoparticulate delivery for its stabilizing properties. PEG chains form a steric barrier at the surface of nanoparticles, which improves stability towards proteases and prevents opsonisation and reduce immunogenicity [23]. In some unique cases it was evident that a combination of one or two strategies, polymers or surfactants in one system resulted in enhanced properties and consequently, improved oral bioavailability. New materials allow the possibility of engineering nanoparticles with precise properties designed better to interact with surface receptors [9].

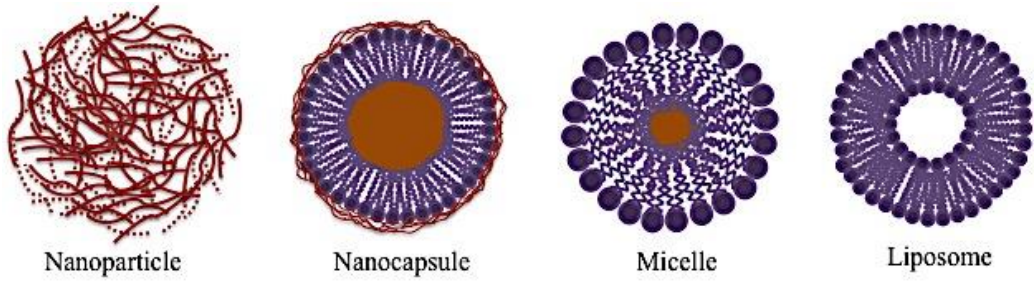


**Figure 3:** Different types of nanocarriers for insulin delivery.

Among the different biomaterials investigated, **polyaminoacids** with a cell-penetration capacity hold promise as a strategy for oral insulin delivery. Polyaminoacids and are polydisperse structures that, contrary to proteins, cannot fold into globular structures. In addition, they can carry versatile reactive functional groups at their side chains (such as carboxylic acids, hydroxyl, amines and thiol groups) that allow for a variety of chemical modifications and they are generally biocompatible and nontoxic [24].

Polyaminoacids have recently been explored to increase the oral bioavailability of peptides and proteins e.g. insulin, by exploiting their ability to interact with the protein molecules, to reduce their degradation by digestive enzymes and to promote their oral absorption [25], a feature also exhibited by cell penetrating peptides (CPPs). Morishita *et al.* pioneered the potential of oligoarginine CPPs as peptides that improve the absorption of poorly membrane permeable macromolecules that otherwise do not cross the cell membrane [26]. In this work, Morishita *et al.* described that arginine-rich CPPs such as octaarginine, have the ability to enhance insulin delivery across mucosal membranes safely by non-covalent cargo interaction strategy, and with highly effective bioavailability [27]. The electrostatic adsorption of octaarginine onto the cell surface proteoglycans and subsequent energy-dependent internalization pathway was associated with the uptake of the CPP itself by epithelial membranes and its enhancing effect on the mucosal insulin absorption.

On the other hand, protamine is a small, arginine-rich, nuclear protein that replace histones late in spermatogenesis and is essential for sperm head condensation and DNA stabilization [28]. When mixed with insulin, protamine slows down the onset and increases the duration of insulin action (see NPH insulin). In gene therapy, protamine's ability to condense plasmid DNA along with its approval by the U.S. Food and Drug Administration (FDA) as drug to neutralize the anti-clotting effects of heparin [29] have made it an appealing candidate to increase transduction rates by both viral and nonviral (e.g. utilizing cationic liposomes) mediated delivery mechanisms [30, 31].

Our group has used arginine-rich biomaterials including polyarginine [32] and protamine [33] for designing drug delivery systems with the ability to deliver different therapeutic compounds. Protamine in particular, was initially used for the preparation of protamine nanoparticles, where Protamine nanoparticles and nanocapsules were developed and showed a great potential to interact with cells and internalize antigens [33]. Thereafter, multi-enveloped nanoparticles were also developed (by the layer-by-layer approach) for the co-delivery of immunostimulants and antigens using the adsorptive polymers, polyarginine and protamine in combination with polysaccharides: dextran sulfate and alginate [34]. Lipid core-shell

protamine nanocapsules containing vitamin E and loaded with curcumin as anti-inflammatory drug were also prepared in our group and remarkably enhanced curcumin permeability in Caco-2 cells [35]. In all cases the use protamine and its positive effects of were attributed to the repetitive sequence of arginine present in the protamine structure (about 70%) (**Figure 4**), which provides this polypeptide with an efficient translocation activity through biological membranes including the intestinal epithelial cells [36]. Protamine molecules are known to translocate through cell membranes mainly by mediated endocytosis following the interaction between the positively charged arginines and the negative charge on the cell membrane surface [37].

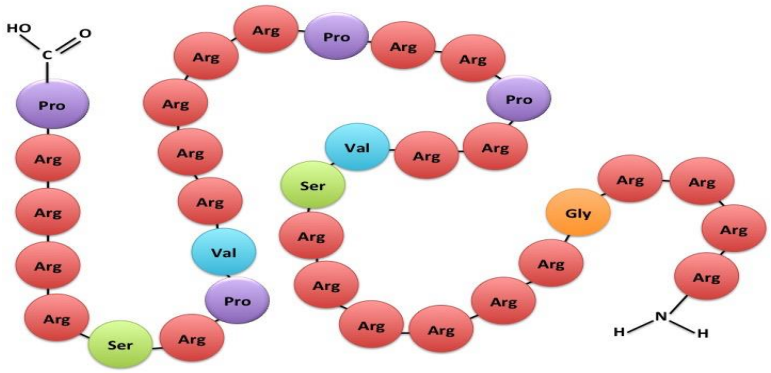


**Figure 4:** A schematic representation of a typical protamine structure.

## 4. Conclusion

Great efforts and progress have been made so far in the development of oral insulin delivery systems, however, the formulation and synthesis of more efficient nanoformulations is still required for commercial significance. Recently, a number of insulin nanocarriers have undergone clinical trials among which only a handful are in the market (see chapter 1 for an elaborated discussion). The use of natural polyaminoacids which are non-toxic and generally regarded as safe (GRAS) and have the highly sought after advantage to promote absorption of oral peptides is a feasible concept. For this goal to materialize in terms of the oral delivery of insulin, the use of polyaminoacids as cell penetrating polymers should be accompanied by the careful consideration of critical aspects for drug delivery such as: optimum particle-sized nanocarriers, the stability of the nanocarriers in biological fluids after *in vivo*

administration, and surface chemical composition.

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# CHAPTER 1

## Emerging delivery platforms for mucosal administration of biopharmaceuticals

# Abstract

Protein and peptide based therapeutics are typically administered by injection due to their poor uptake when administered via non-parenteral routes. Despite the low frequency of clinical breakthroughs with non-invasive protein drug delivery this far, it remains an active research area with renewed interest not only due to its improved therapeutic potential, but also due to the attractive commercial outcomes it offers. Currently, a number of technologies are adopted, including mixtures of penetration enhancers with protease inhibitors and/or nanotechnology-based products are under clinical development. This review provides a critical overview of current strategies of non-invasive mucosal delivery routes for therapeutic proteins, with emphasis on their advantages and limitations. Selected new trends and interesting novel formulations in advanced preclinical and clinical development stages for the pulmonary, nasal and the oral route are discussed for the most relevant peptide and protein drugs in function of their specific requirements and intended therapeutic applications.

## 1. Introduction

Proteins and peptides are building blocks of life and are now evolving as a very promising brand of therapeutic drugs. Once a rarely used subset of medical treatments, therapeutic proteins have increased dramatically in number and frequency of use since the introduction of the first recombinant protein therapeutic; human insulin, more than 33 years ago [1].

Peptides can bind large macromolecular targets with high potency and great selectivity, which translates into fewer off-target side effects and less potential for toxicity than conventional low molecular weight drugs [2]. Unlike other drug molecules, which often trigger side effects by producing toxic metabolites that accumulate in different organs, peptides simply degrade into amino acids, which minimizes the risk of toxicity [3]. These features arise from their macromolecular nature, which provides the structural complexity that is often required for specificity. However, this structural complexity also makes them some of the most challenging molecules to formulate and deliver. Loss of activity due to environmental factors such as moisture or temperature, during storage or in the body, puts a substantial burden on peptide formulation technologies. Whilst, the high molecular mass and hydrophilicity of these peptide/protein drugs creates delivery challenges, including substantial reduction in permeability across biological barriers and mucosal membranes.

## 2. Therapeutic proteins and peptides

Since the 1980s, peptides have been studied as treatments for a wide variety of problematic diseases [4]. Recombinant technology has allowed the production of many potential peptide drugs at an acceptable cost, paving the way for the treatment of severe, chronic and life threatening diseases such as cancer, hypertension, arthritis, and metabolic diseases e.g. diabetes and obesity. Most of currently available peptide drugs are delivered by injection, but several biopharmaceuticals have undergone extensive research towards alternative administration routes, particularly peptide hormones, such as insulin, vasopressin, calcitonin and luteinizing hormone-releasing hormone (LHRH), and small proteins, such as human growth hormone and interferon α [5].

Peptide therapeutics research and development is dynamic, with increasing numbers of candidates entering clinical evaluation in a wide variety of therapeutic categories.

[1]. Overall, there is a notable increase in the number of successfully marketed peptides as compared to the 1980s and 1990s when only 2 % and 11 % of peptide therapeutics were marketed, respectively [8]. Peptides approved in 2013 alone, were notable for their number and diversity of use, with the top two therapeutic areas being oncology (21%) and metabolic diseases (15%) [2]. The clinical pipeline was composed of 128-peptide therapeutics, with 40 in phase I, 74 in phase II and 14 in phase III studies. The number of peptides and diversity of therapeutics areas represented was higher in phase II and I compared to phase III, which indicates an increase and expansion for therapeutic peptide research, with a steady growth of marketed peptides [7]. More than 15 candidates are currently in Phase 3 clinical studies or under regulatory review, which suggests that peptide therapeutic products will continue to be approved at an increasing rate in the near future [7]. This steady increase is an indication of the progress in the use of peptides as therapeutic drug.

Injections (i.e. intravenous, intramuscular or subcutaneous route) remain the most common means for administering protein and peptide drugs. Patient compliance with drug administration regimens by any of these parenteral routes is generally poor and severely restricts the therapeutic value of the drug. Although, direct systemic delivery of these therapeutics overcomes the issue of absorption associated with other routes, other factors limit the chemical potency of peptide and protein therapeutics including: systemic proteases; rapid metabolism; opsonization; conformational changes; dissociation of subunit proteins; and non-covalent complexation with blood products [8]. Moreover, in the specific case of insulin delivery for example, diabetic patients, have to endure multiple injections a day i.e. rapid acting insulin before meals to meet prandial insulin secretion and a supplemental injection of long acting insulin to mimic basal secretion levels. These repeated injections may bring about pain, allergic reactions, infections and nerve damage, which lead to poor patient compliance. Therefore, less invasive options for insulin therapy (and other similar peptides) are highly desirable and represent a priority objective in pharmaceutical innovation [9].

## 3. Mucosal delivery of biopharmaceuticals

Owing to the above detailed limitations of injections, alternative delivery routes including; pulmonary, nasal, oral, vaginal and ocular, for biopharmaceuticals have been explored to increase patient compliance. These specific routes are collectively known as noninvasive mucosal routes of administration and each route offers unique

advantages and limitations [5]. Mucosal delivery of therapeutic peptides and proteins faces formidable barriers. These obstacles to efficient delivery can broadly be categorized as either the enzymatic barriers, which the peptide drug encounters after administration and the physical barriers, which prevent efficient transport of proteins and peptides across epithelial surfaces. Overall these barriers inevitably lead to low bioavailability [10, 11].

The primary barrier (chemical barrier) to mucosal absorption of peptide drugs is the enzymatic barrier in the form of degradation enzymes. Proteolytic enzymes are present all over the human body and they act by hydrolyzing the peptide bonds in polypeptides [12]. Hence, the peptide drugs may degrade, loose their functionality or aggregate prior to reaching the absorption membrane. These peptides are further subjected to digestion by aminopeptidases located in the cell membranes during their transportation through the epithelial layer of cells [13, 14].

The mucosa comprises the epithelial layer covered by a firmly adherent viscous layer of mucus and a less viscous, loosely adherent mucus layer on the surface. The mucus layer covering the epithelium represents the first physical barrier to be encountered by a drug prior to absorption and the thickness of the layer varies greatly throughout the body [15, 16]. Regardless of the localization, the function of the mucus is to lubricate and protect the underlying tissue [11]. The mucus clearance rate also varies between tissues and it has been estimated to be less than 30 minutes at different sites in the respiratory system and around 5 hours in the GI-tract. The detailed composition of mucus is dependent on the specific mucosal site, disease state as well as influenced by variance between individuals. Mucus contains approximately 95 % (w/w) water, 2-5 % (w/w) mucin proteins and a small amount of lipids and electrolytes [15, 16]. In addition to the physical diffusion barrier provided by the mucus network, the hydrophilic nature and the presence of a strong negative charge allow for drug interactions with the mucus components, which have also shown to retard or hinder diffusion and thereby limit drug absorption [17].

**Table 1:** Physical properties and surface area of the different mucosal surfaces

| | Nasal | Pulmonary | Oral |
|---|---|---|---|
| **Surface area** | 150 $cm^2$ | 80-140 $m^2$ | >200 $m^2$ |
| **Histological properties** | Thin mucus layer<br>Ciliated, pseudostratified epithelium (10 μm) | Thin mucus layer<br>Bronchioles: Ciliated columnar pseudostratified epithelium (10-60 μm)<br>Alveoli: squamous epithelial monolayer (<1μm) | Thick mucus layer<br>Columnar epithelial monolayer (10 μm) |

The morphology of the epithelial absorption barrier varies according to the specific anatomical site (**Table 1**). The nasal, lung and intestinal epithelia are commonly comprised of a monolayer of cells interconnected by tight junctions (**Figure 1**) [10]. The mechanism of transport through the epithelial barrier depends mainly on the physiochemical properties of peptide molecules. Common transport mechanisms in all sites include active and passive transport, which can be described by a combination of two processes;

i) *Paracellular transport*: involves the transport of molecules via water filled pores or channels between cells. The paracellular route is restricted to relatively small hydrophilic molecules of <100-200 Da, by the presence of tight junctions or zonula occludins between epithelial cells [18]. Unfortunately, most therapeutic proteins have molecular weights much greater than 500 Da and hence exhibit low permeability.

ii) *Transcellular transport*: involves the diffusion of drug molecules through the apical and basolateral membrane. This route is ideal for lipophilic drugs, which express relatively high affinity for the lipid bilayer of cell membranes. On the contrary, the transport of hydrophilic proteins/peptides through this path poses a significant challenge [19].

Transcytosis, is a specific type of transcellular transport i.e. the manner by which macromolecules or particles are taken up and transported across the cell to the basolateral side and it occurs through different energy dependent endocytic pathways (**Figure 1**) [22], [23]. Phagocytosis is used for uptake of large particles such as bacteria, and is generally the first step in the uptake and degradation of particles larger than 0.5 μm [22]. Clathrin-mediated endocytosis (CME) is the uptake and transport of particles from the cell

surface mediated by clathrin-coated vesicles (100 to 120 nm in diameter) to the basolateral side. Clathrin-coated vesicles (CCVs) are found in virtually all cells and cause the endocytosed material to end up in degradative lysosomes. Whilst caveolae-mediated endocytosis (CvME), involves the formation of small (50 to 100 nm) lipid raft-enriched, flask-shaped pits in the cell membrane surface. Unlike CME, CvME is a highly regulated process involving complex signaling, which may be driven by the cargo itself and it bypasses lysosomes [23].

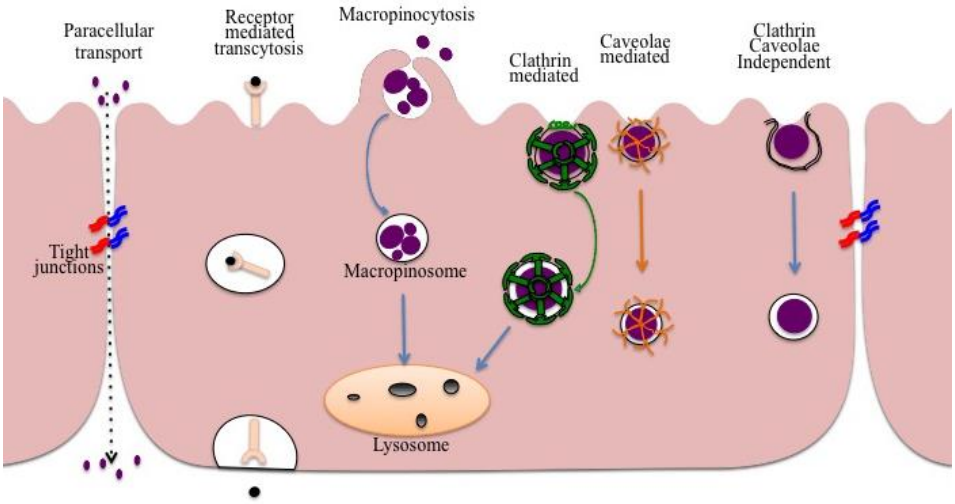


**Figure 1:** Schematic representation of the different pathways employed by molecules to penetrate cells and cross the cell barrier into the systemic circulation.

Tremendous efforts have been dedicated to improving the permeation of the proteins/peptides through the epithelial membranes whilst protecting them against enzymatic degradation. Hence the principal approaches for mucosal delivery include; the use of absorption enhancers [24] and protease inhibitors [25], modification or conjugation of peptides with biological entities that show stabilizing [25–27] and cell-penetrating capabilities [28, 29] and the design of multifunctional [30] and nanoparticulate drug delivery systems [31, 32] that help peptide trafficking across the fore-mentioned epithelial barriers and these strategies have been extensively review elsewhere.

In recent years new delivery strategies including alternative administration routes integrated with emerging nanotechnologies have attracted increased attention in quest of shifting from the parenteral route of administration [33]. Alternative routes for

controlled peptide delivery such as nasal, pulmonary, and oral epithelia have been explored [10]. It is evident that the currently successful and promising peptides are either small, with a molecular weight between 1,000-3,400 Da or they have been adapted by a technology that improves absorption or protection from enzymes [31]. However, despite these advances, the long-awaited dream of "needle-free" delivery of larger peptides such as insulin remains a challenge due to their poor permeability across different barriers and low bioavailability. The next sections are focused on the most common mucosal routes of administrations and recent efforts or advances in this field in terms of peptides/proteins currently on clinical trials.

### 3.1. Nasal delivery

The nasal route has gained importance as a non-invasive, and easily accessible route that offers many advantages for the introduction of drugs into systemic circulation. Compared to other biological membranes the nasal mucosa is a rather porous and thin endothelial basal membrane. It also has a rapid blood flow, with a highly vascularized epithelial layer and a vast absorption area (150 $cm^2$) with ciliated epithelial cells (**Figure 2**) [34], Due to these characteristics, it offers many advantages such as fast absorption of drugs [35]. In addition, nasal delivery avoids gastric degradation, hepatic first-pass effect and may also allow for strategies to circumvent the obstacles for blood-brain barrier (BBB). It has also been considered for the administration of vaccines [36].

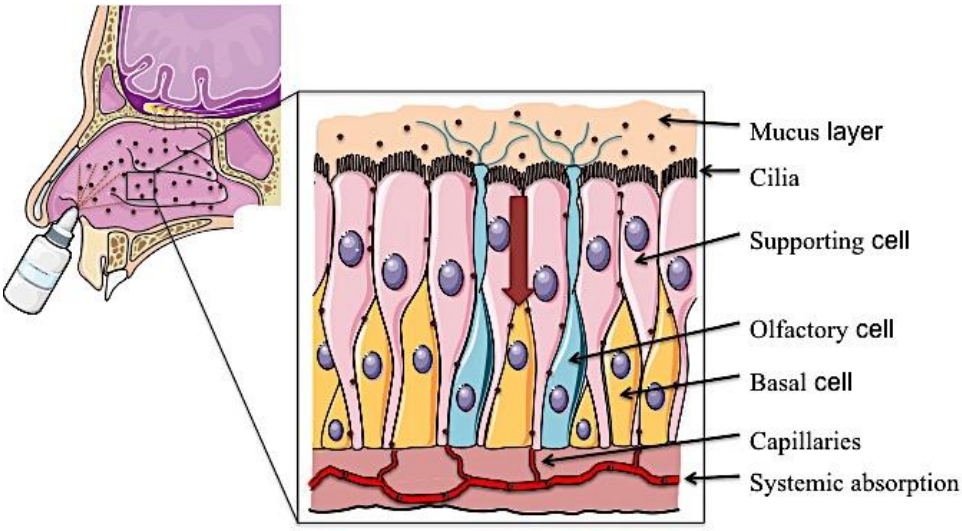


**Figure 2:** Schematic illustration of the nasal mucosa, depicting how a drug is absorbed into the systemic circulation.

Small hydrophilic drugs with low molecular weight are absorbed via the nasal mucosa at a rate that is almost comparable to intravenous application. Nevertheless, the nasal mucosa is an obstacle for the passage of large molecules, particularly for those above 1,000 Da in size, and therefore, the nasal bioavailability of peptides and proteins is usually less than 1%. This low bioavailability of these drugs is associated with the weak mucosal membrane permeability (restricted to paracellular and transcellular transport) and the presence of proteolytic enzymatic activity in the nasal mucosa [35]. Another reason is the mucociliary clearance, through which drugs are rapidly removed from the nasal area [37].

### 3.1.1 Systemic delivery via the nasal route

Intranasal delivery has allowed successful and improved delivery of certain peptide drugs. For instance, Desmospray®, is a solution for nasal use containing desmopressin; an antiduretic hormone used for the treatment of nocturia associated with multiple sclerosis where other treatments have failed. For instance in; i) the diagnosis and treatment of vasopressin-sensitive cranial diabetes insipidus and ii) in establishing renal concentration capacity. Initially, orally administered desmopressin showed minimum absorption from the GI tract (bioavailability = 0.08-0.16%) that improved to 10-20% from nasal mucosa highlighting the increased efficiency for nasal delivery of peptides [38].

Salmon calcitonin is a polypeptide (32 amino acids), which is highly potent in humans for the treatment of bone diseases. Injectable and nasal calcitonins are commercially available for the treatment of osteoporosis and a number of studies are currently on going for the treatment of other medical conditions [39–41]. Shiraz University of Medical Sciences evaluated the effectiveness of long-term administration of intranasal calcitonin for the treatment of central giant cell granuloma of the jaws. After 28 months of treatment, all lesions decreased in size with a high degree of calcification and there were no recurrences (phase II clinical trials), hence intranasal calcitonin spray could be considered as a future alternative to surgery [42].

Aegis, recently used its patented **Intravail® technology** to formulate a novel exenatide formulation, which provides equivalent blood levels using a simple metered nasal spray to replace each of the two daily injections of exenatide [43]. Intravail® comprises a broad class of chemically synthesizable transmucosal absorption enhancing agents (non-disclosed) that open tight junctions without causing any toxicological effects on the mucosal membrane. On the other hand, GLP-1 was incorporated into capsules coated with corn-starch and calcium carbonate with a mean particle diameter of 60 μm by Asubio Pharma Co., Ltd. The capsules were set in a special device (by Shirasu Porous Glass Technology, Miyazaki, Japan) (patent no. 2007-331207) and intranasal administration resulted in rapid peripheral appearance of active GLP-1 in diabetic patients. Furthermore, GLP-1 induced early-phase insulin secretion, inhibited inappropriate glucagon secretion, and improved intermediate-term markers of glycemic control without severe adverse events. Long-term treatment with this new nasal GLP-1 will soon be evaluated in larger trials as a novel treatment for type 2 diabetes [44].

CP024 is a spray-dried nasal powder formulation comprising the human growth hormone (hGH), Solutol® HS15, a gelling agent, and other excipients (non-disclosed), manufactured by Critical Pharmaceuticals in its propriety **CriticalSorb** technology. In Phase 1 clinical trials, CP024 was well tolerated and able to induce insulin-like growth factor-1 (IGF-1) to the same levels as a subcutaneous injection of the marketed product, somatropin. Somatropin is currently available as an injection and not absorbed through nasal mucosa. Interestingly, the CriticalSorb technology enhances the absorption of somatropin and thereby makes it possible to deliver the

drug by nasal spray [45]. CriticalSorb is a novel absorption enhancer based on Solutol® HS15 approved by the FDA as generally regarded as safe (GRAS) and used in currently marketed products as a solubility enhancer for intravenous and oral administration. In preclinical toxicology studies, CriticalSorb has been found to be nontoxic and not irritating to the skin or eyes and was well tolerated by the nasal mucosa. It is available as liquid or powder formulations and promotes absorption of other peptides and proteins such as teriparatide (parathyroid hormone PTH 1-34) and insulin (~6kDa) across the nasal mucosa in phase I clinical trials and preclinical studies, respectively [46].

**Table 2:** Intranasal peptide formulations on the market or under clinical development.

| | Brand name | Drug | Technology | Indication | Phase of development | Company | Ref. |
|---|---|---|---|---|---|---|---|
| **Systemic** | Desmospray | Desmopressin | Nasal spray – high and rapid absorption | Nocturia | Market, 1987 | Ferring pharmaceuticals | [47] |
| | Fortical | Salmon calcitonin | Nasal spray – high and rapid absorption | Osteoporosis | Market, 2005 | Upsher-Smith Laboratories | [47] |
| | Calcitonin | Calcitonin | Nasal spray | Postmenopausal Osteoporotic Women | Phase III | Tarsa Therapeutics, Inc. | [39] |
| | Glucagon | Glucagon | Nasal spray | Hypoglcemia in diabetes mellitus | Phase III | Locemia Solutions ULC | [44] |
| | Exenatide | Exenatide | Intravail® technology | Type II diabetes | Phase I | Aegis Therapeutics LLc | [48] |
| | Calcitonin | Calcitonin | Nasal aerosol | X-linked Hypophosphatemia (XLH) | Phase I | Yale University | [41] |
| | CP024 and CP046 | human growth hormone (hGH) and parathyroid hormone | CriticalSorb technology | Growth failure and osteoporosis | Phase I | Critical Pharmaceuticals in collaboration with the University of Nottingham | [45],[49] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Nose to brain** | Oxytocin | Oxytocin | Nasal Spray | Acute and chronic pain | Phase IV | Northwestern University | [50] |
| | AL-108 | Davunetide | Intranasal aerosol | Schyzophrenia | Phase II | University of California | |
| | Oxytocin | Oxytocin | OptiNose Bi-directional Nasal spray | Autism Spectrum Disorder | Phase II | OptiNose AS | [51] |
| | Insulin | Insulin detemir | ViaNase electronic atomizer | Alzheimer | Phase II | Wake Forest School of Medicine | [52] |
| **Local** | Calcitonin | Calcitonin | Nasal spray | Central Giant Cell Granuloma | Phase II | Shiraz University of Medical Sciences | [53] |
| **Vaccines** | Lytixar™ | Antibiotic peptide (LTX-109) | Intranasal aerosol | Methicillin-resistant/-Sensitive Staphylococcus Aureus (MRSA/MSSA) | Phase II | Lytix Biopharma AS | [54] |
| | GHB01L1 | Influenza Antigen | Intranasal aerosol | Human Influenza A Virus | Phase I | AVIR Green Hills Biotechnology AG | [55] |
| | Vacc-4x | HIV antigen | Lipid-based nanoparticles (Endocine) | HIV immunity | Phase I | Oslo University hospital | [56] |

### 3.1.2. Nose-to-brain delivery

Intranasal delivery provides a practical, non-invasive method of bypassing the blood-brain barrier (BBB) to deliver therapeutic agents to the brain and spinal cord. This route allows drugs that do not cross the BBB to be delivered to the central nervous system within minutes. This is possible because of the unique connections that the olfactory and trigeminal nerves provide between the brain and external environment. A growing body of evidence suggests that insulin plays a role in cognitive processes and that insulin abnormalities may contribute to memory and brain changes associated with Alzheimer disease. Interestingly, the university of Washington recently demonstrated in phase II clinical trials, that regular insulin administered to the nasal cavity is transported within a few minutes into the brain, without affecting blood sugar or insulin levels [57]. A similar study is on going with insulin in phase II trials by Wake Forest School of Medicine [52].

Oxytocin is a small, naturally occurring peptide that acts mainly as a neuromodulator in the brain. Recently the effect of intranasal oxytocin on pain sensitivity and threshold has been evaluated. Participants received nasal sprays into each nostril of 4 Units up to 32 units of oxytocin prior to Thermal Evaluation System Testing and they reported lower pain intensity. This study (currently on phase IV clinical trials, Northwestern University) suggests that oxytocin might represent a novel, safe, and effective analgesic for acutely painful procedures [50, 51]. The university of Alabama at Birmingham is currently undergoing clinical trials for intranasal oxytocin for the treatment of pain associated with interstitial cystitis [58].

Although intranasal delivery does not necessarily require any modification to therapeutic agents the location of deposition of the drug product inside the nasal cavity may affect absorption rate. Hence modification of the administration technique to optimize droplet size, deposition fraction and, thus, volume of administration is crucial in nasal drug delivery [59]. Novel nasal delivery technologies suitable for both liquid and powder peptide drugs are currently being developed. These novel concepts combine knowledge of both functional nasal anatomy and aerodynamics. On this regard, OptiNose undertook a Phase I trial in late 2013 to investigate “nose-to-brain” transport of oxytocin via the patented **OptiNose Bi-Directional™ Breath Powered**

delivery technology. The OptiNose device offers the potential for a more efficient and consistent direct transport of oxytocin into the brain itself, using relatively low doses, which will significantly reduce drug levels in the rest of the body, reducing the risk of side-effects [51]. Other companies such as Kurve technology recently developed the ViaNase electronic atomizer, based on Controlled Particle Dispersion (CPD)® designed to deliver most formulations with efficient nasal cavity saturation and minimal deposition to the lungs and stomach [60]. Other similar devices include; the Teleflex VaxINator™ [61], and the LMA® MAD NasalTM Device [62].

***Intranasal vaccines***

The nasal route has also been proposed as an option for mucosal vaccination, with the possibility to induce both systemic and mucosal immune responses. **Endocine** is a delivery system specifically developed for intranasal vaccines (by Eurocine Vaccines, Sweden). It is a lipid-based (mono-olein and oleic acid) dispersion with particles less than 100 nm. Intranasal administration of Vacc-4x with Endocine (as an adjuvant) was safe and induced dose-dependent T cell responses and both mucosal and systemic humoral responses, in patients on effective antiretroviral therapy (Phase I clinical trials) [56].

### 3.2. Pulmonary delivery

Aerosol administration of therapeutics to the pulmonary epithelium for systemic delivery represents a significant opportunity in the delivery of macromolecules since it allows: (i) rapid absorption into the systemic circulation (this may be especially important for drugs where fast onset of action is critical), and (ii) higher bioavailability than with other non-invasive modes of administration [63]. This can be attributed to the considerable absorptive surface area at the air interface, covered by an extremely small volume of fluid (10–20 ml) and with the entire cardiac output flowing through the underlying capillary network [64]. Due to this physiological and anatomical peculiarity, an inhaled aerosol drug can be widely dispersed and deposited in high concentrations in close proximity to the blood stream (**Figure 3**).

The fate of drugs following inhalation depends on their site of deposition within the lungs. Aerosol particles deposited in the tracheobronchial tree come into contact with

the mucus, and the peptide or protein within the particle dissolves in the mucus. The macromolecules can either be eliminated by the mucociliary clearance towards the gastrointestinal tract or diffuse in the mucus and cross the airway epithelium. On the other hand, particles deposited in the alveolar region initially come into contact with the thin layer of fluid lining of the alveolar epithelium and are able to cross much more efficiently [63–65].

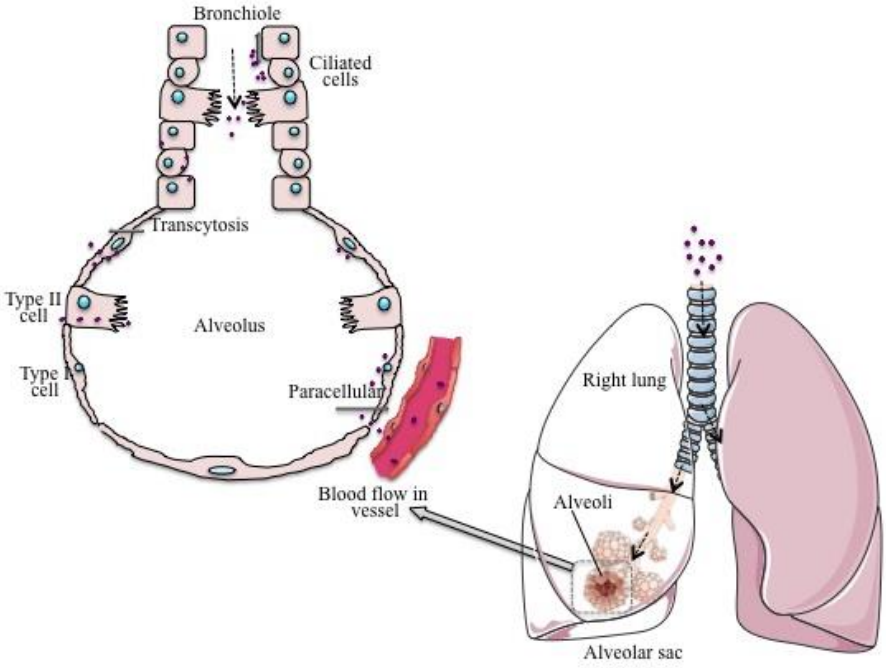


**Figure 3:** Schematic representation of the lung a description of the absorption process in the alveolus.

The presence of proteases in the lung and the barrier between capillary blood and alveolar air (air-blood barrier) and clearance by alveolar macrophages impede pulmonary delivery of peptide drugs. The use of absorption enhancers such as oleic acid, oleyl alcohol, Span 85 and protease inhibitors e.g. sodium glycocholate and surfactin have been reported to be useful approaches for improving pulmonary absorption of biologically active drugs like peptides [11, 66]. Pulmonary technology is the process of making drugs inhalable (e.g. conversion into dry-powder) in order to deliver them to and through the lungs for both systemic and local lung applications whilst PEGylation is their chemical modification to enhance peptide/protein drug performance i.e. improve stability and solubility, increase half-life and reduce immune responses. These technologies form the basis for most industrial products

that have received FDA approval [67].

### 3.2.1. Systemic delivery via the pulmonary route

AFREZZA™ is an FDA approved (Mannkind corporation, 2014) ultra-rapid acting insulin comprising **Technosphere®** insulin powder in unit-dose inhaler cartridges. Technosphere® is a drug delivery system made up of fumaryl diketopiperazine (FDKP), which forms microspheres with 2–5μm sizes. The powder formulation is prepared by precipitating insulin from solution onto the preformed particles, which readily dissolve once in the lung environment, releasing insulin. It rapidly reaches systemic circulation and attains maximum plasma concentration in 15 minutes, which is much earlier compared to injectable insulin (i.e. 1h). AFREZZA® has a relative bioavailability of 21–25% compared to SC regular insulin and is also eliminated quickly (within 2h) from the blood circulation. This pharmacokinetic/dynamic profile mimics what happens with endogenous insulin released in a person without diabetes and the result is significantly less hypoglycemia [68].

Nektar ́s **PEGylation technology™** used in nine approved partnered products for other administration routes, in the U.S. including UCB's Cimzia® for Crohn's Disease, Roche's PEGASYS® for hepatitis C and Amgen's Neulasta® for neutropenia, has also been investigated for lung delivery. This conjugation technology improves peptide stability and reduces immune responses [47]. Nektar also showed that the administration of liposome-encapsulated drugs by aerosols seems to be a feasible way to deliver them via the lungs. Leuprolide acetate, is a potent agonist of luteinizing hormone-releasing hormone (LHRH) currently used for the treatment of prostatic cancer, endometriosis, and precocious puberty, administered solely by injection. In an exploratory study, leuprolide peptide was entrapped in liposomes (3.5 μm in size) containing sorbitan monooleate (Span 80) and administered as a dry powder resulting in a relative bioavailability of 18 %, compared to injectable systemic delivery. The results suggested that clinical doses of leuprolide could be given as once-a-day single inhalation dose, thanks to the liposomes that increased residence time and promoted absorption of the hormone. [55]. However, no further updates have been published on this formulation since 2009.

Baxter Healthcare Corporation, recently completed phase I clinical trials where they investigated the PK and PD properties of recombinant human insulin inhalation powder (RHIIP), manufactured with **PROMAXX® technology**, which allows the formation of protein microspheres of uniform size and shape suitable for inhalation by a temperature-controlled precipitation from an aqueous insulin solution in the presence of polyethylene glycol without the use of absorption enhancers. This microparticulate powder formulation showed a faster onset of action than SC Insulin [69].

In recent years the development of new and improved devices for administration of peptides and proteins has intensified since the site of deposition is crucial for the efficacy of inhaled drugs. Prominent examples of such devices are the AERx® from Aradigm (CA, USA) [70], Respimat® from Boehringer Ingelheim (Ingelheim, Germany) [71] and AeroDose® from Aerogen Inc. (Galway, Ireland) [72]. Some of the most recent developments with these devices are included in **Table 3**. The Aerodose® insulin inhaler for example, resulted in a dose-dependent increase in serum insulin concentration and a corresponding glucose lowering effect that were similar in proportion to that obtained for increasing doses of subcutaneously injected insulin. This novel device is a small hand-held breath-actuated inhaler that contains Aerogen's electronic aerosol generator. When liquid is placed onto the aerosol generator, a micropumping action creates a fine-droplet low-velocity aerosol that is suited for deep lung delivery [72].

### 3.2.2. Local delivery to the lung

Although the efforts made in the field of pulmonary delivery of peptides and proteins have been tremendous, there are still a very limited number of inhaled macromolecules available on the market. As expect, inhaled peptide drugs developed for local therapy are the most promising and the fastest growing group under development [73]. One of the candidates worth mentioning is being developed by the University of Maryland, a new liposomal cyclosporine A (L-CsA) solution consisting of unilamellar liposomes of about 50 nm with a narrow size distribution inhaled through a nebulizer, in phase 1 clinical trials completed in 2015, for the treatment of bronchiolitis obliterans syndrome after lung transplantation. The treatment was well

tolerated, and no drug-related side effects were observed. Once or twice daily dosing of 10 mg aerosol L-CsA would result in a sufficient peripheral lung deposition of approximately 14 and 28 mg/week, respectively signifying the improvement of pharmacokinetics through encapsulation in liposomes [74].

**Table 3:** Inhaled peptide formulations on the market or under clinical development.

| | Brand name | Drug | Technology | Indication | Phase of development | Company | Ref. |
|---|---|---|---|---|---|---|---|
| **Systemic** | AFREZZA™ | Insulin | Technosphere® | Diabetes | Market, 2015 | Mannkind corporation | [68] |
| | Leuprolide | Leuprolide | Inhaler | Prostate cancer and endometriosis | Phase I | Nektar Therapeutics | [75] |
| | **Recombinant human insulin inhalation powder (RHIIP)** | Insulin | PROMAXX® in a dry powder inhaler | Diabetes mellitus | Phase I | Baxter Healthcare Corporation | [69] |
| | Alveair® | Insulin | Alveair™ (Liquid inhaler) | Diabetes | Preclinical trials | Coremed Inc | [76] |
| | Aerodose ® | Insulin | Aerodose inhaler | Diabetes | Preclinical trails | Aerogen | [72] |
| **Local** | Sargramostim | Granulocyte-macrophage colony stimulating factor (GM-CSF) | Nebulizer | Osteaosarcoma (Pulmonary recurrence) | Phase II | Children's Oncology Group & National Cancer Institute (NCI) | [77] |
| | Interferon-gamma | Interferon-gamma | Nebulizer | Cavitary pulmonary tuberculosis | Phase II | New York University School of Medicine | [78] |
| | IL-4R | Recombinant human interleukin-4 receptor (IL-4R) | Aerosol inhaler | Asthma | Phase II | National Institute of Allergy and Infectious Diseases | [79] |

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|---|---|---|---|---|---|---|
| | | | | | (NIAID) | |
| AVONEX® | Interferon β-1a | ANOVEX inhaler | Multiple sclerosis | Phase I | Trio Medicines Ltd | [47] |
| COLI-VLM | Colimycin | Aerosol inhaler | Cystic Fibrosis | Phase I | Poitiers University Hospital | [80] |
| CR002 | Alpha1 Proteinase Inhibitor (API) | Inhalation solution | Cystic Fibrosis | Phase I | CSL Behring | [81] |
| MVA85A | Antigenic peptide | Aerosol inhaler | Tuberculosis Vaccine | Phase I | University of Oxford | [82] |
| Liposomal Cyclosporine (L-CsA) | Cyclosporine A | Aerosol inhaler | Bronchiolitis Obliterans Syndrome | Phase I | University of Maryland | [83] |

### 3.3. Oral delivery

Systemic delivery of drugs via the nasal and pulmonary routes, circumvents the liver and, thus, avoid hepatic first-pass metabolism, which may be advantageously exploited for many peptides drugs [84]. However, the oral route of administration is often preferred due to the easy accessibility of the large surface area available for absorption to the systemic circulation. Furthermore, the pharmacological rationale for administering specific peptides such as insulin via the oral route supports this approach, as the oral delivery of insulin results in absorption directly to the liver (hepatic portal circulation), before it reaches the peripheral tissues (**Figure 4**) [85, 86].

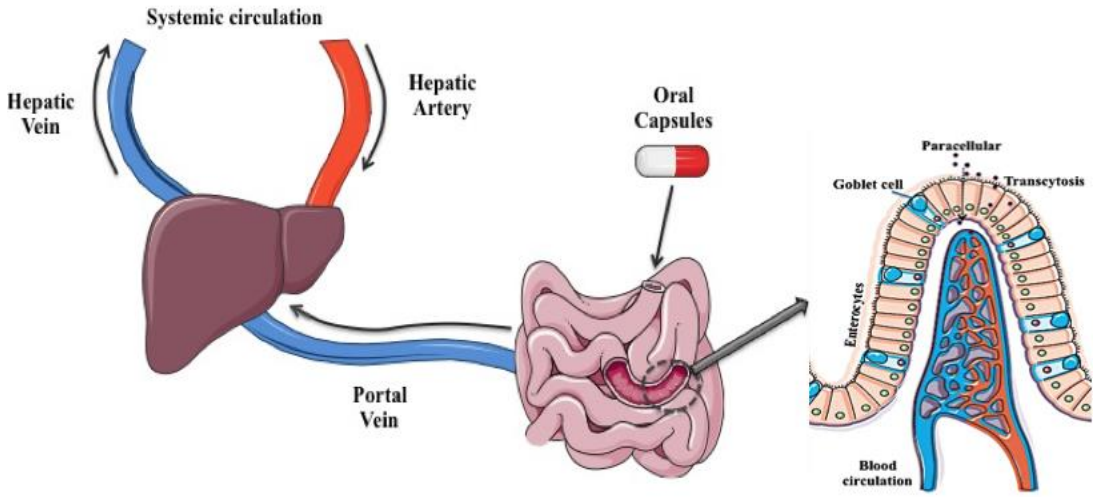


**Figure 4:** Schematic illustration of the large surface area for absorption and first pass metabolism associated with the oral route of administration.

Indeed, when the drug of interest must be used repeatedly administered, the oral route inevitably becomes the route of choice for patients since it is painless and convenient [87]. To achieve successful and satisfactory therapeutic results by the oral route, there must be minimal degradation and significant absorption of the protein molecules in the GIT [32]. Most of current approaches target the GIT barrier in an attempt to overcome its restrictive nature and subsequently increase oral bioavailability [88]. The main challenge is to improve the oral bioavailability to be higher than 1%. New approaches are mostly driven by the necessity to design an approach that not only

protects the protein/peptide from enzymatic degradation but also aids in enhancing its absorption without altering its biological activity [89]. The following tables portrays recent efforts of various pharmaceutical companies in making the transition towards oral peptide delivery by developing innovative technologies and testing multiple drug candidates based on these technologies (**Table 3**). The tables are intended to give a broad overview of the numerous strategies and applications currently in evaluation and are organized from a practical point of view as a function of developmental stage and intended site of action (i.e. systemic and local action). Given the large body of information, the discussion is limited to the most promising and interesting examples.

### 3.3.1. Systemic delivery using the oral route

Desmopressin, is one of the marketed oral peptides. Its oral bioavailability is low (< 1%) but only a very low dose is required to elicit its therapeutic effect [90]. Desmopressin and cyclosporine (another marketed oral peptide), have cyclic structures, which render them stable and less prone to degradation in the GIT. Moreover their small molecular weight allows for faster and less constrained intestinal absorption. Astellas Pharma Inc, also developed new cyclic peptides with anti-hepatitis C virus action, which take advantage of this strategy [91].

The most promising and basic approaches reaching clinical development for oral delivery are enteric-coated formulations that include muco-interactive polymers encapsulating a peptide-drug with enzyme inhibitors and absorption enhancers, capable of rapidly and reversibly increasing epithelial permeability. In this strategy, excipients (chemicals that are added to the formulation that make up capsules, tablets or liquids) are used to shield the native drug chemically or physically and promote absorption. This strategy has been in existence for a while now in pharmaceutical industries and it is being improved progressively by the addition of novel enhancing excipients and polymers [92]. Enteris BioPharma Inc (formerly known as Unigene) developed the clinically proven **Peptelligence™** technology that enables the oral delivery of peptides. This technology entails an enteric-coated tablet with two key components; an organic acid enzyme inhibitor (citric acid in the form of coated beads) and a permeation enhancer (acylcarnitine), which penetrates the mucus layer. The enteric coating prevents the tablet from disintegration within the stomach and enables dissolution within the intestinal pH. The permeation enhancer opens tight junctions and promotes paracellular transport whilst the citric acid transiently decreases the

intestinal pH to prevent degradation of the peptide by intestinal proteases and peptidases, which function optimally at neutral pH. Accordingly, Tarsa therapeutics developed an oral salmon calcitonin formulation, OSTORA®, using the Peptelligence™ technology and completed Phase III clinical trials in post-menopausal osteoporotic patients [93]. The FDA accepted the new drug application (NDA) for review, offering the possibility that OSTORA® (now called TBRIA™) could be cleared for marketing in 2016 [94].

The **Transient Permeation Enhancer (TPE)** technology is an enteric-coated liquid-filled capsule containing an oily suspension of the peptide drug and sodium caprylate (C8) in hydrophilic microparticles that are mixed with castor oil or a medium-chain glyceride and caprylic acid. The FDA approved the orphan status for the Octreotide formulation, Octreolin® developed by Chiasma Pharma, which recently completed Phase III clinical trials with TPE technology. Chiasma claims that the technology protects the peptide from enzymatic digestion and transiently opens tight junctions. It was demonstrated that an oral dose of 20 mg octreotide using TPE technology can achieve similar pharmacokinetics as 0.1 mg octreotide SC (a relative oral bioavailability of less than 1 %), and the safety profiles were also comparable. This product is also expected to be on the market in 2016 [95].

Emisphere Technologies uses SNAC (sodium N-[8-(2-hydroxybenzoyl)amino] caprylate or salcaprozate sodium), 5-CNAC (N-(5-chlorosalicyloyl)-8-aminocaprylic acid), 4-CNAB (4-[(4-chloro2-hydroxy-benzoyl)amino]butanoic acid) and SNAD (N-(10-[2-hydroxybenzoyl]-amino) decanoic acid) as absorption enhancers. These excipients form non-covalent complexes with peptides or proteins, protect them from digestive enzymes and improve the permeation across the intestinal epithelium through transcellular pathway. The Novo Nordisk product candidate, semaglutide (NN9924) is a new glucagon-like peptide-1 (GLP-1) analogue that can help type 2 diabetes patients achieve substantial lowering of blood glucose with a low risk of hypoglycaemia. Semaglutide, is provided in a tablet formulation with the absorption-enhancing excipient, SNAC, included in the **Eligen®** carrier concept. SNAC does not cause histological damage to the intestinal epithelium and successfully completed phase II trials in 2015 [96].

**Table 4 (a):** Oral peptide formulations in clinical use and clinical development (systemic action)

| Brand name | Drug | Technology | Indication | Phase of development | Company | Reference |
|---|---|---|---|---|---|---|
| **Oral-Lyn™** | Insulin | RapidMist™. Buccal delivery | Diabetes | Market, 2013 | Generex Biotechnology Corp | [66] |
| **MINIRIN®** | Desmopressin | Oral tablet (small peptide effective at minimal absorption) | Cranial diabetes/nocturia (multiple sclerosis) | Market, 2008 | Ferring | [97] |
| **Ceredist ®** | Taltirelin hydrate (pyroglutamyl peptide derivative) | Chemical modification | Anti-spinocerebellar degeneration activity | Market, 2009 | Mitsubishi Tanabe Pharma | [98] |
| **OSTORA™** | Salmon calcitonin | Peptelligence technology | Postmenopausal osteoporosis, | New Drug Application (NDA) filed | TARSA Therapeutics | [93] |
| **Octreoline™** | Octreotide | TPE® | Acromegaly and neuroendocrine tumors | Phase III complete | Chiasma Pharma | [99] |
| **Semaglutide** | Glucagon-like peptide- | Eligen® platform | Type 2 Diabetes | Phase IIIa | Novo Nordisk | [6] |

| | | | | | | |
|---|---|---|---|---|---|---|
| **(NN9924)** | 1 (GLP-1) | | | | | |
| **Interferon lozenges** | Interferon | Oral tablet, absorbed along pharyngeal mucosa into lymphatic system | Idiopathic pulmonary fibrosis (IPF) | Phase III | Amarillo Biosciences | [100] |
| **IN-105** | Insulin | Alkylated PEG-insulin conjugate | Diabetes | Phase III | Biocon | [100] |
| **Oral HDV-1** | Insulin | Hepatic-Directed Vesicle insulin (Liposomal tablets) | Diabetes | Phase III | Diasome Pharmaceiticals | [101] |
| **Capsulin** | Insulin | Axcess™ technology | Diabetes | Phase III | Diabetology | [32] |
| **Capsitonin** | Calcitonin | Axcess™ technology | Osteoarthritis | Phase IIb | Proxima Concepts Ltd | [102] |
| **CaPTHymone** | Parathyroid hormone | Axcess™ technology | Osteoporosis | Phase IIb | Proxima Concepts Ltd | [103] |
| **KLH-2109** | Gonadotropin-releasing hormone (GnRH) antagonist | Oral tablet | Endometrosis | Phase IIb | ObsEva & Kisie Pharmaceuticals Co Ltd. | [104] |
| **ORMD-0801** | Insulin | POD™ | Diabetes | Phase IIb | Oramed | [105] |

| | | | | | | |
|---|---|---|---|---|---|---|
| **NN1953** | Long acting insulin | GIPET® platform | Diabetes | Phase II | Novo nordisk | [106] |
| **NN1954** | Long acting insulin | GIPET® platform | Diabetes | Phase II | Novo nordisk | [106] |
| **NN1956** | Long acting insulin | GIPET® platform | Diabetes | Phase II | Novo nordisk | [106] |
| **NN9926** | Glucagon-like peptide-1 (GLP-1) | GIPET® platform | Type 2 Diabetes | Phase II | Novo nordisk | [106] |
| **Nodlin** | Insulin | Nanoparticle Oral Delivery (NOD) tech | Diabetes | Phase II | NOD pharmaceuticals | [107] |
| **Hexyl-insulin monoconjugate (HIM2)** | Insulin | Insulin modification to enhance stability and absorption | Diabetes | Phase II | Nobex | [108] |
| **Oshadi ICP** | Insulin | Oshadi carrier (ICP tech) | Diabetes | Phase II | Oshadi Drug Administration Ltd | [107] |
| **N/D** | HDV-IFN | NANOVES system | Hepatitis | Phase II | TNT Pharma | [109] |
| **Acyline (Mer 104)** | Gonadotropin-releasing hormone (GnRH) antagonist | GIPET® platform | Prostate cancer | Phase I | Merrion | [110] |

| | | | | | | |
|---|---|---|---|---|---|---|
| **NN1952** | Short acting insulin | GIPET® platform | Diabetes | Phase I | Novo nordisk | [106] |
| **hBNP** | Human B-type natriuretic peptide | Covalent link of amphiphilic oligomers | Congestive heart failure | Phase I | Nobex Corporation | [111] |
| **Oradel ™** | Insulin | Vitamin B12 nanoparticles | Diabetes | Passed preclinical | Apollo Life Sciences | [112] |
| **Intesulin** | Insulin | In vivo encapsulated (IVE) nanoparticles | Diabetes | Advanced preclinical | Coremed | [113] |

Proxima concepts, developed an **Axcess™** delivery system based on an oral capsule that contains the protein or peptide as well as undisclosed GRAS excipients such as permeation enhancers and solubilizers to enhance transcellular absorption. Capsulin, is an enteric-coated insulin capsule developed by Diabetology, Ltd based on Proxima ś proprietary Axcess delivery system and is currently under Phase III clinical trials. The same technology is also currently used to develop Capsitonin (calcitonin) and CaPTHymone (parathyroid hormone) for the indications of osteoarthritis and osteoporosis, respectively, both under Phase IIb clinical trials [114].

Oramed is currently using its **Protein Oral Delivery (POD)** technology to develop an insulin pill, now in Phase IIb clinical trials. POD is an enteric-coated capsule containing an oily suspension of the peptide drug, an enzyme inhibitor (soy bean trypsin inhibitor, aprotinin) and an absoprtion enhancer (EDTA or bile salt), in omega-3 fatty acids. Results from Phase IIa clinical trials showed that POD technology significantly reduced gylcemia in a small group of type 1-diabetes patients and this technology was safe and well tolerated [105].

Sodium caprate ($C_{10}$), is one of the main constituents of the **GastroIntestinal Permeation Enhancement Technology (GIPET$^{TM}$)**, an enteric coated solid dosage form currently designed by Merrion Pharmaceuticals to protect the peptide in the acidic gastric medium and ensure its release in the small intestine. It is anticipated that the GIPET has the potential to boost permeation of such peptides as acyline (Mer 104), a gonadotropin-releasing hormone antagonist under phase I clinical trials for treatment of prostate cancer, and is currently licensed to Novo Nordisk for several oral insulin formulations; (NN1952, NN1953, NN1954, NN1956) and GLP-1 agonists (NN9926), which have completed Phase I clinical trials [110].

The **robotic pill** is one of the most recent and novel technology platforms, developed by Rani Therapeutics for the oral delivery of large molecules including peptides, proteins and antibodies. This robotic pill consists of a capsule made of biodegradable material (PLGA), which contains a valve separating citric acid and sodium carbonate in two chambers. The capsule also contains a balloon-like structure containing sugar microneedles preloaded with peptides [115]. The capsule dissolves in the small intestine exposing the valve, causing the citric acid and sodium carbonate to react together releasing carbon dioxide that inflates the balloon. As a result the microneedles loaded with the drug push into the intestinal wall (intra-enteral injection), before detaching from the capsule and slowly dissolving. Rani therapeutics

in partnership with Novartis is currently employing this technology to develop an oral insulin product. Preclinical studies showed very promising results with oral bioavailability over 50 %. Traverso *et al* also recently demonstrated proof-of-concept experiments in swine that microneedle-based delivery has the capacity for improved bioavailability of macromolecules (e.g. insulin). The pill's needles are initially coated by a pH-responsive coating which dissolves in the desired location in the GI tract, revealing the microneedles (solid, drug-containing microneedles fabricated from biocompatible polymers). These detach from the capsule and become lodged in the GI tissue, where they slowly release their payload. Moreover, the authors showed that microneedle-containing devices can be passed and excreted from the GI tract safely [116]. These findings strongly support the potential of implementation of microneedle technology for use in the GI tract.

The **Nanoparticle Oral Delivery** technology, developed by NOD Pharmaceuticals, together with its subsidiary, Biolaxy, comprises enteric coated and bioadhesive calcium phosphate nanoparticles with sizes between 5 – 200 nm in the final dosage form of a capsule. The formulation is obtained by combining the peptide with calcium phosphate in the presence of PEG salts of fatty acids (e.g. caprylate, sodium caprate) and bile salts as precipitating agents. The obtained calcium phosphate nanoparticles are then enteric-coated using cellulose acetate phthalate and a bioadhesive polymer, carbomer. Nodlin is an oral insulin formulation for basal insulin supplementation currently in Phase II trials in China whilst Nodexen (phase I) is an oral exenatide formulation. Both formulations are developed by Shangai Biolaxy using the NOD technology platform [117].

TNT Pharma utilizes the company´s proprietary cell-targeted, bio-nanotechnology-based system called Nanoves® for the oral delivery of HDV-IFN and Parathyroid hormone currently in phase I and preclinical trials, respectively. The NanoVes System is composed of 20 – 50 nanometer sized phospholipid-based bio-nanoparticles that are compatible with both subcutaneous (SC) injection and oral delivery. The active pharmaceutical ingredients are incorporated into the carrier by electrostatic and hydrophobic interactions and are delivered to the desired cellular targets. This level of cell targeting and delivery is supported by a large database of histopathological results that show clear endocytosis of the NanoVes System, *in vitro* and *in vivo*, including human pharmacological data [109]. Oshadi Drug administration developed the Oshadi ICP an oral formulation consisting of: insulin, proinsulin and C-peptide in the **Oshadi**

**carrier**. This invention comprises, pharmacologically inert silica nanoparticles having a hydrophobic surface, a polysaccharide, and a biologically active protein or peptide suspended in an oil. The company carried out Phase II clinical trials in type 1 diabetes patients in 2014.

With regard to **peptide modification**, a polyethylene glycol side chain at position B29 improves the stability and increases the solubility of insulin (IN-105, by Biocon), promoting its rapid absorption. IN-105 is currently under Phase III clinical trials which have showed that timing of the IN-105 administration before meals is crucial to achieve a substantial glucose lowering effect [118].

### 3.3.2. Local delivery via the oral route

Most of the oral peptides in the market or in advanced developmental stage are characterized by local GIT activity. For instance, Constella oral capsules used for the treatment of irritable bowel syndrome (IBS) with constipation (produced by Allergan and Iron wood Pharmaceuticals) contain the peptide linaclotide, which works locally in the gut, where it binds to receptors called guanylate cyclase C receptors, found on the walls of the intestine. It reduces abdominal pain and increase the amount of fluid in the gut, which helps loosen the stools and speed up their movement through the bowel [119].

Taken as a tablet once-a-day, plecanatide designed by Synergy pharmaceuticals, mimics the function of natural uroguanylin by working locally in the upper GI tract to activate and regulate fluid movement required for normal bowel function. Whilst dolcanatide, is designed to be highly stable and resistant to proteolysis in gastric and intestinal fluids but still operate in the same manner as natural uroguanylin. Phase III clinical trials were completed in 2015 with exceptionally good results and NDA were filled in early 2016 [120].

Enzymes such as pancreatic lipases have been routinely administered orally for decades (such as Creon), they are adapted by nature to the GIT environment and there has been a misconception that they do not need any strategic delivery. However, there is also innovation in this area. For example, Sollpura® (Liprotamase, Phase III) and Zenpep®, (marketed, 2009) are formulations intended for pancreatic enzyme replacement therapy (PERT) and for low digestive enzyme levels developed by Anthera and Forest pharmaceuticals, respectively [121].

**Table 4 (b):** Selected oral peptide formulations under clinical development (local GIT action).

| Brand name | Drug | Technology | Indication | Phase of development | Company | Reference |
|---|---|---|---|---|---|---|
| **Constella®** | Linaclotide | Delayed-release capsule | Irritable-bowel syndrome (IBS), | Market, 2012 | Allergan/Iron wood Pharmaceuticals | [119] |
| **ZENPEP** | Pancrelipase | Delayed-release capsule | Low digestive enzyme levels, | Market, 2009 | Forest Pharmaceuticals,Inc | [119] |
| **Sollpura®** | Liprotamase | Delayed-release capsule | Exocrine pancreatic deficiency | Phase III | Anthera Pharmaceuticals | [121] |
| **Plecanatide & Dolcanatide** | Uroguanylin peptide analogs | Modified peptide for resistant against proteolysis | Chronic idiopathic constipation (CIC) & Ulcerative colitis (UC) | Phase III | Synergy pharmaceuticals | [122] |
| **Macrilen™** | Ghrelin antagonist | Chemical modification of peptide | Adult growth hormone deficiency (AGHD) | Phase III | Aeterna Zentaris | [123] |
| **VEN 120** | Recombinant lactoferrin | Modified peptide | Inflammatory Bowel Disease | Phase II | Ventria Bioscience | [124] |
| **ALV003** | Cysteine protease (EP-B2) | Delayed-released capsule | Colitis | Phase IIb | Alvine Pharmaceuticals | [125] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Prolyl endopeptidase (PEP) | | | | | |
| **PLD-116** | Undisclosed peptide | Delayed-release capsule | Inflammatory bowel disease | Phase II | PLIVA | [126] |
| **IL-23R** | IL-23 receptor | Protagonist ś platform | Crohn ś disease | Phase I | Protagonist Therapeutics Inc | [127] |

## 4. General remarks and conclusions

As outlined in this article, it is clear that several pharmaceutical companies have led the efforts to develop “needle free” protein/peptide therapeutics and the most popular route being investigated is the oral route, more especially for metabolic disorders. This is mainly influenced by the fact that from a patient perspective, the oral route is simpler, cost effective and more convenient.

General setbacks, which are common across all mucosal delivery routes, arise due to the excipients added in most of the formulations including penetration enhancers and enzyme inhibitors. Many of these delivery strategies may compromise the barrier function of the epithelia to incoming toxins, and implications of their long-term use in managing chronic disease such as diabetes should be considered. These possible implications raise concerns and cause both the general public and the FDA to be cautious with these innovations; hence their development and advancement into the market are hindered.

Mucosal systemic bioavailabilities should be reproducible and reliable to achieve a regulatory filing, but incompatibility of hydrophilic peptide and protein drugs with hydrophobic delivery carriers can produce uncontrolled drug release which also handicapped the advancement to the market. Moreover, mucosal bioavailabilities are still much lower than those of injected doses, and therefore much higher doses must be administered to have the same effect. For more expensive peptide and protein drugs, this could preclude their mucosal delivery and the toxicity implications of administering relatively large doses of peptide and protein drugs remain a concern.

Needle phobia and stress leading to low patient compliance plus the inconvenience and side effects associated with injecting drugs have encouraged scientists to investigate and exploit all promising noninvasive routes for peptide/protein delivery. In this review we presented the most common and highly sought-after, ‘noninvasive’ mucosal routes, specifically; nasal, pulmonary and oral. Many approaches have been used to study various strategies to overcome the inherent barriers to peptide uptake across mucosal routes. Each of the various routes has its own set of favorable and unfavorable properties and these were outlined. Amongst the three routes discussed here, tremendous work has been done for oral peptide delivery (including insulin). Although extensive human clinical studies are still required for most of the peptides presented here, especially of long-term clinical applications, taken together, the

technologies and developments described here are likely to increase the number of marketed needle free delivered peptides and proteins.

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# Background, Hypothesis and Objectives

## Background

Nanocarriers can overcome critical barriers associated with the oral route of administration including: (i) the harsh gastrointestinal environment (pH range 1.2-7.4) with high concentrations of enzymes specialized in digestion, (ii) the thick mucus barrier (100 μm) subjected to continuous turn-over, and (iii) the intestinal epithelium, which acts as a gatekeeper and is poorly permeable to macromolecules [1, 2]. Our group pioneered the development of a delivery platform, named as polymer nanocapsules, consisting of an oily core surrounded by one or more layers a hydrosoluble polymers [3]. Specific prototypes including chitosan nanocapsules and polyarginine nanocapsules have been found efficient for the delivery of peptides and anti-cancer drugs [4]. These nanosystems portrayed remarkable penetration enhancing properties and stability in biological media, which was further enhanced by introducing PEG-stearate into the shell composition [4, 5].

Protamine is a natural cationic polymer with the intrinsic capacity to translocate through mammalian cell membranes. This ability has been attributed to its arginine-rich sequence, which promotes direct interaction with cell surface domains and subsequently facilitates cellular internalization [6–8]. Protamine is an FDA approved drug indicated for reverting the anticoagulant effects of heparin. It is also present as an excipient, in a parenteral insulin formulation (NPH, Neutral Protamine Hagedorn) [9–12].

Our group has designed a variety of nanocarriers involving protamine. Namely, Protamine nanoparticles and nanocapsules were designed for the delivery of peptides and antigens, whereby they improved their cell internalization[13, 14]. More recently, the group developed multilayered nanoparticles made of protamine and polyarginine in combination with polysaccharides, dextran sulphate and alginate, also intended for intracellular antigen delivery [15].

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**Hypothesis**

3. Protamine could be a promising biomaterial for the development of nanocapsules for the efficient oral delivery of macromolecules such as insulin. The ability of protamine to translocate through biological cell membranes could be combined with the capacity of lipids and surfactants to enhance the intestinal permeation of drugs.
4. The rational selection and combination of protamine with other polymers with stabilizing and muco-penetration properties, including PEG and other polysaccharides, could result in an optimized nanocarrier system able to associate the peptide, protect it from degradation and deliver it across the epithelial barrier.

**Objectives**

Based on the background evidence and hypothesis outlined, the main goal of the present work is to rationally design and develop a new protamine based nanocarrier system for the efficient oral delivery of peptides using insulin as a model peptide drug.

For this purpose the overall aim is split into the following objectives with their specific tasks;

**1) Development of insulin loaded protamine nanocapsules**

1. To prepare and characterise protamine nanocapsules (blank and insulin loaded) with regard to their physicochemical properties, mucodiffusion, stability upon incubation in intestinal biological media and stability during storage.
2. To evaluate their ability to load insulin, prevent the degradation of the peptide by pancreatic enzymes and study the *in vitro* release profile.
3. To demonstrate the stability of insulin loaded protamine nanocapsules in different simulated intestinal media and confirm the conformational structure of insulin after encapsulation.

The results corresponding to the work described here are presented in **Chapter 2:** Rational design and *in vitro* characterization of protamine-based nanocapsules for oral peptide delivery.

**2) Assessment of the *in vitro* mechanistic and toxicity of protamine nanocapsules**

4. To determine the toxicity, penetration and mechanism of action of the nanocapsules using *in vitro* cell models simulating the different mucosal barriers (e.g. enterocytes and mucus) of the intestinal epithelium.
5. To evaluate the toxicity and permeation enhancing property of the nanocapsules *ex vivo* in human intestinal tissue.

The results corresponding to the work described here are presented in **Chapter 3:** The interaction of protamine nanocapsules with the intestinal epithelium

**3) Evaluation of particle interaction with the epithelial barrier including biodistribution and *in vivo* efficacy of the nanocapsules**

6. To elucidate the *in vitro* immunological response of protamine and polysialic acid coated protamine nanocapsules in dendritic cells
7. To study the biodistribution of the fluorescent protamine nanocapsules after their oral administration to mice
8. To determine the *in vivo* efficacy bioactivity of the new nanocarrier in non-diabetic rats.

The results corresponding to the work described here are presented in **Chapter 4**: Protamine nanocapsules: a versatile vehicle for oral insulin delivery.

In addition to these major objectives we have also aimed to explore new technologies for the production of protamine nanocapsules, i.e. the self-emulsifying technique which could exclude the use of organic solvents and consequently simplify the preparation method. However, because this technology was used as a proof of concept

and only applied for a different model peptide (cyclosporine), the corresponding data will be presented in **Annex 1**: Development and characterization of solvent-free protamine nanocapsules as carriers for mucosal delivery of therapeutics.

# CHAPTER 2

## Rational design of protamine nanocapsules as carriers for oral peptide delivery

# Abstract

Peptides represent a promising therapeutic class with the potential to alleviate many severe diseases. Along with the increased understanding of the biological barriers, the development of nanoparticulate carriers, aimed at producing "needle-free" pharmaceutical products represents a challenging but promising strategy. The objective of this work has been the rational design of polymer nanocapsules (NCs) intended for the oral delivery of peptide drugs. For this purpose, we selected insulin as a model peptide. The polymer shell of the NCs was made of protamine, a polypeptide selected for its cell penetration properties. For the formation of the lipid core a variety of components were investigated with respect to their capacity to help the NCs to overcome the intestinal barriers and also to entrap and control the release of insulin. From this screening, we selected specific nanocapsules compositions, which contained PEG stearate 40 (PEGst 40) and sodium glycocholate (SGC), as surfactants. In addition, we studied the potential benefit of a double protamine/polysialic acid (PSA) layer in terms of preserving the stability and controlling the release of the loaded peptide. Insulin-loaded protamine nanocapsules, prepared by the solvent displacement method, exhibited an average size of 300 nm, a neutral surface charge and a spherical shape. Stability studies performed in simulated intestinal media containing enzymes indicated that protamine NCs were stable and able to protect insulin from the harsh intestinal environment and that this capacity could be further enhanced with a double PSA-Protamine layer. Moreover, these NCs were able to control the release of insulin for up to 6 h. Finally, these NCs could be freeze-dried and stored at room temperature without alteration of the colloidal stability upon resuspension in intestinal fluids. In conclusion, both, single layer and double layer protamine/PSA NCs, fulfil a number of key properties that are expected to be critical with regard to their utility for oral peptide delivery.

## 1. Introduction

The number of peptide drugs in the industry pipelines has significantly increased in the last decades due to their high therapeutic efficacy and excellent selectivity. Unfortunately, their utility has been counter-limited due to their difficulties for crossing barriers and, hence the impossibility of being administered by the oral route [1]. Advances in nanomedicine have opened new opportunities for the oral delivery of biomolecules [2]. Indeed, a variety of nanocarriers, among them, liposomes [3], nanogels [4], polymeric nanoparticles (NPs) [5–7] and nanocapsules (NCs) [8], have been reported to reduce the exposure of the associated peptide to the adverse conditions in the GI tract and enhance its transport across the intestinal mucosa. Having as a reference the original work by Damgé *et al*. [9] which showed for the first time the possibility to enhance insulin absorption using poly(cyanoacrylate) NCs our group explored the potential of chitosan NCs for the oral administration of salmon calcitonin [10]. The positive results were attributed not only to the composition of these nanocapsules but also to their structural organization. NCs are composed of a core, which may act as a protective reservoir for drug and a surrounding shell [11], which can be rationally designed in order to confer the NCs with adequate stability, mucodiffusion, and permeability properties [12]. Therefore, such a delivery vehicle offers interesting opportunities for a rational design intended to further enhance the already shown potential for oral peptide delivery

From our perspective, another type of NCs of potential interest for oral peptide delivery is the one making use of the cationic polypeptide protamine. These NCs, which were originally designed in our research group for the delivery of antigens [13], might help enhancing the transport of peptides across the intestinal epithelium. In fact, protamine is known to exhibit membrane-translocation properties attributed to its arginine-rich sequence [14], and because of this special property, protamine has been proposed for enhancing nose-to brain delivery [15] as well as for enhancing insulin intestinal absorption [16]. In this latter work, the authors found that the systemic pharmacological bioavailability of insulin conjugated with low molecular weight protamine (LMWP) increased 5-fold compared to native insulin during in situ loop absorption tests in rats. Moreover, from the toxicological point of view, protamine has the advantage of being part of formulations approved by the FDA. Namely, it is used

to revert the anticoagulant effects of heparin and it is also part of a long-acting injectable insulin formulation [17, 18].

On the other hand, taking into account the cationic charge of protamine, we found it important to explore the value of adding to the nanocapsules and extra-layer of polysiaclic acid (PSA). The rationale was that by virtue of the hydrophilicity of PSA, we could form a "watery cloud" around the nanocarrier system that might hamper the interaction of the NCs with other molecules, e.g. proteolytic enzymes [19, 20]. In fact, the chemical conjugation of PSA to peptides [21] or the decoration of nanocarriers using this polymer [20] are approaches that have been exploited to protect peptide molecules from degradation and improve their pharmacokinetics after parenteral administration. On the other hand, taking into account that PSA is naturally a mucus component, it was presumed that this extra-layer would facilitate the mucodiffusion of the NCs across the intestinal mucus layer.

Based on this information, the aim of this study has been to rationally design and develop protamine and protamine/PSA NCs particularly adapted for oral peptide delivery. With this aim in mind, we have explored formulation strategies intended to facilitate the entrapment and the controlled release of insulin, used as a model peptide, and to provide the NCs with the capacity to overcome the intestinal barriers (degradation, mucodiffusion and interaction with intestinal epithelium). For this, we also selected auxiliary ingredients including stabilizers such as PEG-stearate and sodium glycocholate, which has an additional role as a penetration enhancer [22, 23].

## 2. Materials and methods

### 2.1. Materials

Protamine sulfate of low molecular weight (5 kDa), used in this work was purchased from Yuki Gosei Kogyo, Ltd., (Tokyo, Japan). The stabilizing surfactants, polyoxyethylene 40 and 100 monostearate (PEGst-40 and PEGst-100), were purchased from CRODA Europe Ltd, (Snaith, UK), whilst sodium cholate (SC) and sodium glycocholate (SGC), from Dextra, (Reading, UK). Caprylic/capric triglyceride (Miglyol® 812) was purchased from Cremer, Oleo Division, (Witten, Germany). Colominic acid sodium salt (Polysialic acid, PSA) was purchased from Nacalai

Tesque, INC, (Tokyo, Japan). Insulin glulisine (Apidra®) was obtained from Sanofi (Paris, France). Rhodamine 6G was purchased from empBIOTECH, (Berlin, Germany). Triton x-100 was purchased from Sigma Aldrich (St.Louis, USA), whilst anhydrous monobasic sodium phosphate, maleic acid, calcium chloride, sodium oleate (purity >82%) and acetic acid were purchased from Scharlab (Barcelona, Spain). Pancreatin (8 % USP specification), was purchased from Biozym (Hamburg, Germany). Sodium taurocholate, lecithin, sodium hydroxide and glyceryl monooleate were obtained from Fluka (Buchs, Switzerland). Mucin was purchased from sigma Aldrich (Spain). Organic solvents were of HPLC grade and all other products used were of reagent grade purity or higher.

### 2.2. Preparation of protamine nanocapsules

Blank protamine NCs were prepared by the solvent displacement technique following the procedure described and optimized by our group [24]. Initially, for the core, Miglyol® with a combination of different surfactants (SC, SGC, PEGst-40 and PEGst-100) and different concentrations, were explored and finally for the selected formulation, PEGst-40 (16 mg), SGC (5mg) and Miglyol® (62.5 µL) were dissolved in 3ml ethanol to obtain a clear lipid phase. Acetone (1.95mL) was then added and this organic phase was immediately poured over 10 ml of an aqueous phase containing 0.15% w/v protamine. The elimination of organic solvents was performed by evaporation under vacuum (Rotavapor Heidolph, Germany), to obtain a NC formulation with a constant final volume of 5mL. Subsequently; protamine NCs were isolated by ultracentrifugation (Optima™ L-90K, Ultracentrifuge, Beckman Coulter, USA) at 30000 rpm for 1h (at 15 ℃) and resuspended in ultrapure water to the initial volume.

Optionally, protamine NCs were provided with an additional coating layer consisting of PSA. For this purpose, a volume of 5mL, protamine NCs (concentration 18.66 mg/mL) was incubated with 1ml solution of PSA at a concentration 3 mg/mL. The final Protamine:PSA ratio was of 5:1 (w/w).

Insulin-loaded protamine and PSA-protamine NCs were prepared following the same procedure for blank NCs (section 2.2), by incorporating insulin (1.5 mg, dissolved in 0.01M HCl, 0.05 mL) into the lipid phase before mixing it with the aqueous phase containing 0.15% w/v protamine.

### 2.3. Physicochemical and morphological properties of protamine NCs

The average hydrodynamic diameter, polydispersity index (PDI) and zeta potential of protamine NCs were measured by a Zetasizer® Nano-ZS, Malvern instruments, (Worcestershire, UK) after dilution (50x) with ultrapure water. The transmission electron microscopy (TEM, Joel 2010, 80kV, Philips, Netherlands) was used to analyze the shape and surface properties of the NCs. For TEM analysis samples were deposited on a copper grid and stained with 2% (w/v) ammonium molybdate solution, allowed to dry and then viewed under a the TEM.

### 2.4 Mucodiffusion studies on protamine nanocapsules

To study mucodiffusion, a capillary (0.6 square internal diameter (ID) mm x 0.12 wall (mm), VitroCom, New Jersey), as filled up to three quarters from one end with mucin (5% (w/v) mucin in SIF). Then, rhodamine 6G-labeled protamine nanocapsules were introduced through the opposite end of the capillary using a syringe pump (flow rate = 14 $\mu L.m^{-1}$) in a vertical position without disturbing the mucin-nanocarrier interface. The capillary was then incubated for 10 minutes at 37 ℃, after which it was analysed under a fluorescence microscope (Leica AF6000, Leica microsystems, Germany). The diffusion of the fluorescent nanocarriers through the mucin was monitored by taking snap-shots every 5 seconds. This allowed us to determine the time taken by the nanocarriers to diffuse across 1 mm of the mucin gel. Chitosan (CS) NCs containing exactly the same core as protamine NCs were used as a control since CS is well known for its mucoadhesion property.

### 2.5. Association efficiency (AE%) and loading capacity (LC)

The association efficiency (%) was determined using the direct method, which involves the extraction of insulin from the NCs by the complete disruption of the NCs. Briefly; insulin loaded NCs (0.1 mL) were digested using a combination of acetonitrile (0.1 mL), Triton™ X-100 (0.05 mL) and 0.1 % TFA (0.75 mL). Then the formulation was vortexed at a high speed to obtain a clear aliquot. To corroborate this data, free insulin in the aqueous phase (undernatant) was quantified after isolating the NCs (indirect method). The concentration of insulin in both cases was determined by reverse phase liquid chromatography (HPLC). A 25 µL aliquot of each sample was injected in triplicate onto the HPLC column. Chromatography was performed using a

Agilent 1100 series auto-sampler, equipped with an Agilent 1100 series controller and Dual Absorbance Spectrometer (Agilent 1100 series, Agilent technologies, Germany) with an ATRP11 column (Symmetry C8 5 µm (3.9 x 20 mm)) (Waters, Spain) and a UV detector set at 227 nm. The flow rate was set to 2 mL/min. The gradient was obtained by mixing proportion of phase A (0.1 % TFA) and phase B (acetonitrile:0.1 % TFA, 8:2). Initially, the mobile-phase composition was 25% B; a linear gradient was applied to reach a composition of 25% B after 4min. Quantification was achieved by comparing observed peak area ratios of insulin in the samples to a calibration curve obtained under the same experimental conditions. Linearity was observed in the range from 0.005 to 0.5mg/mL with a correlation coefficient above 0.9995. The detection limit (LOD) was 0.001mg/mL and the quantification limit (LOQ) was 0.005 mg/mL. The association efficiency (AE%) and loading capacity (LC%) were calculated using Equation 1 and 2:

**Equation 1** $$\boldsymbol{AE}\ \% = \frac{\boldsymbol{Insulin\ in\ destructed\ nanocapsules}}{\boldsymbol{Total\ insulin}} \times \mathbf{100}$$

**Equation 2** $$\boldsymbol{LC}\ \% = \frac{\boldsymbol{Total\ insulin \times AE}}{\boldsymbol{Total\ weight\ of\ NCs}} \times \mathbf{100}$$

### 2.6. Conformational stability of loaded insulin

To evaluate the conformational stability of insulin against preparative stress, circular dichroism (CD) spectroscopy was used. For this study, the destruction method used in section 2.4 above, could not be applied since the presence of Triton™ X-100 interfered with the spectrum reading during analysis. For this purpose, protamine and PSA-protamine NCs were completely disrupted using a combination of chloroform, methanol and acidified milliQ water at pH 3.46 to obtain a final concentration of insulin at 0.125 mg/ml. The aliquots were then centrifuged at 14000 rpm for 10 minutes at 25 ℃ to obtain a clear supernatant, which was collected and analysed for insulin conformation by CD. A solution of insulin in the same medium at a concentration of 0.125 mg/ml was used as a control. Spectra were collected at 20 ℃, using a 0.5 nm step size, over a wavelength range of 180–280 nm, using a 1 mm quartz cylindrical cell; a band width of 1 nm and a scanning speed of 500 nm/minute,

with a 0.25 second response time, were applied (Jasco J-1100; Jasco Corp, Tokyo, Japan).

The α-helical content of the protein was estimated according to the following equation:

**Equation 3** $$\%\,\boldsymbol{\alpha - helical\ content} = \frac{\boldsymbol{\theta mrd - 4000}}{\boldsymbol{33000 - 4000}}$$

Where, *θmrd* is the mean molar ellipticity per residue at 208 nm (deg cm2 d/mol). Raw data from the experiment, expressed in terms of θd (the ellipticity in units of mdeg) were converted to mean molar ellipticity (θmrd) per residue, using the following equation:

**Equation 4** $$\boldsymbol{\theta mrd} = \frac{\boldsymbol{\theta d\ M}}{\boldsymbol{10CLN}}$$

Where, *M* is the insulin molecular weight (Da), *C* is the insulin concentration (mg/ mL), *L* is the sample cell path length (cm), and *N* is the number of amino residues [25].

### 2.7. Colloidal stability of protamine/polysialic acid NCs and release studies in simulated biological media

The colloidal stability of the NCs was assessed by monitoring the size, PDI and count rate (particle concentration). Different simulated intestinal media were used (prepared according to the USP XXIV) in order to mimic the intestinal environment after oral delivery. Simulated intestinal fluid (SIF) at pH 6.8 and biorelevant media simulating preprandial and postprandial conditions in the upper small intestine, including fasted-state simulated intestinal fluid (FaSSIF) and fed-state simulated intestinal fluid (FeSSIF), were also employed in this study according to the updated (V2) versions described by Jantratid et al. in order to better mimic in vivo conditions. FaSSIF-V2 has a lower amount of lecithin, whereas FeSSIF-V2 combines the postprandial changes in pH, buffer capacity, osmolality, and bile component concentrations, in addition to lipolysis products; glyceryl monooleate and sodium oleate, with *in vivo* correlating concentrations (**Table 1**) [26, 27].

**Table 1:** Compositions of simulated intestinal fluid (SIF), fasted state, simulated intestinal fluid (FaSSIF-V2) and fed state simulated intestinal fluid (FeSSIF-V2). Adapted from Jantratid *et al*. [27].

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

For the stability studies, insulin loaded protamine NCs were diluted 50x and incubated under moderate shaking at 37 ℃ in the different media: SIF, pH 6.8, FaSSIF-V2, pH 6.5 and FeSSIF-V2, pH 5.8. Samples were collected at times 0, 0.5, 1, 3 and 6 h. The FeSSIF-V2 samples were centrifuged at 5000xg for 5 minutes to eliminate pancreatin aggregates before analysis. The evolution of size distribution, PDI and count rate of the NCs were monitored by dynamic light scattering on a Zetasizer® Nano-ZS, Malvern, UK.

For *in vitro* release studies, insulin loaded NCs were incubated in SIF, pH 6.8 and FaSSIF-V2, pH 6.5 at a ratio of 1:20, NCs to the medium and shaken at 100 rpm at 37 ℃ using a constant-temperature shaker. At specified time intervals (0, 0.25, 0.5, 1, 3 and 6 h); the supernatant was collected by ultracentrifugation (section 2.2). The concentration of insulin in the supernatant was determined by reverse phase HPLC method (section 2.4).

### 2.8. Insulin stability in simulated intestinal fluids containing enzymes

The ability of protamine NCs to protect the associated insulin was evaluated by quantifying the amount of insulin remaining in the NCs after proteolysis. For this purpose, insulin-loaded protamine NCs, PSA-protamine NCs and free insulin solution (as control) were diluted by 1:1 v/v with FeSSIF-V2 then incubated in a 37 ℃ incubator and shaken at 100 rpm for up to 2 hours. At predetermined time intervals (0, 0.25, 0.5, 1 and 2 h), 500 µL aliquots were withdrawn and the enzyme activity of pancreatin in the samples was terminated by the addition of 300 µL ice-cold 0.1 M HCl. The samples were subsequently treated with acetonitrile (0.01 mL) and Triton x-100 (0.01 mL) and vortexed, to disrupt the NCs and extract insulin, which was then analyzed by HPLC to determine the amount of insulin remaining in the NCs.

### 2.9. Stability during storage

The colloidal stability of insulin-loaded NCs was followed during a period of 6 months at different temperatures (4 ℃, 25 ℃ and 40 ℃) and RH conditions, as recommended by ICH guidelines. Samples of the three different batches were withdrawn at predetermined time intervals, followed by determining particle size, zeta potential and insulin leakage coupled with observing the suspension appearance to ensure continued stability.

### 2.10. Freeze-drying studies

Insulin-loaded protamine NCs and PSA-protamine NCs (1% w/v) were lyophilized (Labconco Corp, USA) in presence/absence of trehalose or sucrose at 5 % (w/v) as cryoprotectors. Samples were frozen at -20 ℃ and then subjected to an initial drying step at -40 ℃ followed by a secondary drying at 0 ℃, both steps lasting for 43 hours at a high vacuum atmosphere (200 mTorr). Finally the temperature was increased slowly up to room temperature (+22 ℃) till the end of the process. The freeze-dried formulations were resuspended in ultrapure water by manual shaking and their physicochemical characteristics were evaluated as mentioned in section 2.3.

### 2.11. Statistical

Statistical analysis was performed using GraphPad Prism 5 program (CA, USA). One-way ANOVA in multiple comparisons was applied for comparisons. Differences were considered statistically significant at $*p<0.05$ and all results are expressed as mean ± SD.

## 3. RESULTS AND DISCUSSION

In this study, protamine NCs were rationally designed taking into account different criteria: (i) their capacity to load and control the release of insulin, while protecting it from degradation in intestinal fluids; (ii) their colloidal stability in intestinal fluids; (iii) their penetration across the mucus layer and, (iii) their ability to interact with the underlying epithelium.

### 3.1. Preparation and characterization of protamine and protamine/polysialic nanocapsules

Protamine NCs are reservoir-type systems composed of an oily core and a protamine-coating layer. As indicated earlier, all ingredients and their organization into the nanostructure were rationally selected. The selection of protamine was motivated by its cell penetrating properties [14] and its already shown capacity to enhance the intestinal insulin absorption [16]. On the other hand, a second layer around protamine was formed with PSA, with the idea of enhancing the colloidal stability in the presence of enzymes [21]. The lipid core, i.e. Miglyol® (a medium chain Caprylic/capric triglyceride), of the NCs was expected to help protecting the peptide cargo from degradation as well as to enhance its intestinal permeability [28, 29]. The surfactants selected to disperse the oil in the external water phase were PEG stearate and bile salts. The presence of the PEG molecules oriented towards the external aqueous phase was thought to enhance the stability of the NCs in the presence of enzymes [30, 31], as well as promote their diffusion across the mucus [32, 33]. Two types of PEG-stearates with PEG chain lengths of 40 and 100 units (PEGst-40 and PEGst-100) were initially selected and compared. On the other hand, two different bile salts sodium cholate (SC) and sodium glycocholate (SGC), known for the penetration enhancing properties were also selected and compared. The structural organization of these nanocapsules is illustrated in **Figure 1**.

The results of the physicochemical characterization of prototypes selected from an extensive screening of ingredients in different quantities are presented in **Table 2.** The chosen prototypes emanated from stability studies (discussed later on) and their acceptable physicochemical properties. The results indicate that for the compositions selected (described in the Materials and Methods section 2.2), the size of the NCs is

within the 200-300 nm range (PDI < 0.3) irrespective of their composition. On the other hand, the zeta potential values varied between -6 mV/+30 mV depending on the type of polymer shell (PSA *vs.* protamine) and also on the type of PEG stearate used (PEGst-40 *vs.* PEGst-100), **(Table 2**). The positive charge on protamine NCs, as compared to the negatively charged control nanoemulsions, is an indicator of the presence of the protamine shell around the lipid core. On the other hand, the type of surfactant also influenced the resultant surface charge. Namely, the use of SGC resulted in NCs with a higher negative charge, as compared to that of those produced with SC ($p<0.05$). This result may be attributed to the glycol moiety, which is present in SGC molecule but not in the SC molecule. Surprisingly, the presence of PEGst-100 (Mw 2KDa) gave a higher negative charge in the nanoemulsions and a higher positive charge in the nanocapsules compared to PEGst-40 (Mw 4KDa) ($p<0.05$). This result led us to hypothesize that, as the amount of PEGst added to the formulation was the same, the longer PEGst-100 chains are sparsely dispersed on the surface of the oily cores, thus leaving more bile salt acid molecules exposed to the surface.

In a second step, nanocapsules containing PEGst-40 were provided with a second coating layer of PSA. The positive charge of the nanocapsules was supposed to enable the ionic interaction with PSA. The presence of this extra layer was confirmed by the charge inversion of the NCs containing PSA and protamine, as compared to those having only the protamine layer (**Table 2**). The chemical structure of PSA and the schematic representation of the components and their arrangement in the NCs are illustrated in **Figure 1 and 2**, respectively. The double layer PSA/protamine nanocapsules was expected to help improving the stability of the nanocapsules in the presence of proteolytic enzymes. This expected role, could be explained by two different mechanistic perspectives. First, it is known that both, macromolecules and nanocarriers coated/linked to hydrophilic polymers, i.e. PSA, are shielded from opsonisation [19, 20, 35], and this shielding results in their greater stability in the blood circulation. On the other hand, it has been reported that the presence of negatively charged functional groups, i.e. carboxylic moieties, in polymeric materials may result on the inhibition of the proteolytic enzymatic activity by chelating the Ca2+ ions required for normal enzymatic activity [35]. Despite these attractive properties of PSA, to our knowledge, there is no work reported, on the use of PSA-based nanocarriers for oral peptide delivery.

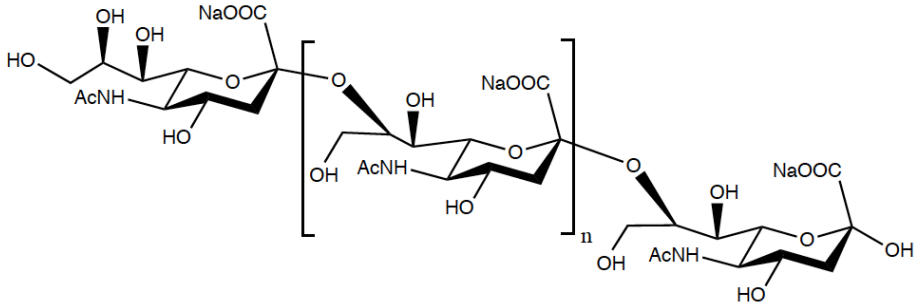



**Figure 1:** Chemical structure of polysialic acid (PSA), also named as colominic acid

**Table 2:** Physicochemical properties of protamine and PSA-coated protamine NCs with their corresponding nanoemulsions, (mean ± SD, n=3, #*p<0.05 significant difference between nanoemulsions containing PEGst40 and PEGst100, and SC and SGC, respectively).

| | | **Control nanoemulsions** | | | **Protamine NCs** | | | **PSA-Protamine NCs** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **PEG** | **Bile salt** | **Size (nm)** | **PdI** | **ζ-pot (mV)** | **Size (nm)** | **PdI** | **ζ-pot (mV)** | **Size (nm)** | **PdI** | **ζ-pot (mV)** |
| **40** | SC | 198 ±7 | 0.1 | -13 ±5 | 207 ±18 | 0.1 | +6 ±5 | 200 ±16 | 0.2 | -4 ±1* |
| | SGC | 214 ±15 | 0.2 | -21 ±2* | 288 ±20 | 0.2 | +8 ±4 | 261 ±24 | 0.2 | -6 ±4* |
| **#100** | SC | 205 ±16 | 0.2 | -25 ±2 | 210 ±25 | 0.2 | +28 ±5 | - | - | - |
| | SGC | 221 ±13 | 0.2 | -32 ±1* | 238 ±16 | 0.3 | +30 ±2 | - | - | - |

The size of the nanocapsules was confirmed by TEM analysis. The images presented in **Figure 3,** also illustrate the spherical shape of the NCs and different appearance of the single layer protamine nanocapsules as compared to the double layer PSA/protamine nanocapsules.

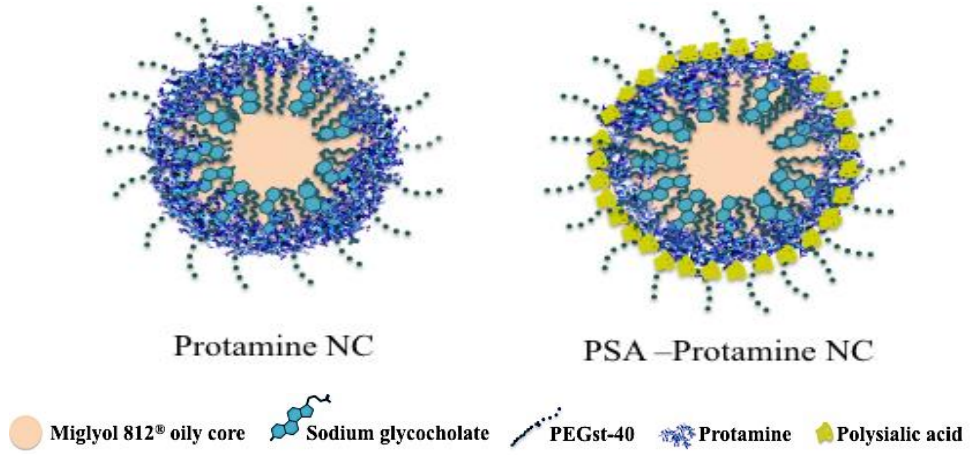



Figure 2: Schematic representation of protamine NCs and PSA coated protamine NCs.

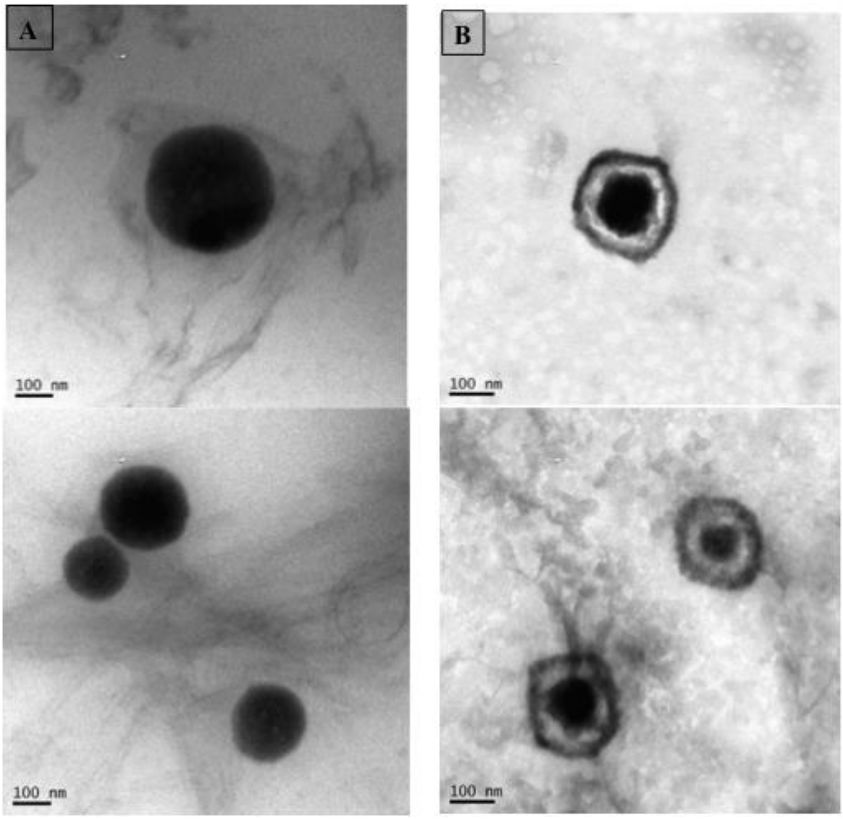



**Figure 3:** TEM images of blank protamine NCs (A) and PSA-coated protamine NCs (B).

### 3.2. Mucodiffusion studies on protamine nanocapsules

To check if the NCs are able to penetrate through mucus, their diffusion rate in mucin (SIF + 5% mucin) was studied in a preliminary mucodiffusion study where the

mucodiffusion enhancing property of PEGst-40 was compared to that of PEGst-100. Herein, protamine NCs containing PEGst-40 were found to diffuse more rapidly through the mucin gel compared to chitosan NCs. Interestingly, the substitution of PEGst-40 with PEGst-100 in the nanostructure led to a clear reduction in the mucodiffusion rate of the NCs. Protamine NCs containing PEGst-100 (Mw 5KDa) took twice the time (300 seconds) taken by protamine NCs containing PEGst-40 (Mw 2KDa) (150 seconds) to diffuse across 1 mm of the mucin gel. These results are in agreement with the higher positive charge observed for protamine nanocapsules containing PEGst 100, which may hamper the diffusion of these nanocapsules. As explained, the hypothesis is that PGEst-40-containing nanocapsules would have a more dense coating of low molecular weight PEG, as compared to those containing PEGst-100, and, would, thereby facilitate the mucodiffusion of the nanocapsules [36]. Similar results have been reported for polystyrene particles (200 and 500 nm). Those coated with 2 KDa PEG were found to diffuse faster through mucus compared to the same NPs with higher MW PEG [37].

### 3.3. Insulin association and stability

Taking into account that insulin is a hydrosoluble polypeptide with and IP of 4.75, the strategy for its entrapment within the oily core was based on tuning its solubility and ionization degree using different pH media. As expected, the results shown in **Table 3**, indicate that both, the pH of the insulin solution and the presence of cholate bile salts influence the insulin association efficiency to the NCs (*$p<0.05$). The highest AE % values were obtained when insulin was dissolved in 0.01M NaOH, pH 9.2 in the absence of a bile salt and also when insulin was dissolved in 0.01M HCl, pH 2 These, apparently contradictory results could be explained as follows. At pH 9.2, insulin has an important net negative charge, which may help its interaction with the positively charged protamine and, thus its encapsulation is favoured in the absence of a bile salt. On the contrary, association to the NCs was highly dependent on the type of bile salt. In this case, the positively charged insulin was supposed to interact with the negatively charged bile salt surfactants in the lipid core. The higher association observed for the nanocapsules containing SGC as compared to those containing SC, might be due to the presence of the glycol moiety in the SGC molecule. In fact, the longer hydrophilic side chain has been associated with higher interaction and activity of SGC compared to SC. [38].

**Table 3:** Association efficiency (AE%) and physicochemical properties of insulin loaded protamine and PSA-coated protamine NCs with insulin (IP=4.75) at different pH, (mean ± SD, n=3, $p<0.05$ statistical difference in #size and in *AE).

| Variations | | Protamine NCs | | | | PSA-protamine NCs | | | |
|---|---|---|---|---|---|---|---|---|---|
| Bile salts | pH of insulin | Size (nm) | ζ-pot (mV) | AE% | Final pH | Size (nm) | ζ-pot (mV) | AE% | Final pH |
| No cholate | 9.2 | #425 ±82 | +2 ±1 | 76 ±9 | 6.2 | 364 ±43 | -1 ±1 | 48 ±7 | 5.0 |
| | 2.0 | 298 ±83 | +1 ±2 | *22 ±5 | 5.6 | - | | | |
| SC | 9.2 | 287 ±46 | +3 ±2 | 27 ±5 | 5.3 | - | | | |
| | 2.0 | 266 ±75 | +8 ±3 | 38 ±4 | 5.1 | 206 ±14 | -5 ±3 | 32 ±4 | 4.7 |
| SGC | 9.2 | 295 ±56 | +4 ±1 | 29 ±2 | 6.0 | - | | | |
| | 2.0 | #382 ±29 | +6 ±3 | *62 ±16 | 5.2 | 321 ±33 | -4 ±1 | 51 ±6 | 4.8 |

Regarding the effects of the amount of each of the components in the NCs on the AE % of insulin, it was observed that whilst the presence of SGC and PEGst-40 confers advantageous properties to the system, such as enhanced stability, high amounts of these components compromise the insulin AE. We found that there is an optimum balance between the amounts of the components used that favoured the loading of insulin.

Although the HPLC analysis used to quantify the association efficiency provided a good indication of the insulin stability (100% insulin was recovered after analysis of associated and non-associated insulin), a subsequent circular dichroism (CD) analysis was performed as the conformation of insulin is critical for its optimal therapeutic effect. Since α-helices are one of the elements of the secondary structure, the quantitative analysis of the structural change of insulin could be evaluated by the content of the preserved α-helices. **Figure 5** shows the CD spectra of insulin (glulisine) secondary structures before and after the formulation process. The far-UV CD band at 208 nm primarily arises from the α-helix structure. The calculated percentage of α-helix in insulin glulisine (control) and insulin glulisine entrapped in protamine NCs and PSA-protamine NCs are 38.0, 36.8 and 38.4 % respectively. In other words, no significant conformation change was noted after loading insulin into protamine based NCs.

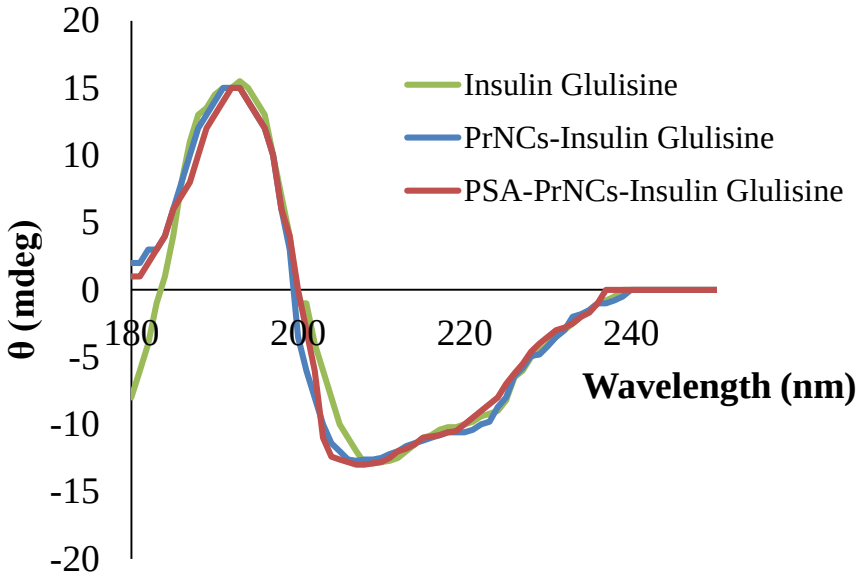



**Figure 5:** CD spectra of free insulin glulisine and insulin glulisine associated in protamine NCs and PSA-protamine NCs after the formulation process. The insulin concentration was kept constant at 0.125 mg/ml at pH3.46 in all aliquots. (Mean ± SD, n=3, *p<0.05).

### 3.4. Colloidal stability of protamine-based NCs in biological fluids

To ascertain that the NCs preserve their physicochemical properties under physiological conditions we evaluated the colloidal stability of nanocarriers in simulated intestinal fluids including simple SIF, FaSSIF-v2 and FeSSIF-v2. The stability of the NCs was determined by monitoring the size, PDI, and count rate of the NCs using the DLS technique. Preliminary stability studies revealed that PEGst-40 conferred a greater stability to the NCs than PEGst-100 in SIF and this was attributed, as indicated in section 3.1, to the possibility to form a more uniform coating on the shell as compared to PEGst-100. Hence, the PEGst-40 was selected for further analysis. Moreover, since the surface properties and composition of the NCs is known toplay an important role in their stability properties, the influence of the type of bile salt and polymer coating on the stability of the NCs in the presence of enzymes was investigated. For comparison purposes a formulation without any cholic acid salt (PrNCs no Cholate) was also investigated. The results showed that the three protamine NCs were stable in SIF (data not shown). However, a steady increase in size and a decrease in count rate was observed upon incubation of the NCs in FaSSIF-V2 and FeSSIF-V2, reaching a 50% size increase after 6 h. T is small size increase

could be attributed to the high ionic strength and/or to the interaction of the surfactants (sodium taurocholate and lecithin) present in the medium (**Figure 6 and 7A**).

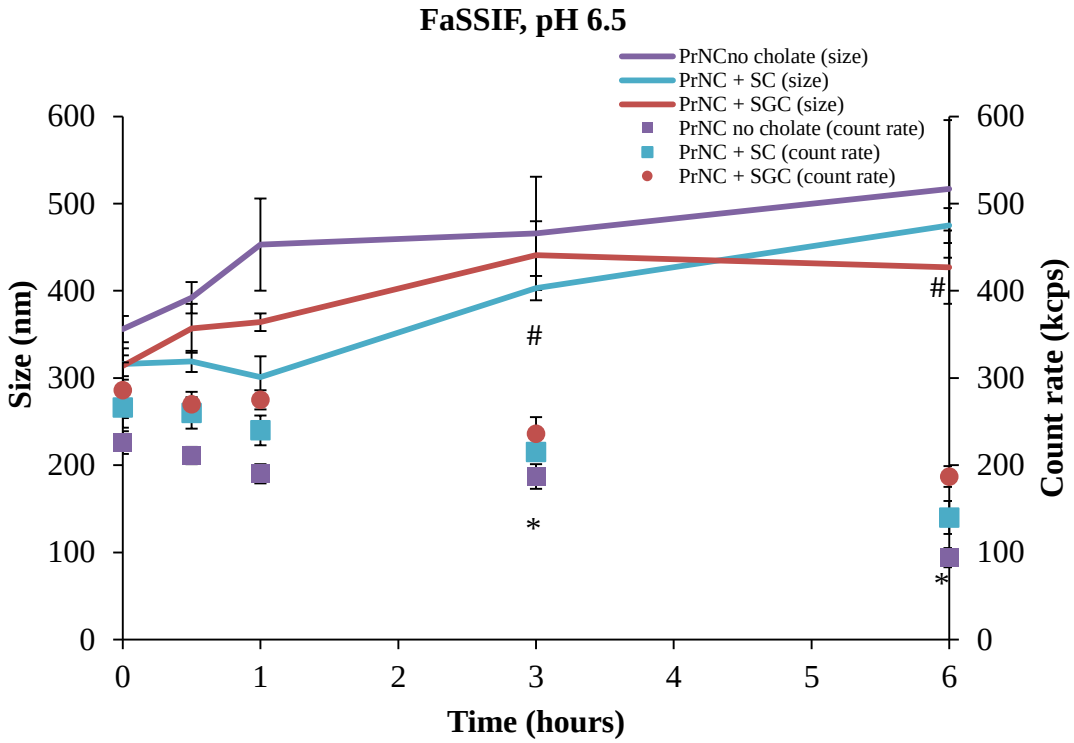



**Figure 6:** Evolution of the particle size (continuous line) and count rate of insulin loaded Protamine NCs without bile salts, with SC and SGC following incubation in FaSSIF-V2 (mean ± SD, n=3, $p<0.05$ significant difference $^{\#}$size and *count rate for all formulations compared to their size and count rate at time 0 h).

The stability of the NCs provided with an extra coating layer of PSA is shown in **Figure 7(B).** The results indicate that the stability of the NCs was improved by the presence of PSA. Moreover, the formulation containing SGC showed enhanced stability compared to the formulation containing SC and the formulation without cholate, since the count rate of this formulation remained constant, which suggests that the number of particles in suspension was maintained throughout the experiment. From these observations, it can be concluded that PSA and SGC have a synergetic effect on improving the stability of the NCs by providing a protective shell and because of their enzyme inhibition property. The order of stability of the PSA-coated formulations can be ranked as follows: PrNCs+SGC > PrNCs+SC > PrNCs-no Cholate in all cases.

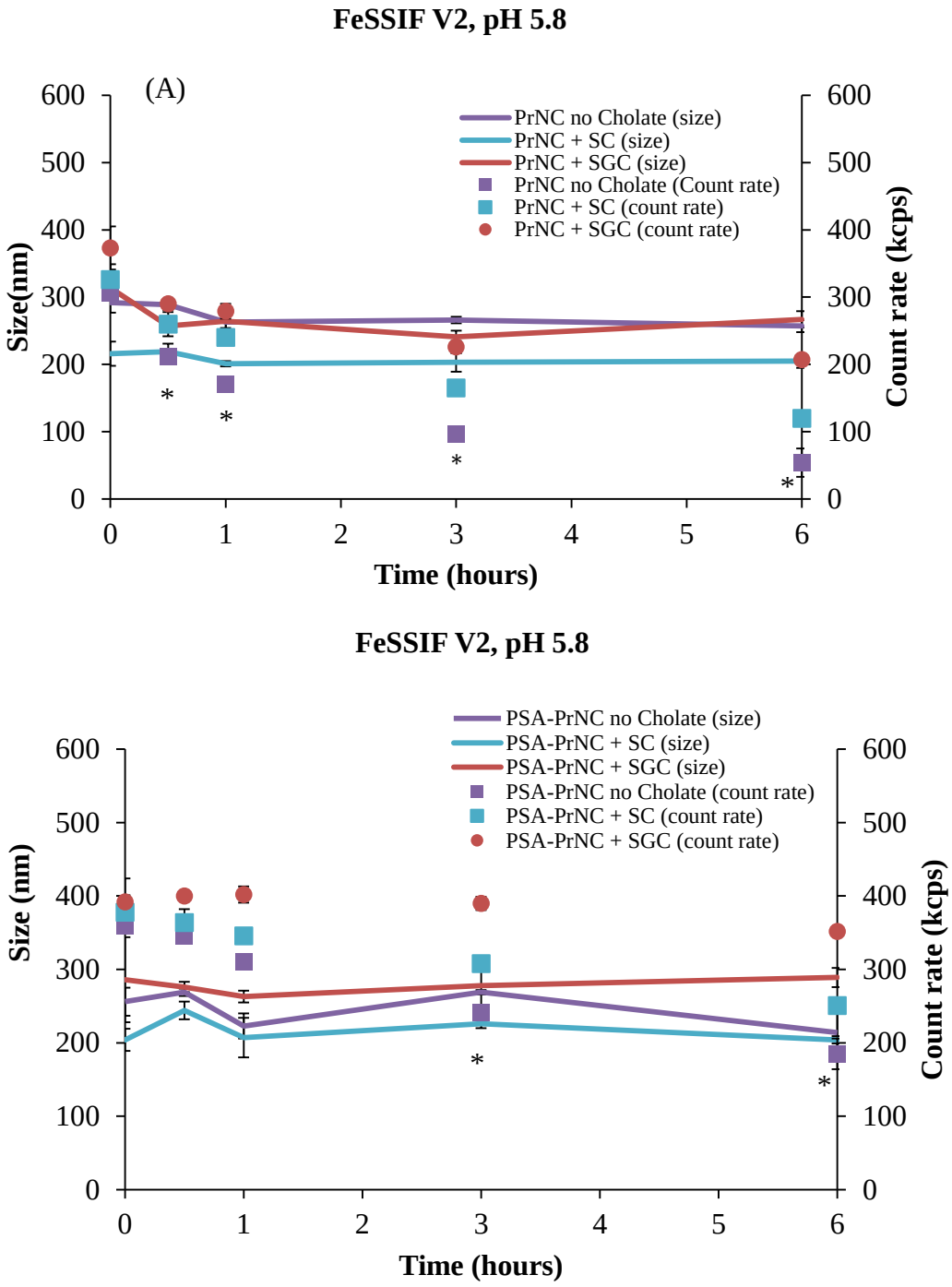
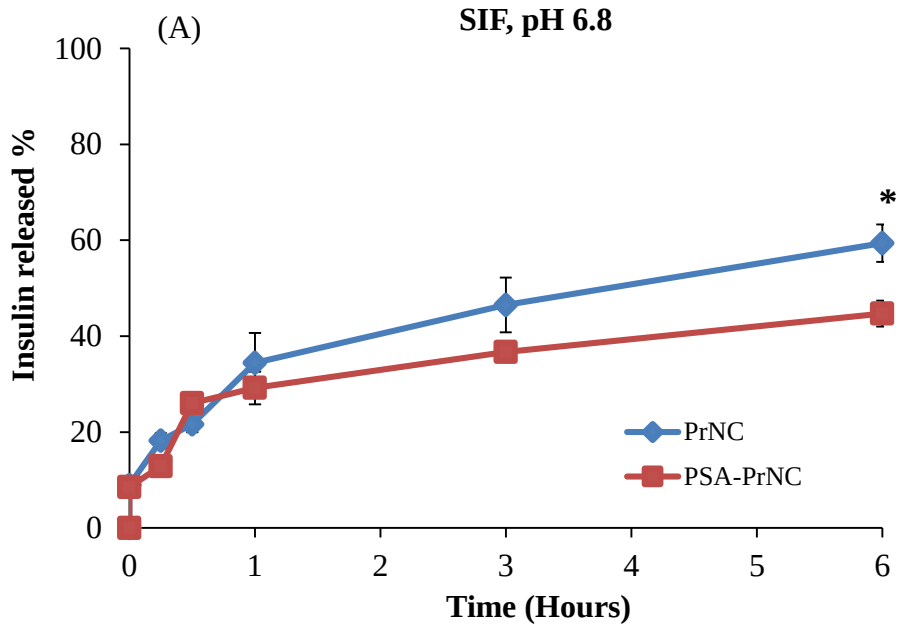



**Figure 7:** Evolution of the particle size (continuous line) and count rate of insulin loaded protamine NCs (A) and PSA-coated protamine NCs (B), without bile salts, with SC and SGC, following incubation in FeSSIF-V2 containing pancreatin, (mean ± SD, n=3, *p<0.05 significant difference in count rate for all formulations in (A) and for only the formulations with SC and without cholate in (B) compared to their count rate at time 0 h).

### 3.5. *In vitro* release studies in simulated intestinal fluids

**Figure 8 A and B** illustrate the *in vitro* release behaviour of protamine NCs and PSA-coated protamine NCs (containing PEGst-40 and SGC) in SIF at pH 6.8 and FaSSIF-V2 at pH 6.5. In both media a biphasic release pattern with a rapid and constant insulin release, followed by a slow sustained release over 4 hours was observed. The initial release phase could correspond to the release or diffusion of some insulin loosely associated to the shell of the NCs. This kind of biphasic profile has been previously reported for solid lipid nanoparticles (SLNs) and chitosan nanocapsules [39, 40]. Protamine NCs post coated with PSA showed improved controlled release of the insulin in both media, however, this difference was not found to be statistically significant ($p>0.05$). Comparing the release profile from the two different media it was observed that the insulin release was slower in simple SIF, compared to FaSSIF-V2, this being particularly noticeable after one-hour incubation. This could be due to the fact that the FaSSIF medium has higher ionic strength and a higher concentration of surfactants (lecithin, sodium taurocholate), which might contribute to the alteration of the alteration of the NCs protective shell.



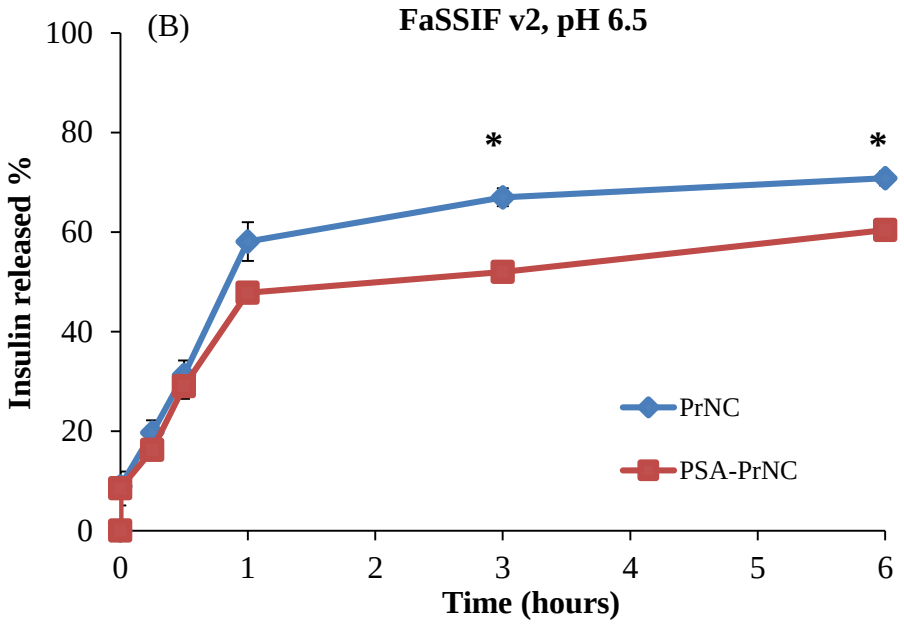



**Figure 8:** *In vitro* release profiles of insulin from protamine NCs and PSA-protamine NCs incubated in SIF (A) and in FaSSIF-V2 (B), (mean ± SD, n=3, *p<0.05).

### 3.6. Protection of the insulin from proteolysis

The ability of the NCs to preserve the associated insulin against degradation by enzymes was studied by incubating insulin-loaded protamine NCs and PSA-protamine NCs and free insulin solution (control) in FeSSIF-V2. The results in **Figure 9A** indicate that free insulin, was totally degraded in the first 15 minutes. In contrast, insulin entrapped into protamine NCs was highly preserved from degradation, maintain between 55 – 75 % insulin in the same period of time (15 min). Moreover, the presence of bile salts, and notable SGC, significantly contributed to enhance the stability of the encapsulated insulin. This stabilizing role of the nanocapsules was even more intense when they were provided with the external coating of PSA (**Figure 9B**). This behaviour, expected in the rational design of the nanocapsules, was attributed to the shielding effect of the PSA layer, which could be able to reduce the attachment of the degrading enzymes [20]. Moreover, both PSA and SGC could have a role in terms of inhibiting the activity of proteases [41].

Overall, protamine-based NCs containing bile salts showed a potential for the effective protection of insulin and this property was enhanced by the presence of an

additional coating layer of PSA. It should also be taken into account that some of the observed insulin degradation could be attributed to its release in the medium as observed in section 3.4.

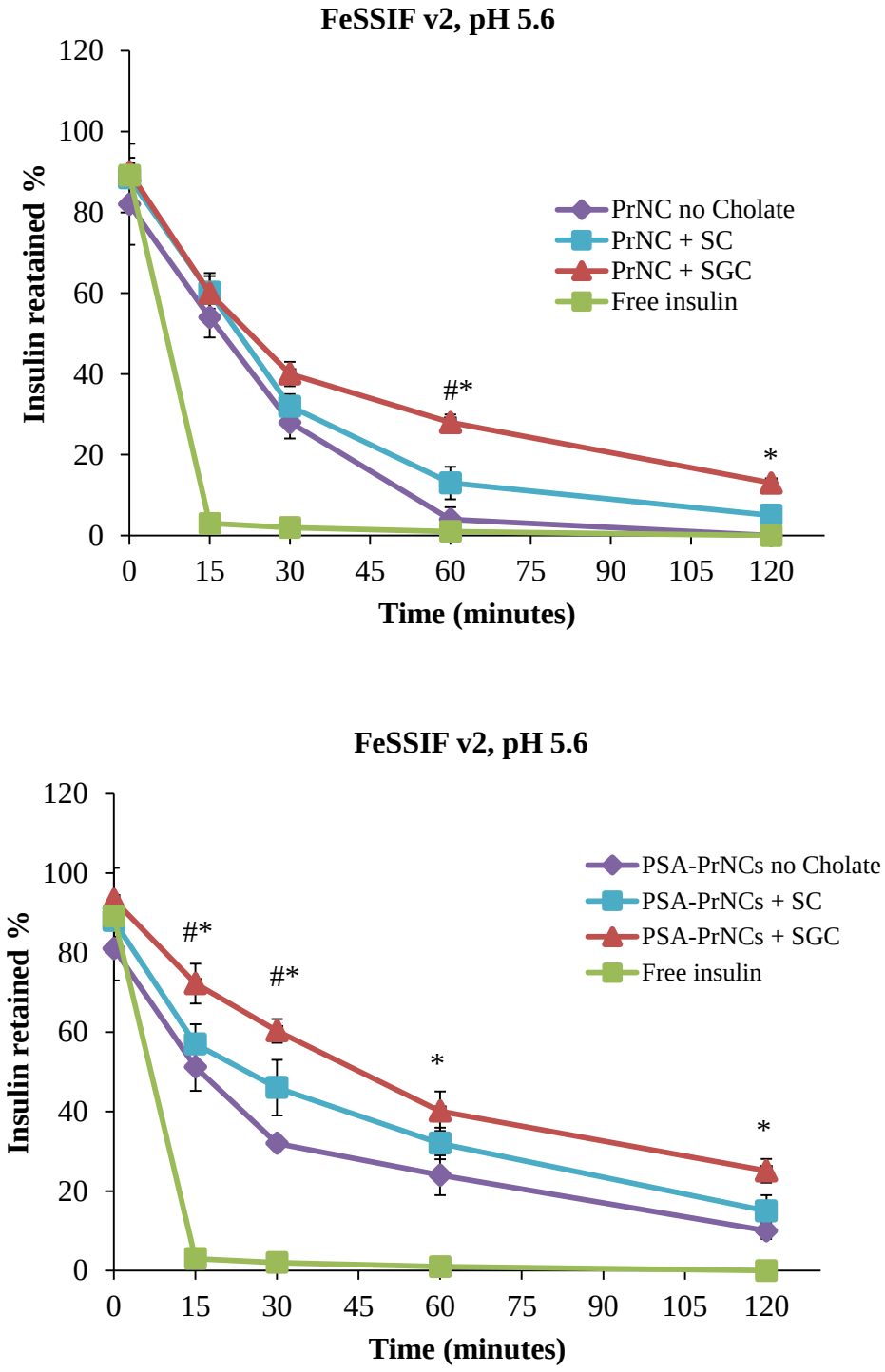



**Figure 9:** Stability profiles of the associated insulin after incubation of (A) Protamine NCs and (B) PSA coated Protamine NCs in FeSSIF-V2 (mean ± SD, n=3,

$p<0.05$ significant difference of PSA-PrNC+SGC compared to #PSA-PrNC+SC and *PSA-PrNC no Cholate).

### 3.7. Stability during storage

To determine the stability of the NCs under storage conditions, insulin-loaded protamine NC suspensions were stored at 4 ℃ and 25 ℃ and size and insulin leakage (AE %), were monitored for a period of 6 months. **Table 4** below shows the variations of size and AE of protamine NCs and PSA-protamine NCs over a period of 6 months. Both formulations maintained their nanometric size (and count rate) for the duration of the study at 4 ℃. However, both formulations were only stable for 7 days at room temperature (25 ℃).

**Table 4:** Physicochemical properties and insulin leakage from protamine NCs and PSA-protamine NCs during storage at 4 ℃, (mean ± SD, n=3).

| | **Initial** | | **1 month** | | **3 months** | | **6 months** | |
|---|---|---|---|---|---|---|---|---|
| | **Size ±SD nm** | **AE ± SD %** | **Size ±SD nm** | **AE ± SD %** | **Size ±SD nm** | **AE ± SD %** | **Size ±SD nm** | **AE ± SD %** |
| PrNCs | 396 ±24 | 68 ±8 | 394 ±41 | 52 ±5 | 344 ±63 | 41 ±4 | 374 ±47 | 31 ±4 |
| PSA-PrNCs | 341 ±54 | 59 ±6 | 314 ±32 | 50 ±3 | 278 ±45 | 43 ±2 | 277 ±21 | 37 ±5 |

### 3.8. Freeze drying studies

The conversion of the colloidal NCs into a dried state was considered as a strategy to further incorporate the nanocapsules into a solid dosage form [42]. It has been previously reported that the addition of cryoprotectants to nanoformulations is necessary in order to protect them during the freezing process. In this study, different concentrations of insulin-loaded protamine NCs were freeze-dried in presence and/or absence of cryoprotectants (trehalose or sucrose at 5 %). As shown in **Figure 10A and B**, the reconstituted powders of both formulations protamine NCs and PSA-protamine NCs, maintain their initial nanometric properties even without the use of cryoprotectants. Therefore, the presence of the protamine coat and the combined PSA-protamine coat around the oily core of the NCs play an important protective role during freeze-drying [43]. This is a quite unique behaviour for the nanocapsules with important technological consequences as it avoids the dry dilution and, thus, the final drug loading of insulin in the powder.

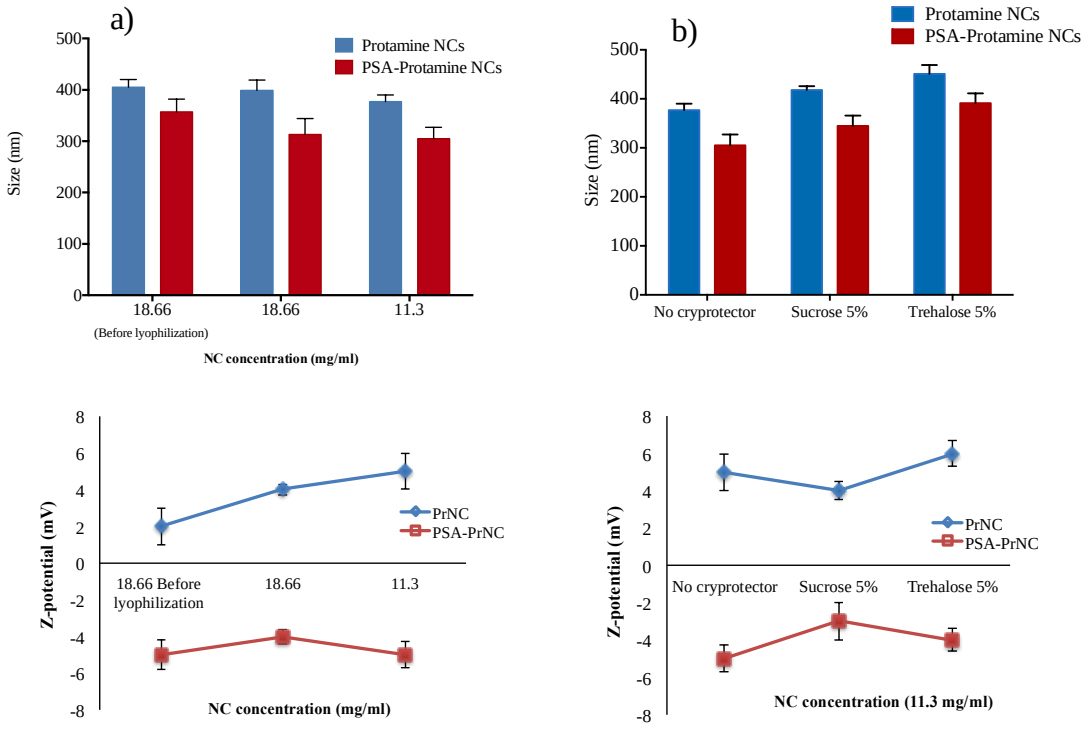



**Figure 10:** Physicochemical properties (Particle size and zeta potential) of protamine NCs and PSA-protamine NCs after lyophilization at different NC concentrations (a) and at 11.3 mg/ml NC concentration in the presence of 5% sucrose or 5 % trehalose (b), after resuspension in water (mean ± SD, n=3).

The results of the *in vitro* release of insulin from freeze-dried NCs was also determined. It is evident from **Figure 11** that converting protamine based NCs into powder also improved their *in vitro* release profile by promoting more controlled release and reducing the amount of insulin initially release in the first 30 minutes (compared to NCs in suspension). This change in the release behaviour could be associated to the compaction of the NC's shell during the freeze-drying process. This is a very promising feature for the nanocarrier system as it shows that converting the NCs into powder enhances the properties of the NCs by promoting controlled release and stability, which are advantages sought by employing the concept of nanomedicine.

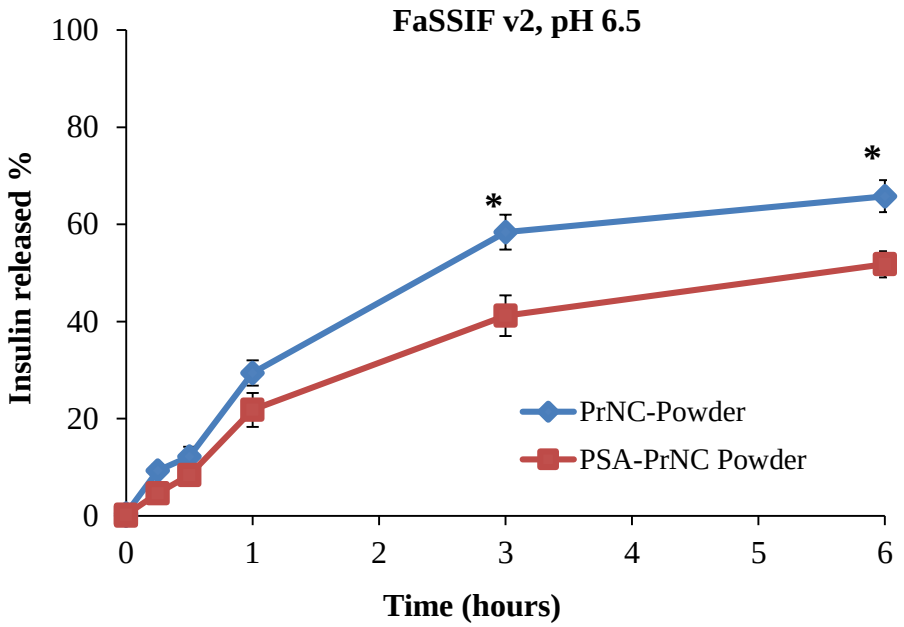



**Figure 11:** *In vitro* release profiles in FaSSIF-V2 (B) of lyophilized Protamine NCs and PSA-Protamine NCs, resuspended in milliQ water to the initial volume. (Mean ± SD, n=3, *p<0.05).

Finally, the effect of storage temperature and relative humidity on the stability of freeze-dried NCs was tested through an accelerated stability study, according to ICH conditions and guidelines. Freeze-dried insulin loaded protamine NCs and PSA-protamine NCs were kept at 25 ℃ ± 2 ℃ / 60% ± 5% RH, and their physicochemical properties were monitored and recorded over a period of 6 months. **Figure 12** shows that the physicochemical properties of the NCs did not change during a period of the study. These results affirm the concept that freeze drying improves the stability of colloidal systems since the NCs stability which was initially compromised at room temperature for the NC suspensions was preserved by converting the NCs into dry powder. Moreover, the freeze-dried NCs were also stable in simulated intestinal media.

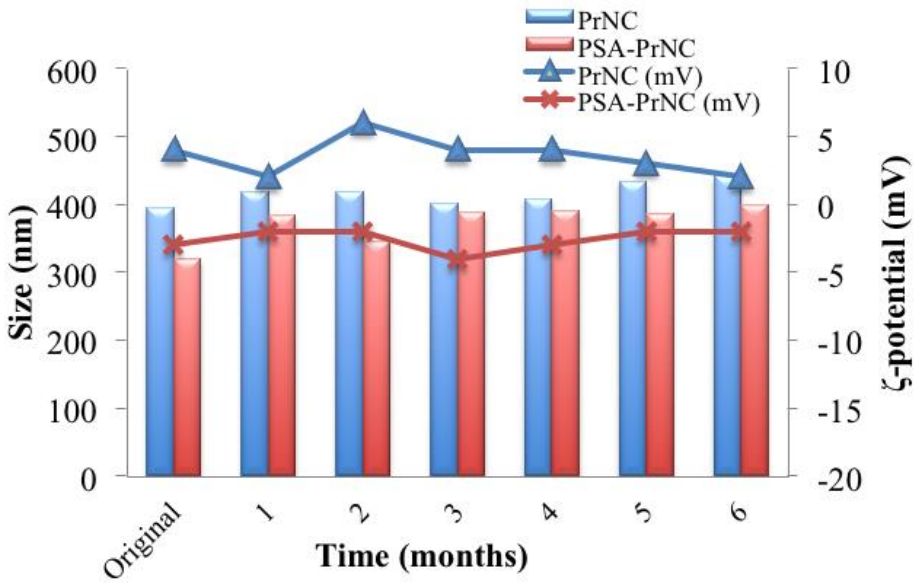



**Figure 12:** Physicochemical properties of freeze-dried protamine NCs and PSA-protamine NCs stored at 25 ℃ ± 2 ℃ / 60% ± 5% RH over a period of 6 months, (mean ± SD, n=3, *p<0.05).

## 4. CONCLUSIONS

In this work we report for the first time NCs consisting of an oily core and double layer PSA/protamine as a candidate delivery technology for the oral administration of peptides. These NCs, which were rationally designed in order to confer them with the capacity to withstand the multiple barriers associated to the intestinal tract, were also able to load a significant amount of insulin and control its release in simulated intestinal media containing bile salts and proteolytic enzymes. Moreover, the conversion of the NCs into a powder led to an improvement of their stability at room temperature and of their controlled release capacity. Therefore, this novel nanocarrier system could be considered as a promising candidate for improving the oral delivery of peptide drugs, such as insulin. *In vitro* and *in vivo* mechanistic studies presented in the next chapter were aimed to elucidate the interaction of these nanocapsules with the intestinal epithelial barrier.

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# CHAPTER 3

## The interaction of protamine nanocapsules with the intestinal epithelium

This work was done in collaboration with:

1 Veneto Nanotech, Italy (Dr. F. Bennetti).

2 Uppsala University, Sweden (Dr. P. Lundquist, Prof. P. Artursson).

# Abstract

Single-layer protamine and double layer polysialic acid (PSA)/protamine nanocapsules (NCs) were designed in order to be used as carriers to facilitate the transport of macromolecules across the intestinal epithelium. The rational for the design of these NCs was based on that protamine is a non-toxic yet potent cell-penetrating peptide, which is capable of translocating protein cargos through cell membranes. On the other hand, PSA is a low molecular weight polysaccharide, which has been used to enhance the stability of macromolecules and nanocarriers. The objective of this work was to study *in vitro* the mechanism of interaction of these NCs with different cell models, (Caco-2, Caco-2/Raji and Caco-2/HT29-MTX) and also with colonic human tissue. For this, the fluorescent marker, TAMRA was covalently linked to protamine. The interaction and transport of the NCs with the Caco-2 cells (measured by fluorescence) was found to be concentration, temperature and size dependent. In all cases, the double layer PSA-protamine NCs exhibited a significantly higher transport compared to protamine NCs. On the other hand, the transport of the NCs was significantly higher in the co-culture (Caco-2/Raji monolayer) compared to the monoculture model (Caco-2 monolayer), implying that *M-cells* are involved in the transport of these nanosystems. On the contrary, no significant differences in the amount of fluorescence transported in Caco-2/HT29-MTX compared to Caco-2 monolayer was observed, thus making evident the mucodiffusion properties of the NCs. Moreover, both systems were observed within the epithelium of human intestinal tissue without any cytotoxicity effects, thus reflecting their safe interaction with the intestinal tissue.

## 1. Introduction

The intestinal mucosa is a large interface that consists of a monolayer of epithelial cells connected by tight junctions and protected by a thick mucus layer. Although this mucosa is highly specialized in the absorption of nutrients, i.e. aminoacids, it represents a phenomenal barrier for the absorption of large molecules, e.g. peptide drugs [1]. As a consequence, the development of strategies to make the oral administration of peptides feasible has become a primary goal in the biopharmaceutical world [2]. The possibility to deliver peptides by the oral route is particularly critical in the case of drugs used in the treatment of chronic diseases. In the specific case of anti-diabetic drugs (e.g. insulin and glucagon-like peptide (GLP-1), their administration by the oral route has the additional advantage of simulating the normal physiological pathway i.e. *via* hepatic portal circulation [3]. Indeed, insulin transported across the intestinal epithelium would directly reach the liver, the target organ for pancreas-secreted insulin, whereas injected insulin goes directly into the systemic circulation and only a limited amount (20 % of injected dose) becomes effective at the target site.

A variety of strategies attempting to provide structural protection and improved absorption of peptide drugs have been disclosed. These include the use of enzyme inhibitors [4] and absorption enhancers [2], chemical modification [5, 6] and encapsulation of peptide drugs within microspheres [7], nanoparticles [8] and nanocapsules [9]. Some of these nanocarriers have been shown to protect peptides from degradation by intestinal enzymes [10, 11]. The subsequent barrier, the mucus layer, has been found to be more difficult to overcome and, hence, the development of muco-diffusive nanocarriers has become a crucial challenge in the area of nanoparticle-based oral peptide delivery [12, 13]. In addition to this, the interaction of nanocarriers and transport of the associated peptide across the intestinal epithelium, which is supposedly highly dependent on the nature of the nanocarrier, remains poorly understood.

Based on this background information, the aim of this work was to study the mechanism of interaction and transport of single layer protamine and double layer

PSA/protamine NCs across the intestinal epithelium, using insulin as a model peptide. The oily core of these NCs is composed of Miglyol® oil, and it contains insulin together with the penetration enhancer, sodium glycocholate (SGC). The shell of the NCs is made of protamine, which has the role of facilitating the interaction with the epithelium and PEG-stearate, which is supposed to facilitate the transport of the NCs across the mucus layer [14]. In addition, an extra layer of PSA was added to the NCs with the aim of preventing their interaction with proteolytic enzymes followed by the degradation of the associated insulin. PSA, a polysaccharide that forms part of the mucus in mammals, is a non-toxic and biodegradable polymer [15] that has already been used to improve the stability and lengthen the circulatory half-life of proteins [16]. For example, in a recent study, polysialylated insulin was found to exhibit a 2-fold longer half-life compared to normal insulin following subcutaneous injection in mice [17]. Moreover peptide loaded nanocarriers have been decorated with PSA to protect the peptide from degradation and to improve their function following parenteral administration. For these reasons, we hypothesized that coating the nanosystem with PSA may render it with muco-penetrative properties allowing it to penetrate the intestinal mucus layer.

To achieve the above-indicated objective different *in vitro* cell monolayer models simulating the different mucosal barriers (e.g. enterocytes and mucus) of the intestinal epithelium were adopted [18], [19]. The influence of parameters that could potentially affect the toxicity and mechanism of interaction with the monolayers, including particle size, surface composition, particle concentration as well as time and temperature of exposure were investigated. Finally, the results in terms of cytotoxicity and mechanism of interaction were compared with those obtained upon contact of the NCs with human intestinal tissue mounted on a Ussing chamber.

## 2. Materials and methods

### 2.1. Materials

Protamine sulfate of low molecular weight (5 kDa), used in this work was purchased from Yuki Gosei Kogyo, Ltd., (Tokyo, Japan). The stabilizing surfactants, polyoxyethylene 40 monostearate, Croda Europe Ltd, (Snaith, UK) and sodium

glycocholate (SGC), Dextra, (Reading, UK). Caprylic/capric triglyceride (Miglyol®) 840, Cremer, Oleo Division, (Witten, Germany). Colominic acid sodium salt (Polysialic acid, PSA, Nacalai tesque INC, (Tokyo, Japan). Insulin (insulin glulisine) was obtained from Sanofi (Paris, France). 5-TAMRA, SE (5-carboxytetramethylrhodamine, succinimidylester, single isomer), was purchased from emp Biotech, (Berlin, Germany). Alexafluor® 488 phalloidin was purchased from Life Technologies, (Eugene, USA). Triton X-100, chlorpromazine, filipin III and Alcian blue were purchased from Sigma-Aldrich, (Leuven, Belgium). All cell culture media and reagents were purchased from Invitrogen (Merelbeke, Belgium). Organic solvents used were of HPLC grade. All other products used were of high purity or reagent grade.

## 2.2. Preparation and characterization of protamine nanocapsules

### 2.2.1 Preparation of insulin-loaded protamine nanocapsules

Insulin loaded protamine NCs were prepared by the solvent displacement technique following the procedure described by our group [14]. Briefly, PEGstearate-40 (16 mg), sodium glycocholate (5 mg) and Miglyol® (62.5 µL) were dissolved in 3 ml ethanol. Acetone (1.95 mL) was then added to this lipid phase followed by the addition of 1.5 mg insulin dissolved in 50 µL 0.01M HCl. This organic phase was immediately poured over 10 ml of an aqueous phase containing 0.15 % w/v protamine. The elimination of organic solvents was performed by evaporation under vacuum (Rotavapor Heidolph, Germany), until a final volume of 5 mL. Finally the nanoparticles were isolated by ultracentrifugation (Avanti® J-E, Ultracentrifuge, Beckman Coulter, USA) at 30,000 rpm for 1 h (at 15 ℃).

Nanocapsules with a double protamine/polysialic acid polymer layer were obtained upon addition of 0.1 mL of PSA solution (concentration: 3 mg/mL) to a volume of 0.5 ml of NCs (concentration: 18.6 mg/mL) under magnetic stirring for up to half an hour. The final Protamine:PSA ratio was of 5:1 w/w).

### 2.2.2. Preparation of TAMRA-labelled protamine nanocapsules

For cell uptake studies, fluorescent protamine NCs were prepared with TAMRA-labelled protamine (TAMRA-protamine). Protamine (10 mg) was dissolved in 0.1 M

sodium bicarbonate buffer (1 mL, pH 8,60) and TAMRA (10 mg/mL in DMSO) was slowly added under mild stirring. After 1 h incubation (mild stirring) at room temperature, the labelled protamine was dialyzed for 72 h to remove free TAMRA (SnakeSkin, cellulose membrane MW 3.5 KDa, Thermo, Spain). The obtained polymer conjugate (TAMRA-protamine) was freeze-dried and NCs were prepared according to the procedure described in 2.2.

### 2.2.3. Determination of particle size and zeta potential distribution

The mean size and polydispersity index (PDI) of the particle size distribution of protamine NCs were measured after dilution with ultrapure water by dynamic light scattering using a Zetasizer Nano series DTS 1060 (Malvern instruments, Malvern, UK). The zeta potential was measured by laser-Doppler anemometry after diluting the samples in water (Zetasizer®, NanoZS, Malvern Instruments, Malvern, UK).

### 2.2.4. Insulin association efficiency (AE %) and loading capacity (LC %) of the nanocapsules

The association efficiency (AE %) of insulin was determined after isolation of the NCs. The amount of free insulin in the aqueous phase (indirect method) and also that associated to the NCs (direct method) was determined by HPLC (described below). For the extraction of insulin from the NCs, 0.1 mL of the NCs suspension (concentration: 18.6 mg/mL) were mixed with acetonitrile (0.1 mL), 0.1 % trifluoroacetic acid (TFA) (0.75 mL) and Triton™ X-100 (0.05 mL). The mixture was vortexed at a high speed to obtain a clear solution.

*Quantification of insulin by HPLC*

The amount of insulin loaded into the NCs was quantified by HPLC (Agilent model 1100 series LC and a diode-array detector set at 214 nm). The chromatographic system was equipped with a reversed-phase 125 × 4 mm Supersphere®100- RP-18e-125-4 column (particle size 4 µm). The mobile phase, eluted at 1 mL/min, was a mixture of phosphate buffer (0.1 M, pH 2.3) and acetonitrile (44:56, v/v). The column was set at 35 ℃ and the injection volume was 10 µL. Calibration curves ranging from 5 µg/mL up to 1,050 µg/mL ($r^2$=0.999) were obtained. The limit of quantification (LOQ) and limit of detection (LOD) were 200 µg/mL and 80 µg/mL, respectively.

Samples were transferred into auto-sampler vials, capped and placed in the HPLC auto-sampler. Each sample was assayed in triplicate. The concentration of insulin in the aliquots was used to calculate the association efficiency (AE %) using equation 1

**Equation 5** $$AE\ \% = \frac{Amount\ of\ insulin\ in\ destructed\ NCs}{Total\ insulin} \times 100$$

### 2.2.5. Assessment of *in vitro* behaviour of nanocapsules in cell culture media: insulin release, TAMRA release and colloidal stability

Insulin loaded TAMRA-labelled NCs were incubated in Hank´s Balanced Solution Salt (HBSS), used for *in vitro* cellular assays, at a dilution ratio of 1:50, NCs:HBSS (v/v), a final NC concentration of 0.37 mg/mL, and shaken at 100 rpm at 37 ℃. At specified time intervals (0, 0.5, 1, 2, 4 and 6 h), insulin released was evaluated after isolating the NCs by ultracentrifugation (Avanti® J-E, Ultracentrifuge, Beckman Coulter, USA) at 30,000 rpm for 1 h (at 15 ℃). The concentrations of insulin in the aqueous medium were determined by reverse phase HPLC method (section 2.2.3.). The stability of the NCs was determined in cell culture medium DMEM and transport buffer HBSS. Briefly, the NCs were diluted 50x with the relevant medium, DMEM or HBSS (NC concentration: 0.37 mg/mL) and shaken at 100 rpm at 37 ℃. The size and count rate (number of particles) of the NCs were monitored at specific time intervals (0, 0.5, 1, 2, 4 and 6 h).

The absence of leakage of TAMRA from the NCs during the transport studies was also evaluated. Insulin loaded TAMRA-labelled NCs (1 mL of 2 mg/mL) were placed in dialysis devices (Spectra/Por Float-A-Lyzer G2, MWCO: 5KD, Spectrum Laboratories, USA) and introduced into 50 mL of HBSS, maintained under magnetic stirring at 37 ℃. At pre-determined time points (0, 0.5, 1, 2 and 4 h), samples were withdrawn from the medium and analysed using the fluorescence reader, (SpectraMax M3, Molecular devices) at 583 nm wavelength. The free TAMRA and TAMRA-protamine conjugate were also used as controls.

## 2.3. *In vitro* cell culture studies

### 2.3.1. Cell cultures

Caco-2 cells (clone 1) were kindly provided by Dr Federico Benetti (Veneto nanotech, Venice, Italy) and used from passage 18 to 28. Human Burkitt's lymphoma Raji B cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and used between passages x+5 to x+10. Caco-2 cells were grown in DMEM supplemented with 10% (v/v) inactivated fetal bovine serum, 1% (v/v) non-essential amino-acids (NEAA), 1% (v/v) L-glutamine, and 1% (v/v) of penicillin–streptomycin (PEST) at 37 °C under a 10% $CO_2$/90% air atmosphere. Raji cells were grown in suspension, cultivated in RPMI medium supplemented with 10% (v/v) inactivated fetal bovine serum, 1% (v/v) NEAA, 1% (v/v) L-glutamine, and 1% (v/v) PEST, at 37 °C in a 5% $CO_2$/95% air atmosphere. HT29-MTX cells were kindly provided by Dr T Lessufleur (INSERM UMR S 938, Paris, FR) and used between passages x+16 and x+20 [20]. HT29-MTX cells were grown in DMEM supplemented with 10 % (v/v) heat inactivated fetal bovine serum, 1% (v/v) NEAA, 1% (v/v) L-glutamine, and 1% (v/v) PEST in a humidified incubator with 5 % $CO_2$ atmosphere at 37 °C.

### 2.3.2. Cytotoxicity studies

Cell viability was assessed after the co-incubation of 20,000 Caco-2 cells/well on a 96-well tissue culture plate (Costar® Corning® CellBIND Surface) with the formulations, protamine NCs and PSA-protamine NCs in dispersion in culture medium (concentration range: 0.5 to 8 mg/mL). After 2, 6 and 24 h of incubation, the cells were rinsed with PBS and incubated at 37 °C for 3 h with 100 µL 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-(2,5-diphenyltetrazolium bromide) solution (Sigma-Aldrich, Belgium) (MTS assay). 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 by 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 (LDH assay) (Roche Diagnostics Belgium, Vilvoorde, Belgium), and incubated for 20 min at room temperature in the dark. The reaction was stopped by adding 25 µL of stop solution, and the amount of produced formazan was measured by recording

absorbance at 500 nm with Synergy 4 microplate reader [21]. The $IC_{50s}$ for the different formulations were calculated using the GraphPad Prism 5 program (California, USA).

### 2.3.3. Interaction of TAMRA-Protamine nanocapsules with Caco-2 cell monolayers

The interaction of TAMRA-protamine NCs and TAMRA-PSA-protamine NCs with Caco-2 cells was quantitatively and qualitatively studied using flow cytometry (FACS) and confocal laser scanning microscopy (CLSM), respectively. 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 48 h until confluency. For the transport studies, cells were co-incubated with a-400 μL of a TAMRA-protamine NCs or TAMRA-PSA-protamine NCs suspension in transport buffer (NC concentration 1, 1.5 or 2 mg/mL) corresponding to a final insulin concentration of 4, 8 and 12 μg/mL, respectively.

After 2 h of co-incubation, cells were washed three times with PBS and detached from the plates by trypsinization. Cells were then centrifuged at 1,500 x g (Eppendorf centrifuge 5804 R, Daigger scientific Inc., USA) the supernatant was discarded and the cells were resuspended in PBS. Fluorescence was measured using a BD FACS Verse flow cytometer and BD CellQuest software (Becton Dickinson Biosciences, San Jose, CA, US). Cell fluorescence was quantified by measuring the fluorescence of TAMRA (red) at 583 nm. For cell viability measurements, the TOPRO-3 reagent was employed (blue). The reagent was added to each sample at a final concentration of 10 μg/mL, and, after 10 min of incubation, the fluorescence corresponding to dead cells was measured at 642 nm. For each sample, 10,000 events were collected. The data were subsequently analysed using the FlowJo data analysis software package (TreeStar, USA).

For subsequent imaging, transwell inserts were fixed in paraformaldehyde (PFA) 4 % were gently washed in HBSS. Actin was stained with 200 μL of Alexafluor® 488 phalloidin (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.* [22]. Cell nuclei were stained with DAPI (1:20). Subsequently, inserts were washed in HBSS, cut and mounted on glass slides. Images were captured using a Zeiss™ confocal microscope (LSM 150).

Data were analysed by the Axio Vision Software (version 4.8) to obtain *y-z*, *x-z* and *x-y* views of the cell monolayers.

## 2.4. Transport across intestinal cell monolayers Cell culture models

### 2.4.1. Caco-2 and Raji cell monolayers (Follicle-associated epithelium model)

Caco-2 cells were seeded at a density of 5 x $10^5$ cells/well onto Matrigel® (BD Biosciences, Belgium) (10 µL/mL in DMEM)-coated Transwell® polycarbonate inserts (12 mm insert diameter, 3 µm pore size) (Corning Costar, Cambridge, U.K.) and cultivated over 21 days. The medium was replaced every second day. The inverted follicle-associated epithelium (FAE) model was obtained by co-culturing Raji and Caco-2 cells as previously reported by des Rieux *et al.* and Beloqui *et al.* [1, 23]. Briefly, after 3 to 5 days of Caco-2 seeding, inserts were inverted, a piece of silicone tube was placed into the inserts and maintained until day 21 in large Petri dishes. The medium was replaced every other day, until day 9 to 11 when Raji cells were then added on top of the Caco-2 cells for the conversion of the Caco-2 cells into M cells at a density of 2.5 x $10^5$ cells/well [23].

For the assessment of FAE model functionality, in each experiment transport studies were conducted under the aforementioned conditions with commercial fluorescent carboxylated nanoparticles (0.2 µm) (Gentaur, Belgium). A nanoparticle suspension (400 µL at a concentration of 4.5x$10^9$ NPs/mL) was added on the apical side and inserts were incubated at 37 ℃ for 2 h. After this incubation time, basolateral solutions were then sampled and the number of transported nanoparticles was measured by FACS (BD FACS Verse). Nanoparticle transport was expressed as mean ± SD.

### 2.4.2. Caco-2 and Caco-2/HT29-MTXcell monolayers (mucus model)

The influence of the mucus was studied by co-culturing Caco-2 and HT29-MTX, seeded at a density of 5 x $10^5$ cells/well in a 3:1 ratio (Caco-2:HT29-MTX) and maintained until day 21 [24]. The medium was replaced every after two days.

The Alcian blue staining technique was employed to demonstrate the presence of acid mucins and sulfated mucosubstances on the surface of the Caco-2/HT29-MTX co-

culture [19]. For this purpose, both the Caco-2 monoculture and the Caco-2/HT29-MTX co-culture were grown and seeded as described above. After confirming the integrity of the monolayer by measuring the TEER, cells were washed 2 x with HBSS and fixed with 4 % Paraformaldehyde (PFA) for 30 min, then rinsed 3x with PBS and stained with 1 % Alcian blue at pH 2.5 for 1 h. They were then washed 3x with PBS, mounted on slides and viewed under a microscope (Axioskop 40, Carl Zeiss Microscopy, LLC, New York, United States) equipped with the Zen lite 2012 software for capturing images.

In all cases the integrity of the monolayers was corroborated by measuring the trans-epithelial electrical resistance (TEER), before and after the transport studies on day 21. These measurements were carried out at 37 ℃ using an epithelial voltohm meter (EVOM, World Precision Instruments, Berlin, DE). TEER values over 250 $\Omega cm^2$ for both Caco-2 and Caco-2/HT29-MTX monolayers and values above 160 $\Omega cm^2$ for Caco-2/Raji monolayers were used. TEER values after transport studies were not significantly different to initial values unless otherwise stated.

All transport experiments were conducted at 37 ℃ (or 4 ℃) by adding a volume of 400 µL at 12 µg/mL insulin concentration in HBSS on the apical side and 1 mL of HBSS on the basolateral side. After the specific incubation time (1, 2 or 4 h), samples were collected from the basolateral side and the amount of fluorescence (TAMRA) was measured using a fluorescence plate reader ($\lambda_{Ex}$=540 and $\lambda_{Em}$=570).
The absence of cytotoxicity in the presence of the NCs was assessed by measuring the LDH activity released from the cytosol of damaged cells in the apical medium after each transport experiment (procedure in section 2.3.2). The cell monolayers were then washed twice in cold HBSS to stop endocytosis or uptake of NCs and fixed in PFA 4% for subsequent staining and imaging.

### 2.5. Mechanism of interaction of insulin–loaded protamine NCs with Caco-2 and Caco-2/Raji monolayers

To evaluate the mechanism of interaction of protamine and PSA-protamine NCs with Caco-2 cells and Caco-2/Raji cells, the monolayers were pre-incubated for 1h at 37 ℃ with 400 µL of a solution endocytosis inhibitors in HBSS. Namely,

chlorpromazine at 10 μg/mL was used as an inhibitor of receptor-mediated and clathrin-mediated endocytosis [25, 26] and Filipin III (1 μg/mL), was used for the inhibition of caveolae and clathrin-mediated pathways by cholesterol depletion [27, 28]. After 1 h of incubation with the inhibitors formulations were added onto the apical side of the monolayer and co-incubated for 2 h. Control transport studies were carried out in transport buffer without inhibitor solutions.

The absence of cytotoxicity in the presence of inhibitors was assessed by measuring the LDH activity released from the cytosol of damaged cells into the apical medium after the interaction experiments following the same procedure in section 2.3.2.

## 2.6. Interaction of TAMRA-protamine nanocapsules with human intestinal tissue

Intestinal tissues collected immediately after the surgery, were immediately transferred into a vessel containing ice cold, oxygenated Krebs-Ringer buffer (KRB) and quickly transported to the laboratory. Upon arrival, the epithelium was dissected away from subepithelial tissues and mounted in horizontally as well as vertically oriented Ussing chambers with 9 mm openings between the two chambers. The basolateral chamber was filled with glucose containing KRB, while the glucose in the apical chamber was substituted with mannitol in order to avoid SGLT-induced tight junction opening. The chambers were kept at 37°C and bubbled with 95% $O_2$ /5% $CO_2$ for the duration of the experiment. To assure continued tissue viability, the electrophysiology of the tissues was monitored throughout the experiment. After mounting the tissues were allowed to equilibrate for 40 min with two medium exchanges [29].

Protamine NCs and PSA-protamine NCs (5 mg/mL) were then added to the donor chambers. Aliquots of 20-100 μL, were withdrawn at 0, 30, 60, 90 and 120 min from the receiver and donor compartments. The sample volume in the receiver compartment was replaced with fresh KBR. 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 Cli channels, the response was monitored as changes in potential difference and short-circuit current over the epithelium. Permeability of fluorescently

labelled NCs was analysed by quantifying the amount of the fluorescence dye found in the receiver chamber using a plate fluorescence reader ($\lambda_{Ex}$=540 and $\lambda_{Em}$=570) [29]. For imaging, the tissue specimens were rinsed and fixed in 4% formalin for 24h. Then they were embedded in paraffin and sectioned to a thickness of 5μm. Nanocapsules in each section were visualized by LSCM using the fluorescent marker TAMRA.

### 2.7. Statistics

Statistical analysis was performed using the GraphPad Prism 5 program (CA, USA). Normal distribution was assessed with the Shapiro–Wilk normality test. One-way ANOVA in multiple comparisons followed by Tukey's post-hoc test was applied according to the result of the Bartlett's test of homogeneity of variances for the 37 ℃ and 4 ℃ transport comparisons. All other analyses were performed using a Student's t-test. Differences were considered statistically significant at *$p<0.05$. Results are expressed as mean ± SD.

## 3. Results and discussion

In this work, we studied the interaction of protamine-based NCs with the intestinal epithelial cells and the mucus layer as important barriers to successful oral peptide delivery. Preceding work showed that NCs consisting of an oily core and a poly(alkyl cyanoacrylate) coating are potential carriers for improving insulin absorption [9]. The NCs studied here present the same structure, although the components were rationally selected and organized in order to overcome specific biological barriers, i.e. the mucus layer and the underlying epithelium. The selection of protamine was based on its its ability to penetrate cell membranes, as it is an arginine rich polypeptide [30, 31]. On the other hand, the formation of a double layer with PSA was aimed at increasing stability and enhance the penetration of the NCs through the mucus layer by avoiding the interaction with the extracellular matrix including proteins [32]. Finally, the surfactants, SGC and PEG-stearate were added to the nano-composition, with the objective of enhancing the penetration properties of the nanocarrier as well as their colloidal stability and mucodiffusion properties.

### 3.1. Physicochemical properties, stability and release behavior of protamine nanocapsules

Insulin-loaded protamine NCs were successfully prepared using the simple solvent

displacement technique, a mild and easily scalable preparation technique. The particle size, zeta potential and association efficiency of the two formulations are summarized in **Table 1**. The particle size range for both formulations is between 300 – 400 nm. The zeta potential for protamine NCs was +6 ± 3 mV whilst PSA coated protamine NCs showed a slightly negative zeta potential (-4 ± 1 mV), confirming the presence of PSA on the outer shell. The insulin association efficiency was 62 ± 16 % and 51 ± 9 % for protamine and PSA-protamine NCs respectively.

**Table 1:** Characteristics of protamine NCs and PSA-protamine NCs (mean ± SD, n=9, *p<0.05)

| Formulation | Size (nm) | ζ-potential (mV) | AE % | LC% | Stability in HBSS |
|---|---|---|---|---|---|
| **Protamine NCs** | 382 ± 69 | +6 ± 3 | 62 ± 16 | 1.0 ± 0.03 | Stable (6h) |
| **PSA protamine NCs** | 301 ± 84 | -4 ± 1 | 51 ± 9 | 0.8 ± 0.01 | Stable (6h) |

*In vitro* stability and insulin release studies were performed to ensure that the transport buffer, HBSS, does not compromise the stability of the NCs and does not cause a burst release of the associated insulin. The results shown in **table 1**, indicate that the stability of the NCs was not compromised upon incubation in the transport buffer medium (HBSS) during 6 h at 37 ℃. On the other hand, for both, protamine and PSA-protamine NCs, less than 30 % of the associated insulin was released in the same period of time (6 h). Comparable data were obtained when the NCs were incubated in SIF and SGF for 6 h, confirming their stability in simulated gastrointestinal media. Moreover, no TAMRA leakage from the NCs was observed over the period of 0 to 6h in HBSS.

### 3.2. Cytotoxicity of protamine nanocapsules in Caco-2 cells

The cytotoxicity of the different types of NCs was studied in order to estimate the highest safe concentration of NCs that can be used for subsequent mechanistic studies. Two different incubation times, 2 h and 6 h were tested as they fall within the average transit time in the intestinal tract. As shown in **Figure 1**, protamine NCs were found to induce no significant cytotoxicity (p>0.05) after 6 h incubation at concentration range of 0.5 to 2 mg/mL. However, the highest concentration tested, 8 mg/ml, significantly decreased cell viability of the Caco-2 cells for both formulations

regardless of the incubation time, implying that cytotoxicity of protamine NCs is concentration dependent.

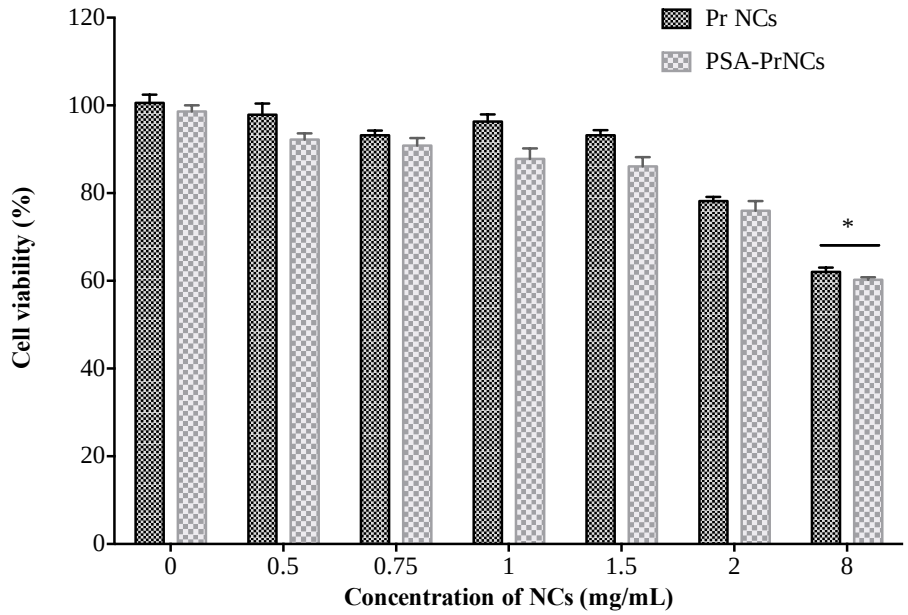



**Figure 1:** Cytotoxicity of insulin-loaded protamine (PrNCs) and polysialic acid coated PSA-PrNCs on Caco-2 cell viability measured by MTS assay (mean ± SD, n=3, N=2, *p>0.05).

### 3.3. *In vitro* evaluation of nanocapsule interaction with the intestinal barrier

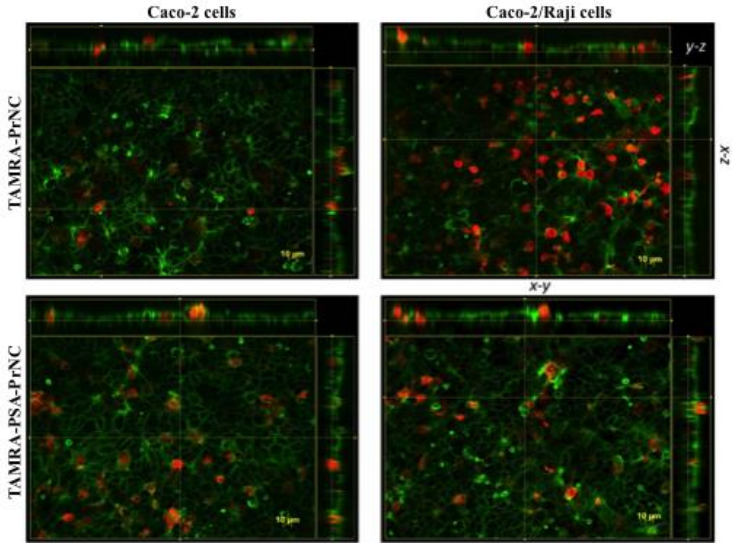
#### 3.3.1. Intracellular uptake in Caco-2 and Caco-2/Raji monolayers

The adherence and internalization of the fluorescent NCs into the Caco-2 monolayers was studied using confocal microscopy and flow cytometry. **Figure 2(a)** represents the *y-z*, *x-z* and *x-y* view of Caco-2 and FAE monolayers after 2 h incubation with TAMRA-labelled protamine NCs and TAMRA-labelled PSA-protamine NCs. The images show that the two formulations were internalized by the cells up to a certain extent, although a significant amount was also localized on the surface of the cell monolayers. Based on this, it could be expected that the NCs would release the associated insulin on the surface or into the enterocytes

The interaction of the nanocapsules with the Caco-2 monolayers was also quantitatively analyzed by flow cytometry. In this study, the TOPRO-3 reagent was employed to measure cell viability and untreated cells were used as controls. As shown **Figure 2(b),** the cellular uptake of both, protamine NCs and PSA-protamine

NCs, was concentration (dose) dependent. This observation is consistent with des Rieux *et al.*, who found that transport of yellow-green carboxylated particles (Fluospheres®) was concentration dependent [22]. Interestingly, as observed by confocal microscopy, at all concentrations PSA-protamine NCs exhibited significantly higher count values ($p<0.05$) than protamine NCs. Initially, this result was surprising as, in general, the cell internalization of positively charged nanoparticles is more important than that of negatively charged ones [31, 33]. However, there is also the possibility that the PSA coating minimizes the interaction of the NCs with the proteins present in the cell culture medium, thereby enhancing their internalization [15].

(a)



(b)

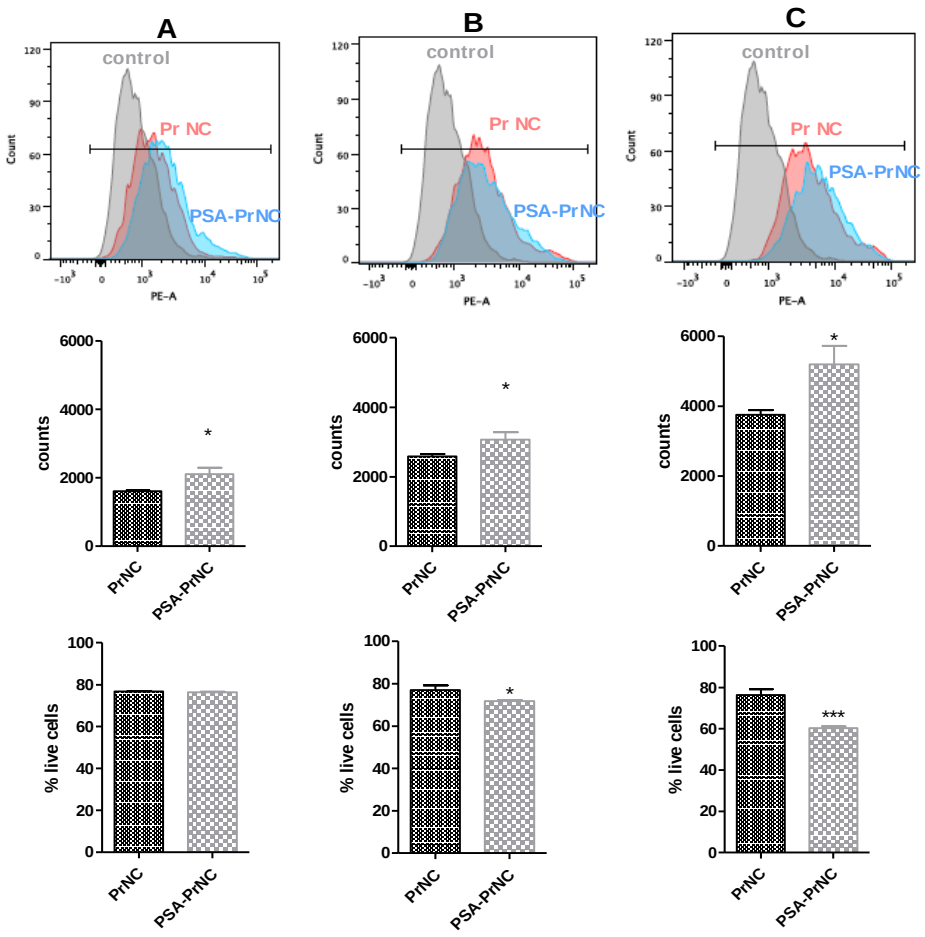



**Figure 2:** (a) CLSM images showing *y-z*, *x-z* and *x-y* sections of Tamra-labeled protamine formulations (red). Cell membranes are stained in green with Alexafluor® 488 phalloidin. (b) Cellular uptake of Tamra-protamine NCs and PSA-protamine NCs at different NC concentrations, 1 (A), 1.5 (B) and 2 (C) mg/ml in Caco-2 cells, measured by flow cytometry. (Mean ± SD, n=3, N=3, *p<0.05, ***p<0.001)

### 3.3.2. Permeability across model cell monolayers

#### *3.3.2.1. Caco-2 vs Caco-2/Raji cell monolayers*

The ultimate goal of this work was to evaluate the potential of protamine and PSA/protamine NCs to transport protein drugs such as insulin across the epithelial barrier. For this, the enterocyte-like model (Caco-2 monolayers) and the FAE model (Caco-2/Raji co-culture), containing M cells, were employed. The conversion of Caco-2 cells into M-cells in the FAE model was confirmed by measuring the number of fluorescent commercial carboxylated particles transported using flow cytometry [1, 23]. The results of this control study indicated that the number of transported nanoparticles was significantly higher in the FAE model than in the Caco-2 model (9

532 ±133 *vs.* 108 ±91 nanoparticles, respectively n=6, *p<0.05)

The ability of the NCs to cross the cell monolayers was estimated by quantifying the amount of fluorescent maker covalently linked to protamine, detected in the basolateral side compared to the initial amount added in the apical side of the monolayer [22, 34]. **Figure 3** represents the fluorescence detected in the basolateral side of Caco-2 and FAE monolayers, after 1, 2 and 4 h incubation time. In agreement with previous work [22, 35]. Overall, the results show that the transport of both protamine NCs and PSA-protamine NCs, measured in terms of fluorescence, was increasing over the time in both types of monolayers and that this transport was significantly higher in the FAE monolayer as compared to the Cacco-2 monolayer (*p<0.05). Moreover, as observed in confocal microscopy studies (section 3.3.1), the transport was significantly higher for the NCs with a PSA layer.

The explanation of the higher transport observed in the FAE monolayer, as compared to the Caco-2 monolayer, could be found in the intrinsic nature of the M cells, which unlike the neighbouring absorptive enterocytes, lack a highly organized apical brush border and glycocalyx, and are poorly equipped with digestive enzymes [36]. They also possess a high transcytotic capacity and are able to transport a broad range of materials, including nanoparticles [37]. Moreover, protamine has been shown to enter primary human dendritic cells (DCs) in a dose-dependent manner [38]. The transport profile observed in this study correlated with other similar studies where less than 10 % of the NPs added to the apical side were transported to the basolateral side [22]. The authors also confirmed that NP transport depends on the surface composition of the nanocarriers.

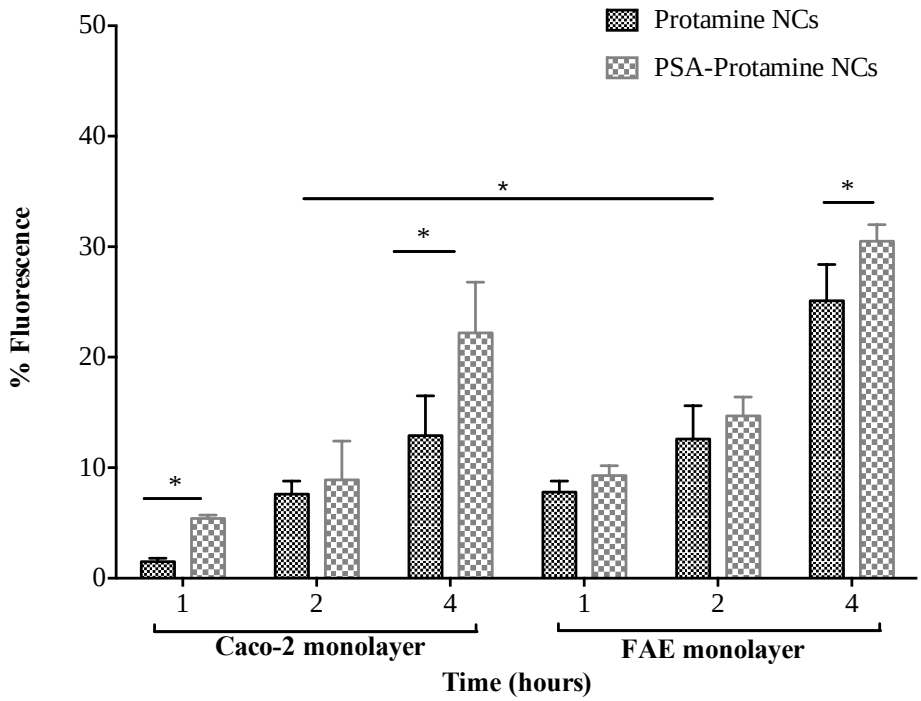



**Figure 3:** Influence of NCs composition in their transport over the time across the Caco-2 and FAE monolayers. Fluorescence (%) represents the amount of fluorescence transported to the basolateral side in comparison to that initially added in the apical side, (mean ± SD, n=3, N=2; *p<0.05).

Subsequently, the NCs were evaluated for their ability to enhance the intestinal paracellular transport by monitoring the alternation of TEER, which correlates to the tightness of the cell layer. **Figure 4** shows that the prototypes tested resulted in an significant reduction of TEER after 4 h incubation time (*p < 0.05). However, after the removal of the NCs and the replacement with fresh medium, the initial TEER was completely recovered. These results suggest that protamine-based NCs could induce a transient opening of tight junctions (TJs) between Caco-2 cells, especially as an effect of time. This phenomenon was mainly associated to the presence of protamine in the nanocapsules. In fact, the permeabilizing effect of protamine has already been reported by several authors using a fish model [39], a rat brain perfusion model [40] and the intestinal perfusion model in rats [41] and the effect was found to be dose and/or time dependent. Although the mechanism is still unclear, the authors presumed that protamine might interact with intracellular calcium transfer or cause disruption of the microfilaments on the tight junction proteins. In addition, this effect was also the hypothetical mechanism by which the intestinal absorption of insulin was promoted

by its conjugation to protamine [5]. On the other hand, the fact that the effect on TEER was transient and reversible is very important since loss of the tight junction barrier function is undesirable as it may lead to the penetration of pathogens and other harmful immunoreactive antigens into the mucosa. The results of this study also showed that the extra layer of PSA does not alter the penetration enhancing properties of the NCs. Consequently, it could be inferred that somehow, the hidden protamine layer interacts with the anions on the cell surface and the TJ proteins along the cell membranes [42].

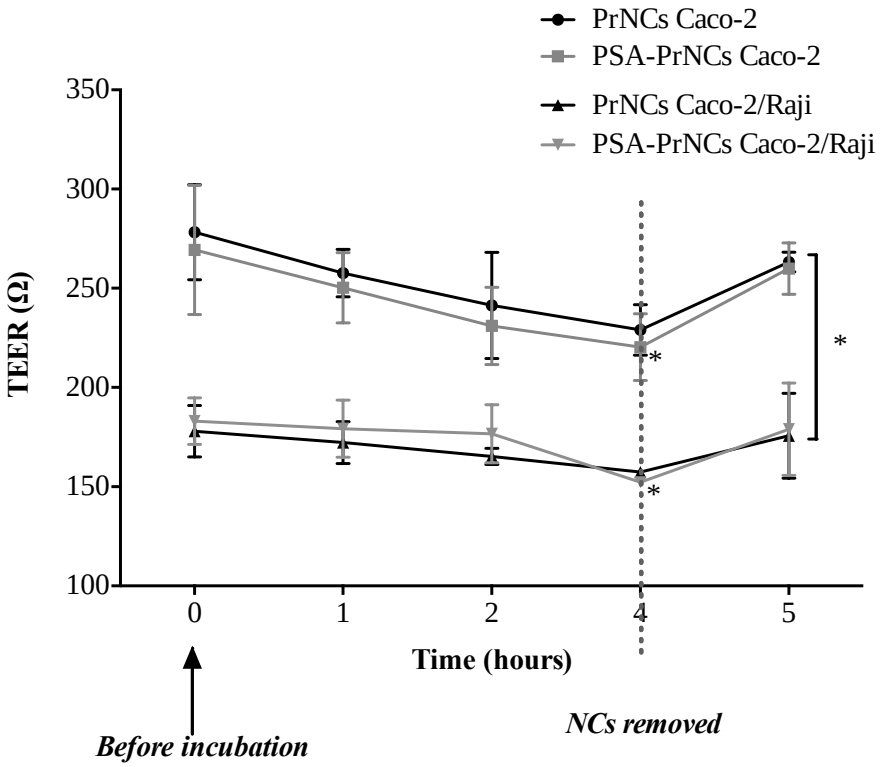


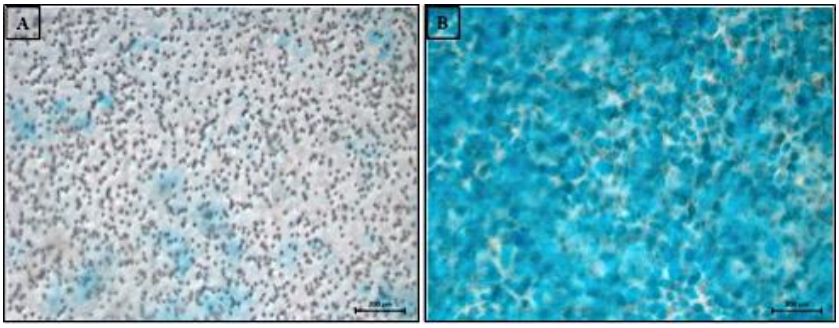

**Figure 4:** Effects of protamine NCs and PSA-protamine NCs on the transepithelial-electrical-resistance (TEER) values of Caco-2 and Caco-2/Raji monolayers (mean ± SD, n=3, N=3; **p<0.05).

*3.3.2.2. Caco-2 vs Caco-2/HT29-MTX cell monolayers*

Caco-2 and HT29-MTX cells represent the two most abundant cell populations in the intestinal epithelium, enterocytes and goblet cells, respectively. Therefore, co-cultures of Caco-2 cells and mucus-producing goblet cells HT29-MTX would provide a drug absorption model incorporating the mucus barrier [19]. Acid mucins produced by goblet cells were stained with Alcian blue to verify the presence of mucus on the surface of the Caco-2/HT29-MTX monolayers. Caco-2:HT29-MTX cells seeded at a

3:1 proportion exhibited a homogenous mucus layer, depicted by the monodispersed blue layer on the surface of the co-culture corresponding to the mucus layer produced by the HT29-MTX cells (**Figure 5B**). This blue layer is not observed in the Caco-2 monoculture ratifying the absence of mucus (**Figure 5A**). Hence the Caco-2:HT29-MTX co-culture in a 3:1 proportion used in this study is a reliable model to predict the behavior of the NCs when in close contact to intestinal mucosa as it maintains better *in vivo*/*in vitro* correlation relevance [43].

The permeability profiles of the NCs, measured in terms of fluorescence, are shown in **Figure 5C**. The statistical analysis of these data indicated that there are not significantly different in the amount of fluorescence transported in Caco-2/HT29-MTX compared to Caco-2 monolayer for either protamine NCs or PSA/protamine NCs. This observation suggests that the presence of mucus did not hinder the permeation enhancing property of the nanocarriers. In a similar study, Shan *et al.*, found no significant difference in the transport of nanoparticles (NPs) across the Caco-2/HT29-MTX-E12 co-culture in the presence or absence of mucus, implying that the presence of the mucus did not hinder the internalization of these NPs. This observation was attributed to the structure of the NPs as they possessed a nanocomplex core composed of insulin and a cell penetrating peptide (CPP), coated with a dissociable hydrophilic, mucodiffusive polymer [44].



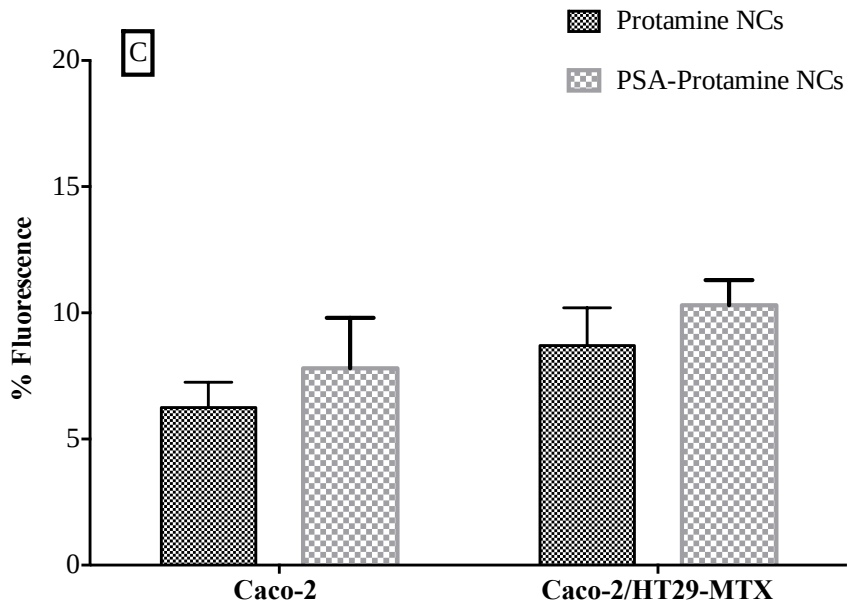



**Figure 5:** Caco-2 monolayer (A) and Caco-2/HT29-MTX (B) stained with Alcian blue, demonstrating the presence of mucus (magnification 400x) and Transport of PSA coated and uncoated protamine NCs in Caco-2 and Caco-2/HT29-MTX monolayers after 2 h incubation (C) (mean ± SD, n=3, N=3)

## 3.4. Mechanistic study of NC transport across Caco-2 cells

### 3.4.1. Influence of temperature

Nanoparticles have been reported to predominantly enter cells actively by pinocytic or endocytic uptake at 37 ℃ [45]. Conformably, we observed a significant reduction of transport at 4 ℃ which implies that the membrane is involved during transport of these formulations since membrane fluidity is highly restrictive at 4 ℃ (**Figure 6**). It is known that several proteins and enzymes are sensitive to temperature, thus active processes are inhibited by lowered temperatures [25]. Further studies were carried out to investigate the specific nature of endocytosis involved.

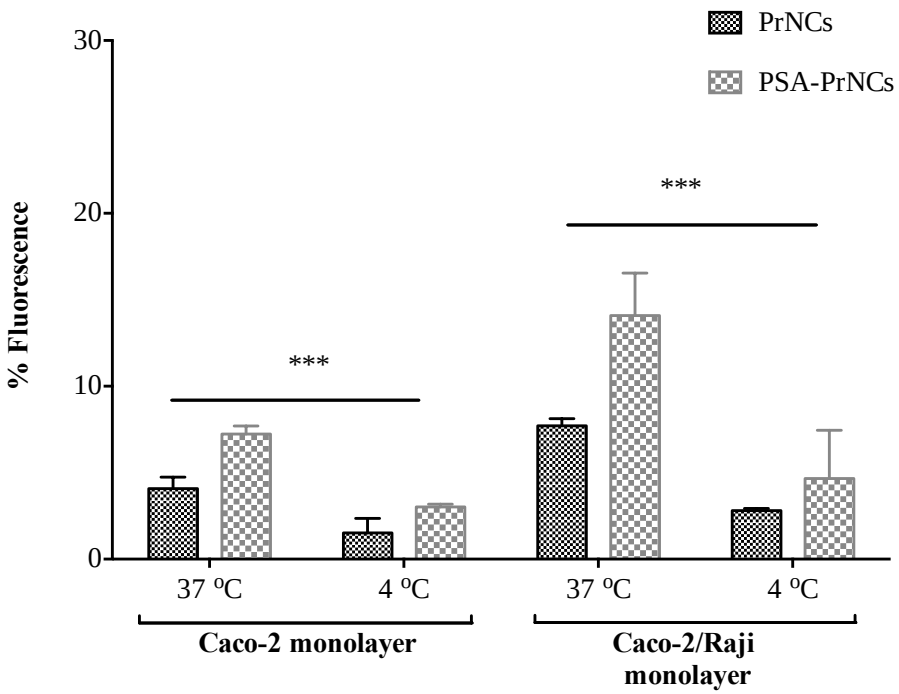



**Figure 6:** Influence of temperature on transport in Caco-2 and FAE monolayers after 2 h of incubation at 37 ℃ and 4 ℃ (mean ± SD, n=3, N=2; ***p<0.001).

### 3.4.2. Characterization of NC endocytosis mechanism

In order to elucidate the specific mechanisms involved in the uptake of protamine-based NCs and the transcytosis of the cargo, transport studies were performed in the presence of inhibitors and compared to transport without inhibitors (controls). The formulations were co-incubated with chlorpromazine (10 μg/mL), which reduces the number of coated pits associated receptors at the cell surface by disrupting the assembly of clathrin [46]. Under this particular condition a reduction (30 %) in fluorescence internalized into the cells and transported to the basolateral side was observed (**p<0.01, ***p<0.001) (**Figure 7)**. This implies that clathrin is involved in the transcytosis of protamine NCs and cargo. Caveolae-mediated endocytosis was evaluated by incubating cells with the NCs in the presence of 1 μg/mL Filipin III. Filipin disrupts the caveolae structure by binding to sterols such as cholesterol and disorganizing caveolae proteins [46]. A significant decrease (approximately 80 %) in fluorescence transported to the basolateral side was observed in the presence of Filipin III (***p<0.001) (**Figure 7**).

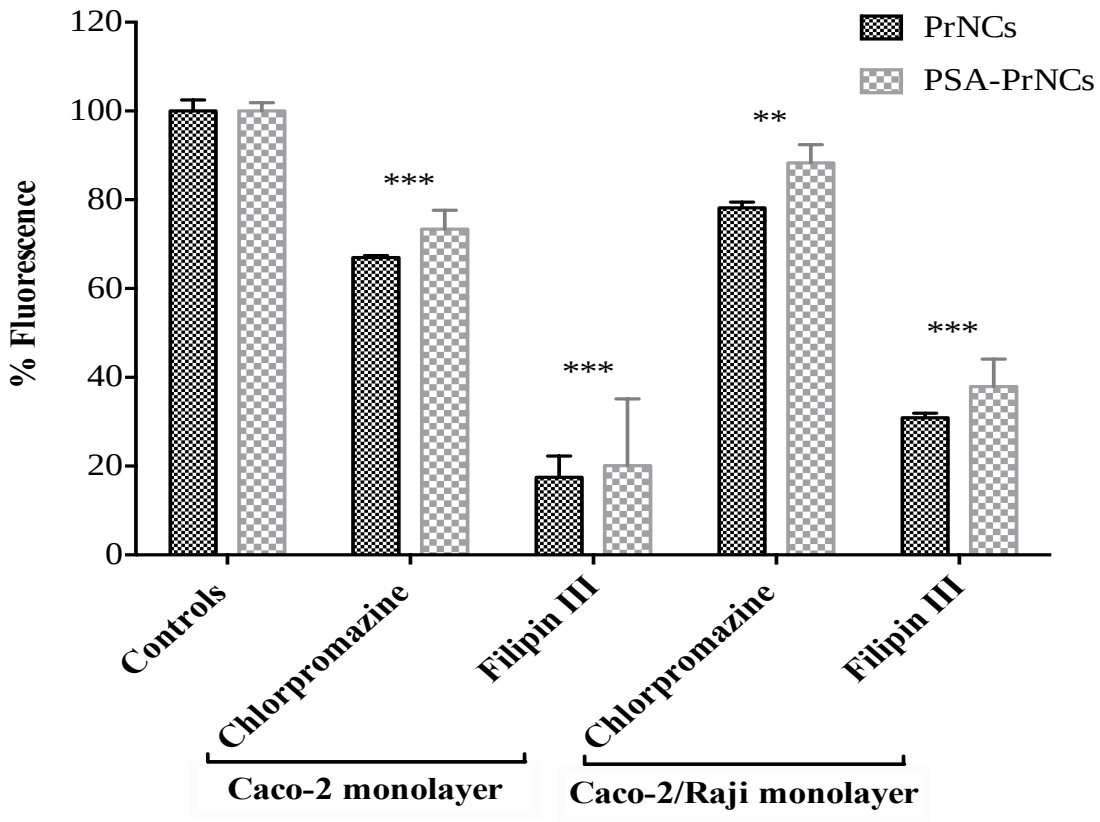



**Figure 7:** Comparison of transport under clathrin and caveolae inhibitors, (chlorpromazine and Filipin III respectively), in Caco-2 and FAE monolayers after 2 h incubation with inhibitors. Untreated cells used as controls and fluorescence (%) represents the amount of fluorescence transported to the basolateral side in comparison to that in the basolateral side of the respective control, (mean ± SD, n=3, N=3; *p<0.05,**p<0.01, ***p<0.001).

Collectively, the data presented here suggest that caveolae and clathrin are both involved in the transcellular transport of cargo by protamine-based NCs, although caveolae (80% decrease) transport is predominantly higher than clathrin (30% decrease). Caveolae-mediated endocytosis involves clustering of lipid raft components on the plasma membrane into flask-shaped invaginations formed by the interaction of different proteins mainly caveolin with the cellular membrane [47]. Roger *et al.*, reported that caveolae was predominantly involved in the transcytosis of paticlaxel (Ptx)-loaded LNCs when they found a significant decrease in the amount of Ptx transported across Caco-2 cells to the basolateral after co-incubation LNCs with filipin [28]. These results are in agreement with Rejman *et al.*, who suggested that caveolae flask-shaped invaginations are able to internalize larger particles more easily than clathrin coated pits [47]. Dos Santos *et al.*, also found that none of the inhibitors

used could completely inhibit NP-uptake in different cell types and concluded that there is a possibility that multiple pathways are used simultaneously to internalize the same NP [25]. Moreover, the ability of protamine to enhance membrane translocation has been reported [42]. Different mechanisms have been postulated for this property, including the fact that the basic guanidine group of the arginine forms stable hydrogen bonds with sulfates or phosphate present in biological membranes. This interaction is considered essential for membrane translocation and can consequently improve the cellular uptake of protamine-based nanosystems [50].

The implications and fate of internalized nanoparticles is debatable. Hillaireau *et al* described polymeric nanoparticles as efficient vehicles that can be modified to ferry their cargo to the target site. In this manner, it is predicted that internalized nanoparticles are degraded and hence release the drug into the enzymatic environment (lysosomes) or directly into the cell cytoplasm [51]. In this study, it was observed that both formulations used multiple endocytosis pathways to enter the cells, which implies that once within the cells the cargo can be released in different organelles i.e. within the cytoplasm, the Golgi complex, endoplasmic reticulum or even in the lysosomes, depending on the specific pathway. Hence the amount of fluorescence detected on the basolateral compartment could be the TAMRA-protamine or free TAMRA, transcytosised at the basolateral as a product of the nanocarrier degradation that occurs intracellularly [39], [47]. Moreover, a considerable amount of insulin was detected in the cell monolayers (intracellular) and in the basolateral receiving chamber but this data are not conclusive and are pending validation.

### 3.5. Transport of TAMRA-protamine NCs in human intestinal tissue

The permeation enhancing property of protamine NCs was further explored in human intestinal tissue, estimated by quantifying the amount of fluorescence accumulated in the basolateral chamber. **Figure 8** shows the accumulation of fluorescence in the basolateral side over a period of time obtained from the vertical Ussing chamber as the permeation effect was more pronounced in this chamber compared to the horizontal chamber. It was observed the amount of fluorescence that crossed to the receiving chamber increased over time, for both prototypes. The confocal microscopy was used to visualize how the nanocarriers interact with the intestinal epithelium. The

images show fluorescence protamine nanocapsules attached mainly on the surface of the epithelium with a low amount of fluorescence located within the epithelium. In addition, some fluorescence, which could be attributed to the labelled polymer or simply to the fluorescent marker, was found to be across the tissue and transported to the basolateral side (**Figure 9**). However, dialysis of the transcytosed material in a 3.5 KDa cut-off dialysis device showed that the majority of the detected fluorescence displayed a size below this cut-off, suggesting significant break-down of the nanoparticles during transit of the intestinal tissue. In addition, efforts to quantify the amount of insulin transported to the receiving chamber are on going and at this point, no insulin was detected in this model. This could be attributed to the high enzyme activity associated with the intestinal tissue and the low amount of insulin used.

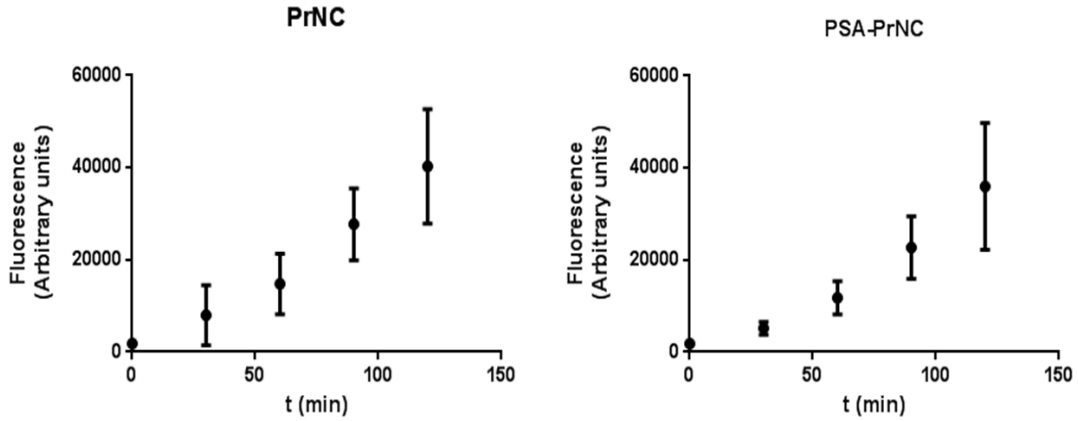



**Figure 8:** Transport of protamine NCs and PSA coated protamine NCs in human intestinal epithelia after 2 h incubation. Fluorescence (arbitrary units) represents the detected amount of fluorescence transported to the basolateral side measured as a function of time (mean ± SD, n=3 *P<0.05).

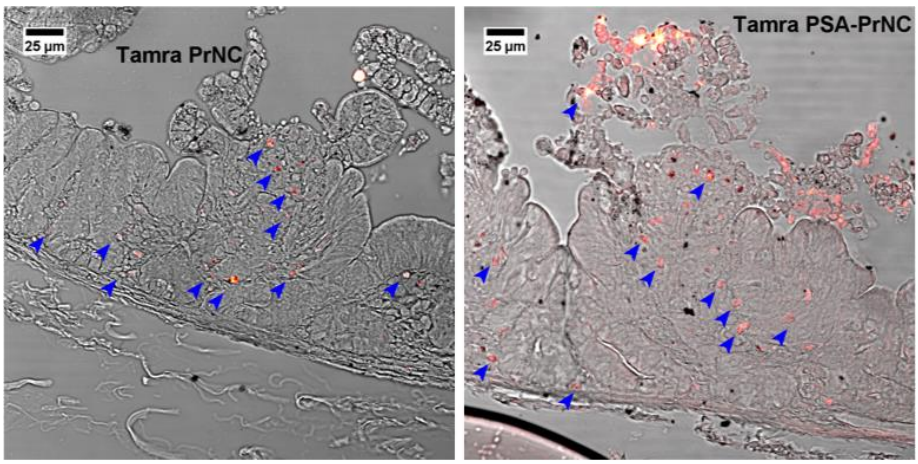


**Figure 9:** Confocal images showing the transport of protamine NCs (a) and PSA-protamine NCs (b) in human intestinal epithelia.

It is critical to assure the viability of the tissue during and even after the experiment. In this study forskolin response was used to estimate tissue physiological function after the experiments. After adding the cAMP agonist forskolin to the tissues, viable tissue that is able to perform oxidative metabolism will form cAMP in response and stimulate increased short-circuit current (Isc) due to CFTR $Cl^-$ channel activation [52]. As shown in **Figure 10**, no apparent change in forskolin response was observed in the tissues after incubating them with protamine NCs and PSA-protamine NCs. Moreover, there was no LDH leakage observed after incubating the tissues with the nanocarriers (data not shown). This study shows protamine NCs have the capacity to transport cargo across intestinal tissue without compromising the viability, confirming the safety and non-toxicity of these nanocarriers even in this more realistic *in vivo* condition and using a higher concentration of the NCs (5 mg/ml).

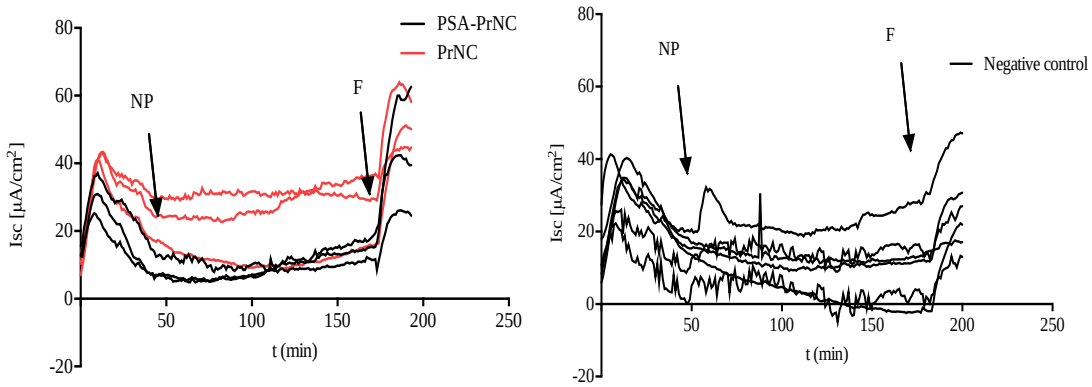



**Figure 10:** Forskolin response of human intestinal tissues after 2 h incubation with protamine NCs and PSA-protamine NCs at 5 mg/ml NC concentration.

## 4. Conclusion

The results of this work have led to the conclusion that insulin-loaded into NCs with a single protamine or a double PSA/protamine layer exhibit a very low toxicity in the Caco-2 cell culture models and also in human intestinal tissue. The NCs interacted with the monolayers and affected both, the transcellular and paracellular mechanisms of transport. With respect to the first one, irrespective of their composition, the NCs were internalized in a dose and time dependent manner mainly *via* the caveolae and clathrin endocytic pathway. With respect to the second one, NCs led to a significant

but reversible decrease in the TEER values of the monolayers On the other hand, he presence of raji cells in the monolayer led to a significant increase in the permeation properties of NCs, whereas the presence of mucus-secreting cells did not interfere with the NCs internalization permeability was not affected by the presence of mucus. All in all, based on these *in vitro* studies, protamine-based NCs exhibit a promising profile as oral peptide delivery carriers.

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# CHAPTER 4

## Protamine nanocapsules: *in vitro* immunotoxicity and in vivo efficacy studies

This work was done in collaboration with:

1 Veneto Institute of Oncology, Italy (Dr. I. Marigo, Prof. V. Bronte).

2 University of Santiago de Compostela, Spain (Dr. S.Tovar and Prof. C. Dieguez).

# Abstract

Nanocarriers made of polycationic polymers with penetration enhancing properties are gaining increasing attention for oral peptide delivery. It has been hypothesized that the combination of these polymers with other biomaterials with additional penetration properties, controlled release and stabilizing properties i.e. lipids and surfactants may further increase the potential value of these nanocarriers. Protamine-based nanocapsules (NCs) made of one single protamine layer or a double polysialic (PSA)/protamine layer were shown to exhibit these properties. The aim of this work was to evaluate the *in vitro* immunotoxicity and the *in vivo* biodistribution and efficacy of these nanocapsules for oral insulin delivery. Results showed that protamine nanocapsules did not induce any toxicity or immunological response in dendritic cells (DCs) at the concentration range of 0 to 16 mg/mL. The biodistribution profile of the fluorescently nanocapsules following oral administration to healthy mice showed that the nanocapsules are retained in the gastrointestinal tract (GIT) for at least 6 h. On the other hand, the pharmacological studies indicated that the subcutaneous (s.c.) injection of insulin-loaded protamine nanocapsules resulted in a glucose reduction (30%) with a delayed peak (2 h) and longer duration (3 h) compared to the control (free insulin solution) with a peak at 0.5 h and 2 h duration. The same formulation, administered intra-duodenally to healthy rats (4 h fasting) resulted in a moderate reduction of the glucose levels (20% reduction), which lasted for up to 7 h. This work raises prospects that protamine based nanocapsules may have the potential as oral peptide delivery nanocarriers.

## 1. Introduction

Insulin is one of the most important therapeutic drugs for the treatment of diabetes mellitus. It is commonly administered subcutaneously, which can lead to pain, allergic reactions, hyperinsulinemia, and even failed glycemic control due to non-compliance [1]. Consequently, obtaining an oral insulin formulation is one the highest priorities in a number of pharmaceutical industries. This interest relies on the fact that oral insulin administration would not only avoid the inconveniences associated with injections, but could also mimic the physiological fate of insulin hence provide better glucose homeostasis [2]. However, in order to make oral delivery for insulin possible, two major obstacles must be overcome, namely, the lack of insulin stability in the gastrointestinal tract (GIT) and its poor intestinal permeability. A variety of strategies have been investigated to achieve this goal [3], however, so far the success in terms of insulin reaching the clinical development phase has been very limited [4].

The use of nanotechnologies to develop nanocarriers for oral insulin delivery is one of the strategies that has received significant attention. The basis for this development has been that nanocarriers can protect the peptides against the harsh gastric environment including enzymatic degradation, while controlling drug release and increasing their absorption in the small intestine [5]. Due to their high surface-to-volume ratio, nanocarriers can significantly increase their cellular contact with the intestinal epithelium, thereby offering more chances for the drug to get across this epithelium [6][7]. Among the different nanosystems investigated for oral insulin delivery, it is important to highlight the pioneering work by Damgé *et al.*, who showed that poly(alkylcyanoacrylate) nanocapsules were able to increase the oral absorption of insulin [8, 9]. Since that time until now, the biopolymers that have received the greatest deal of attention for oral insulin delivery are chitosan [10–12], PLGA [13–16] and polyanhydrides [17–19]. Our group has contributed to this field through the design of nanocarriers made of chitosan and lipids [20, 21], which were shown to enhance the absorption of insulin [22] and salmon calcitonin [23, 24]. More recently our group introduced the use of polyarginine (PArg) nanocapsules for oral peptide delivery, as they were found to decrease the trans-epithelial resistance and closely interact with the intestinal epithelium (Lollo *et al.,* Submitted). This positive interaction was attributed to the positively charged arginine residues.

Protamine nanocarriers have also been designed in our group with the initial idea of using them for enhancing the transport of antigens across the nasal mucosa [25]. This development was based on the known capacity of protamine to enhance the penetration of proteins across cell membranes [26]. This study and others showing the possibility to enhance insulin absorption using protamine [27] portrayed protamine nanocapsules as a potential nanocarrier candidate for enhancing insulin absorption. In addition to this, it is worth to mention protamine has been approved by the FDA as one of the excipients in an insulin formulation [28].

Based on these premises, the main aim of this work was to elucidate the potential of protamine-based nanocapsules as an effective oral drug delivery system for insulin (and other similar peptides). Nanocapsules consisting of an oily core containing sodium glycocholate as a penetration enhancer, and coated with a single protamine layer or a double PSA/protamine layers were prepared and characterized with respect to their toxicity profile and the capacity to induce immunological response in dendritic cells (DCs). Biodistribution studies were also carried out in healthy mice after oral administration to provide information about the specific interaction and retention time of the nanocarriers in the different regions of the gastrointestinal mucosa. Finally, *in vivo* studies were performed in healthy rats in order to elucidate the pharmacological activity of insulin loaded protamine based nanocapsules after subcutaneous (s.c.) injection and intra-duodenal administration.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Protamine sulfate of low molecular weight (5 kDa, pharma grade), was purchased from Yuki Gosei Kogyo, Ltd., (Japan). Insulin glulisine (Apidra®, Mw 5823 Da) was kindly provided by Sanofi S.A. (Paris, France). Polyoxyethylene 40 monostearate (PEGst-40, pharma grade), was purchased from CRODA Europe Ltd, (UK), whilst sodium glycocholate (SGC, pharma grade), was obtained from Dextra, (UK). Caprylic/capric triglyceride (Miglyol® 812, pharma grade) was purchased from Cremer, Oleo Division, (Witten). DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) was supplied by Invitrogen (Spain).

Organic solvents were of HPLC grade and all other products used were of reagent grade purity or higher.

## 2.2. Preparation and characterization of insulin loaded protamine nanocapsules

The surfactants, PEGst-40 (16 mg) and sodium glycocholate (5 mg) together with Miglyol® (62.5 µL) were dissolved in 3 mL ethanol to obtain a clear lipid phase. Acetone (1.95 ml) was added to this organic phase followed by insulin glulisine (1 mg, dissolved in 0.01M HCl, 0.05 ml). This mixture was immediately poured over 10 ml of an aqueous phase composed of 0.15 % w/v protamine. The organic solvents were removed by evaporation under vacuum (Rotavapor Heidolph, Germany), to obtain a nanocapsule formulation with a constant final volume of 5 ml. Finally, protamine nanocapsules were isolated by ultracentrifugation (Optima™ L-90K, Ultracentrifuge, Beckman Coulter, USA) at 30 000 rpm for 1h (at 15 ℃) and resuspended in ultrapure water to the desired NC concentration. Optionally, protamine NCs were post-coated with polysialic acid (PSA) to improve their stability properties against enzymatic degradation of the loaded peptide. For this purpose, a volume of 5 ml, isolated protamine nanocapsules (concentration 18.66 mg/ml) was incubated with a volume of 1 ml solution of PSA at a concentration 3 mg/ml. The final protamine:PSA ratio was of 5:1 (w/w). The concentration of nanocapsules was determined taking into account all components in the formulation.

The amount of insulin associated to the NCs was quantified by reverse phase liquid chromatography (HPLC). For this purpose, the nanocapsules were digested using a combination of acetonitrile and Triton™ X-100 (2:1, v/v) and the amount of insulin associated to the nanocapsules was measured by HPLC (Chapter 2, section 2.2). Particle size, polydispersity index (PDI) and zeta potential of the NCs were determined by Dynamic Light Scattering (DLS) using Malvern Zeta-Sizer (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) fitted with a red laser light beam ($\lambda$=632.8 nm), at 25 ℃ after dilution with ultrapure water.

The nanocapsules loaded with the fluorescent dye DiD, used for *in vivo* imaging studies, were prepared as described above. In this case, 0.02 ml of DiD stock solution

in ethanol (2.5 mg/ml) was added to the organic phase before nanocapsules preparation. The final concentration of DiD in the formulation was 10 µg/ml. To determine the encapsulation efficiency of DiD-loaded nanocapsules, the system was centrifuged (30 000 rpm, 1h, 15°C) to eliminate the non-encapsulated dye. After this step, the encapsulation efficiency and release were calculated indirectly by the difference between the total amount of DiD in the formulation and the free dye in the undernatant. These samples were analyzed a λ = 646 nm, by fluorescence spectroscopy (EnVision Multilabel Plate Reader, Perkin Elmer, Massachusetts, MA, USA).

**2.3. Cell culture: murine bone marrow-derived dendritic cells (BM-DCs)**

Tibias and femurs from C57BL/6 mice were removed using sterile techniques and bone marrow (BM) was flushed. Red blood cells were lysed with ammonium chloride. $5x10^5$ BM-cells were plated in a 6-well plate, in a final volume of 3 ml of medium supplemented with 20ng/mL mouse recombinant GM-CSF and 100ng/mL of IL-4. The cultures were maintained at 37 ℃ in 5% $CO_2$-humidified atmosphere. On day 3 of culture, floating cells were gently removed, and fresh medium with cytokines was replaced. Culture medium was RPMI 1640 (Euroclone) with 2mM L-glutamine, 10mM HEPES, 20 µM 2-ME, 150U/mL streptomycin, 200U/mL penicillin, and 10% heat-inactivated FBS (Biochrom).

On day 6 protamine nanocapsules and PSA-coated protamine nanocapsules were added at different concentrations (0, 0.5, 1, 2, 4, 8, 16, 25 mg/mL) to the DC culture and incubated for 1 day with the aim of studying their potential effects on DC viability and maturation. Cultures of DCs treated with 1µg/mL of lipopolysaccharide (LPS, Sigma) were used as a positive control for DC maturation and untreated cells were used as the negative control. On day 7, DCs were recovered from plates, stained with Trypan Blue (Sigma) for cell count or stained for flow cytometric analysis. Cell vitality was evaluated by using 7-AAD Viability Staining Solution (eBioscience) as outlined in the manufacturer's instructions.

For FACS analysis Fcγ receptors were blocked with IgG2b from HB-197 hybridoma (LGC PromoChem) for 10 min at 4 ℃ and surface markers of interest were stained for 20 min at 4 ℃ with the following rat anti-mouse conjugated antibodies: anti-MHC-II

Ia-Ie FITC (BDBiosciences, 2G9 clone), anti-CD11c PE (BioLegend, N418 clone) and biotinylated anti-CD86 (eBioscience, GL1 clone). Then, samples were incubated with APC-conjugated streptavidin 1:1000 (BD Biosciences) for 30 min at 4 ℃. MHC-II and CD86 were considered as markers for DC maturation and reported as mean fluorescence intensity (MFI) [29]. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and then analyzed by using the softwares FlowJo v10 (Tree Star, Inc.) and Excel (Microsoft). Final data were normalized on the non-treated sample (negative control).

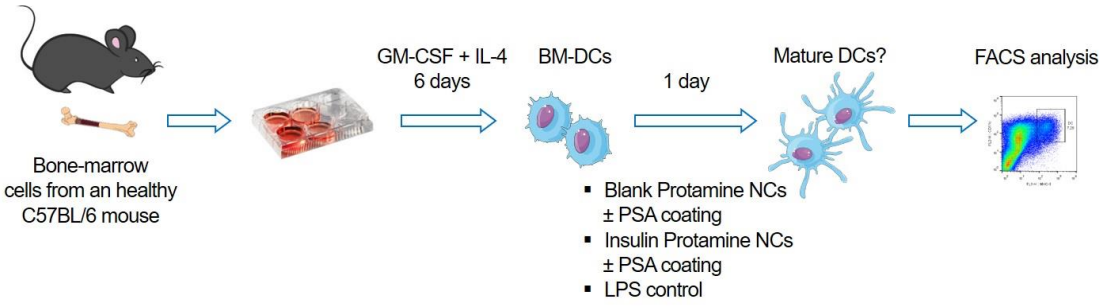


**Figure 1:** Schematic representation of experimental steps to determine the effects of protamine-based nanocapsules on DC viability and maturation.

After a 24-h incubation time with NCs at two different concentrations (1 and 4 mg/ml) DCs culture media were harvested, centrifuged to remove cells and frozen at -80 ℃. The concentration of TNF-α, IL-1β, IL-12 p70, IL-10 and IFN-γ released by DCs was assessed by ELISA following eBioscience's protocol. Briefly, flat-bottomed 96-well MaxiSorp plates (Nunc) were coated with capture antibody overnight at 4 ℃. Plates were rinsed three times with PBS-0.05% Tween-20 (Sigma) and blocked with assay diluent for 1 h at RT. After one wash, serially-diluted standards and samples were added and incubated 2 h at RT. Then, plates were rinsed three times and the biotinylated detection antibody was added and kept for 1 h at RT. Wells were washed three times, incubated with horse radish peroxidase-conjugated avidin for 30 min at RT, washed again and incubated with the chromogenic substrate TMB (eBioscience) until colour was fully developed but not yet saturated. Finally, the reaction was stopped with phosphoric acid 1 M. Measurements were made at a wavelength of 450 nm subtracting the specific emission at 540 nm on a Victor microtiter plate reader

(Perkin Elmer). The concentration of each cytokine was calculated by applying the sample absorbance to the standard's calibration curve.

### 2.4. *In vivo* biodistribution study

One week before the experiment BALB/c mice were placed on a low manganese diet to reduce auto-fluorescence. Moreover, abdominal fur was removed by depilation and the animals were fasted 24 h before administration of the formulation. Then, 0.2 ml of isolated DiD-labelled protamine NCs (NC concentration: 18.66 mg/mL and DiD concentration of 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 h) on isoflurane/oxygen-anaesthetized animals, using the Optix MX2 scanner (ART Inc., Saint-Laurent, Canada). Bioluminescent images were acquired with the Optix OptiviewTM (ART Inc.) acquisition software.

To further examine the interaction of NCs with the intestinal epithelium by confocal microscopy the intestines were dissected after treatment, washed with PBS and fixed in 4% PFA for 3 to 4 hours at 4 ℃. Thereafter they were treated and stored at -80 ℃ for subsequent cryostate processing. Samples were then cut into and 10 μm thick cryoslices using a Leica CM 1850 CM cryostat and transferred to slides (SuperFrost Plus, Thermo Scientific). Confocal images were acquired with Leica TCS SP5 II microscope and further processed using ImageJ software.

### 2.5. *In vivo* efficacy studies in non-diabetic rats

All animal experiments were reviewed and approved by the ethics committee of the University of Santiago de Compostela (ref. 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. Male Sprague–Dawley rats (250–300 g) were obtained from the Central Animals House, University of Santiago de Compostela (Spain). They were kept under 12 h light/12 h dark cycles and were fed a standard laboratory rodent diet (Panlab A04, Panlab laboratories).

### *2.5.1. Subcutaneous injection*

Male non-diabetic Sprague-Dawley rats (average weight 260 ± 12g) were fasted for 4 h prior to the experiment, with free access to water. Protamine NCs were administered subcutaneously to the rats at an insulin dose of 1IU/kg. An equivalent dose of free insulin solution was administered to a different group of rats as a control. Blood samples were collected from the tail vein 30 min prior to the administration, to establish the baseline blood glucose level. At time points 30 min, 1, 1.5, 2, 3, 4, 5, 6, 7 h after administration, the blood samples were collected to monitor the change in glucose levels following insulin-loaded protamine NCs or free insulin administration. The glucose levels were measured using a hand-held glucometer (Glucocard™ G+ meter, Arkray Factory, Japan). Administration and blood sampling were conducted without anaesthesia.

### *2.5.2. Intra-intestinal administration study*

Male non-diabetic Sprague-Dawley rats (average weight 267 ± 11g), used for the intra-duodenal (ID) and intra-jejunal (IJ) administration underwent an operation where, a catheter was implanted surgically into the duodenum or jejunum of each rat with the proximal end of the cannula tunneled subcutaneously to exit at the back of the neck, and sutured. The rats were allowed to recover for 6 days and were monitored for recovery and weighed everyday. They were fasted for 4 hours before the experiment with free access to water. Formulations (insulin-loaded protamine nanocapsules and blank protamine nanocapsules) were injected intraduodenally at a constant volume of 300 μL at a dose of 50 IU/kg. Before administration (0h), blood samples were withdrawn and initial blood glucose levels were measured using a hand-held glucometer (Glucocard™ G+ meter, Arkray Factory, Japan). During the experiment, 0.2 mL aliquots of blood were collected from the jugular vein at pre-determined time intervals (2, 4, 6 and 8h) whilst glucose levels were measured every hour. The pharmacological availability (PA) of oral insulin loaded protamine-based NCs was determined based on a 100% availability of the control insulin solution administered subcutaneously to the healthy rats at a dose of 1 IU/kg. Plasma glucose levels were plotted against time, and the area above the curve (AAC) below the 100% cut-off line was determined using the trapezoidal method (Origins 8.5).

**Equation 6**: $PA\% = \frac{AAC\ oral\ NC \div Dose}{AAC\ sc\ insulin \div Dose} \times 100$

### 2.6. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 program (CA, USA). All analyses were performed using the Student's t-test and differences were considered statistically significant at *$p<0.05$, unless stated otherwise.

## 3. Results and discussion

The nanocarriers investigated in this work were NCs consisting of an oily core, which contained insulin and the penetration enhancer SGC. These oily cores were stabilized with the surfactant PEG-st and also with a polymer coating made of a single protamine layer or a double PSA/protamine layer. The selection of protamine was motivated by previous works, which indicated the capacity of arginine-rich peptides to enhance the absorption of peptides through different mucosa [27]. Furthermore, the inclusion of a lipid core was based on the recognized ability of lipids to protect macromolecules, as well as to enhance their intestinal permeability. The obtained protamine NCs, prepared by the solvent displacement technique, had a size below 400 nm, and a low positive or negative zeta potential, for protamine or PSA/protamine NCs, respectively. This almost neutral charge was attributed to the presence of PEG-st around the oily nanodroplets (**Table 1**). The association of insulin resulted in a size increase whilst there were no significant variations after the labelling of the nanocapsules with DiD.

**Table 1:** Physicochemical properties, insulin association efficiency (AE) and loading capacity (LC) of the nanoemulsion (NE), Protamine nanocapsules, PSA-Protamine nanocapsules and DiD-labelled protamine nanocapsules. (Mean ± SD, n=3).

| Formulation | | Size ± SD (nm) | PDI | Z-pot ± SD (mV) | AE ± SD (%) | LC (%) |
|---|---|---|---|---|---|---|
| **Protamine NCs** | Blank | 264 ± 18 | 0.2 | +8 ± 2 | - | - |
| | Insulin loaded | 391 ± 35 | 0.3 | +5 ± 2 | 65 ± 9 | 1.0 |
| **PSA-Protamine NCs** | Blank | 215 ± 12 | 0.2 | -6 ± 3 | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Insulin loaded | 366 ±27 | 0.2 | -3 ±4 | 52 ±11 | 0.8 |
| **DiD-Protamine NCs** | Blank | 302 ±21 | 0.2 | +6 ±3 | - | - |

### 3.2. *In vitro* toxicity and immunological evaluation

#### *3.2.1.* Toxicity of nanocarriers in murine bone marrow-derived dendritic cells (BM-DCs)

The therapeutic use of peptide and protein drugs, especially when repeated administrations over prolonged periods are required, is often compromised by the possibility of immunotoxicity through the activation of dendritic cells (DCs) [30]. Therapeutics should not induce DC mortality or maturation and therefore we found it crucial to evaluate the interaction of protamine nanocapsules with immune cells (DCs). The results shown in **Figure 2, panel A** indicate that protamine and PSA/protamine NCs are non-toxic to DCs at concentrations up to 16 mg/mL (insulin concentration, 0.23 mg/mL**)**. However, while protamine nanocapsules did not activate DC maturation, PSA/protamine/ nanocapsules did. This is shown in (**Figure 2, panel B)** which depicts the percentage of activated DCs (those DCs that expressed maturation markers).

Under conditions of infection or inflammation, DCs encounter activating signals that may mature and activate DCs simultaneously. DC activators include pro-inflammatory cytokines and bacterial or viral products such as LPS, CpG motifs, and double-stranded RNA. They induce the maturation and activation of DCs, allowing DCs to present antigens to the immune system [31]. Activated DCs can be distinguished from resting and immature DCs by the expression of higher levels of MHC class II and the expression of co-stimulatory molecules such as CD80 and CD86 or for the production of cytokines such as interleukin (IL)-12.

The effect of protamine-based nanocapsules on DC maturation was further evaluated by measuring the expression of two DC markers; MHC-II and CD86. The results shown in **Figure 2, panel C, D, E** indicate that 24h after incubation with protamine nanocapsules, DCs expressed relatively low levels of surface MHC-II proteins and co-stimulatory molecule CD86 compared to PSA-coated protamine nanocapsules. The comparison between blank protamine nanocapsules (white bars) with insulin-loaded

protamine nanocapsules (lighter grey bars) shows that the presence of insulin inside nanocapsules had no additional effects on DC maturation. However, the levels were significantly higher for the nanocapsules containing PSA, these values being remarkable even at low concentrations.

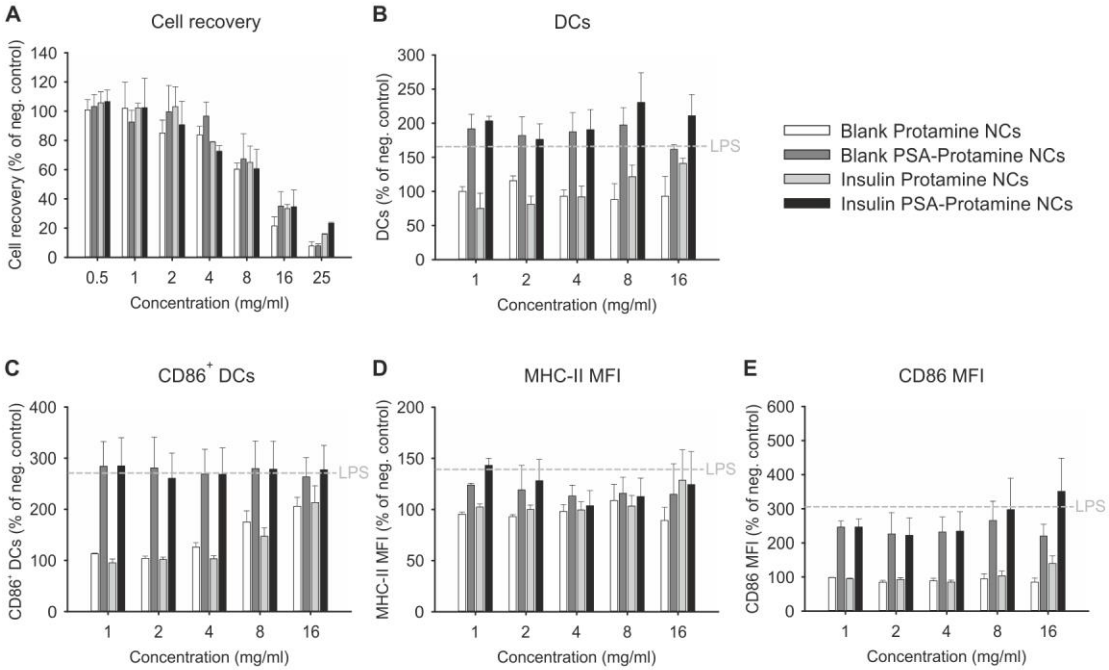


**Figure 2:** Effects of protamine and polysialic acid (PSA/protamine NCs on DC vitality (A) and maturation (B). Vitality of cell cultures was evaluated by Trypan blue staining. Percentages of matured DCs obtained (alive) and their MFI (mean fluorescence intensity) were determined by FACS analysis on DCs (C,D,E). MHC-II and CD86 are used as markers of DC maturation (mean ± SE, n=3).

In agreement with the previous data, the analysis by ELISA shows that the presence of protamine nanocapsules in the cultures did not induce the release of the inflammatory cytokines TNF-α, IL-1β, IL-12 (p70) and IL-10 in the culture medium compared with negative control (**Figure 3**). However, blank and insulin-loaded-PSA-protamine nanocapsules induced a higher cytokine release, which was comparable to the positive control in the case of TNF-α and IL-12 (**Figure 3**). Altogether, these observations show that the PSA-coating can stimulate proliferation and maturation of *in vitro*-differentiated murine DCs. These data are in contradiction with the fact that polysialylation has been used to abrogate both the immunogenicity and antigenicity of injected peptides and proteins whilst preventing proteolytic degradation and prolonging their half lives [17]. However, some concerns remain pertaining to the

utility of PSA coatings for oral peptide delivery, since its binding to the surface of the carrier system may alter the immunological response. It is also worth mentioning that all excipients used in this formulation are pharmaceutical grade, except for the PSA polymer. Hence, one of the next steps include more studies to understand if these effects depend on PSA molecule *per se* or on some contaminants remaining from its synthesis/association to protamine nanocapsules.

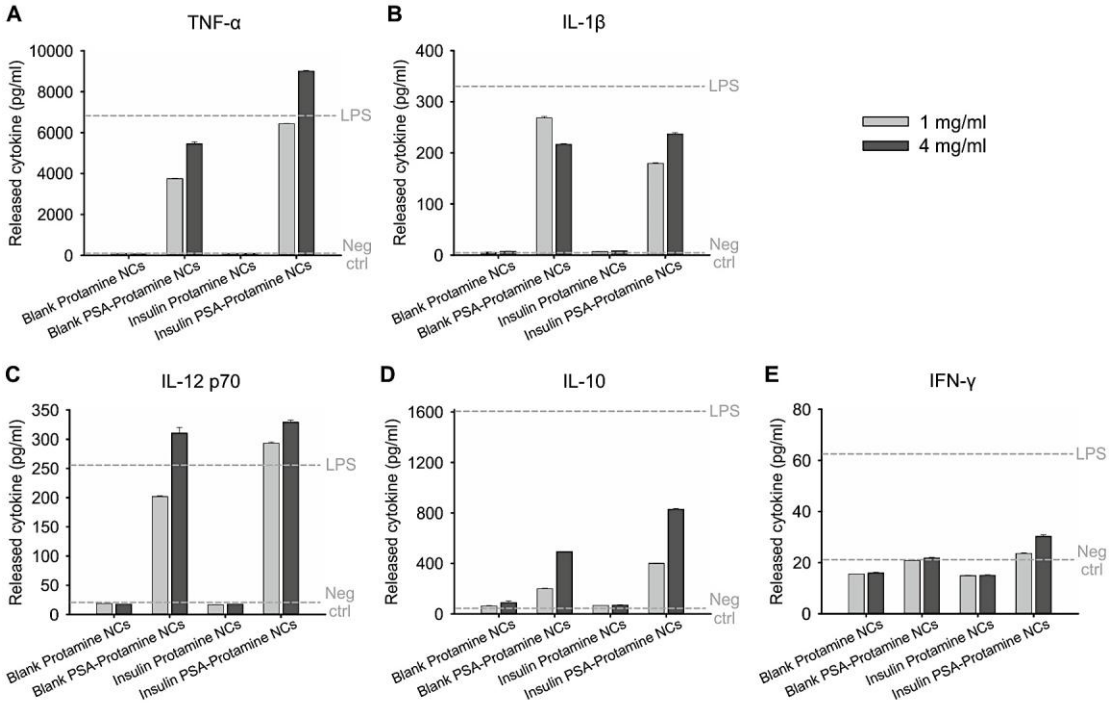


**Figure 3:** Release of different inflammatory cytokines evaluated in the supernatant of DC cultures either in presence or absence of protamine and polysialic acid/protamine nanocapsules at two different concentrations assessed by ELISA (mean ± SE, n=3).

Overall, this study shows that protamine-based nanocapsules are non-toxic to DCs, and that PSA coated NCs induce DC maturation, which may imply that this formulation could cause inflammation of the epithelium. It would be interesting to investigate *in vivo* if this inflammation occurs and if it causes any damage or irritation to the intestinal epithelial membrane.

### 3.3. *In vivo* studies

#### *3.3.1.* Biodistribution of protamine nanocapsules

The total body scanning following oral administration discloses that the DiD-labelled protamine nanocapsules produced a signal that remained confined mainly to the stomach and the small intestine, with an emission peak at one hour post-administration, and a decrease at 24 h after administration (**Figure 4A**). The histogram on the left shows the mean of total photons emitted from regions of interest (ROI) around the peritoneal cavity (**Figure 4B**). Consequently, the number of photons was high after the first hour of administration and gradually decreased over time. The prolonged retention of the protamine nanocapsules could be related to their potential interaction with the GI mucosa, which in turn increases the prospects for these nanocapsules to improve permeability of the loaded peptide across the mucosa. Similar results were reported for polyarginine nanocapsules (Lollo *et al*., submitted).

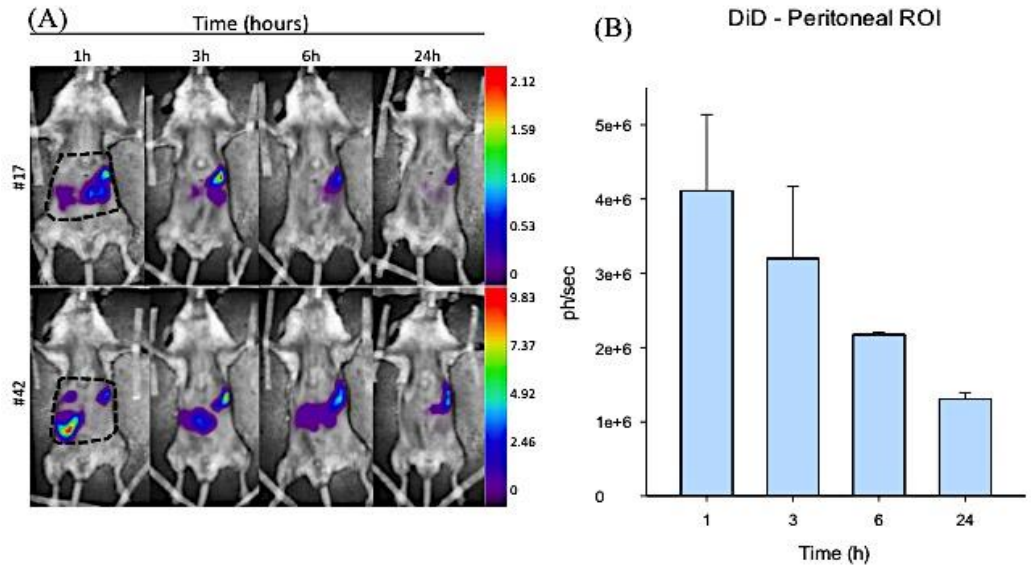


**Figure 4:** Representative fluorescence images of mice at 1h, 3h, 6h and 24h following oral administration of DiD-labelled protamine NCs (A), and photons emitted from regions of interest (ROI) around the peritoneal cavity (B), Mean n=3 ± SD.

A more in depth analysis of the interaction of the fluorescent NCs in the intestinal mucosa was performed by dissecting the intestines of mice at 1 h after oral administration of the NCs. As shown in **Figure 4 C**, at this time point (after 1h) protamine NCs appear to be localized in the mucus layer covering the intestinal villi. This effect may imply that protamine NCs do not diffuse through the mucus. This

interpretation would disagree with the results observed in the Caco-2/HT29-MTX co-culture (mucus-secreting cells) model. Nevertheless, this observation was made only one hour after administration and, hence further studies are needed to have a more concrete understanding of the fate of the NCs [32].

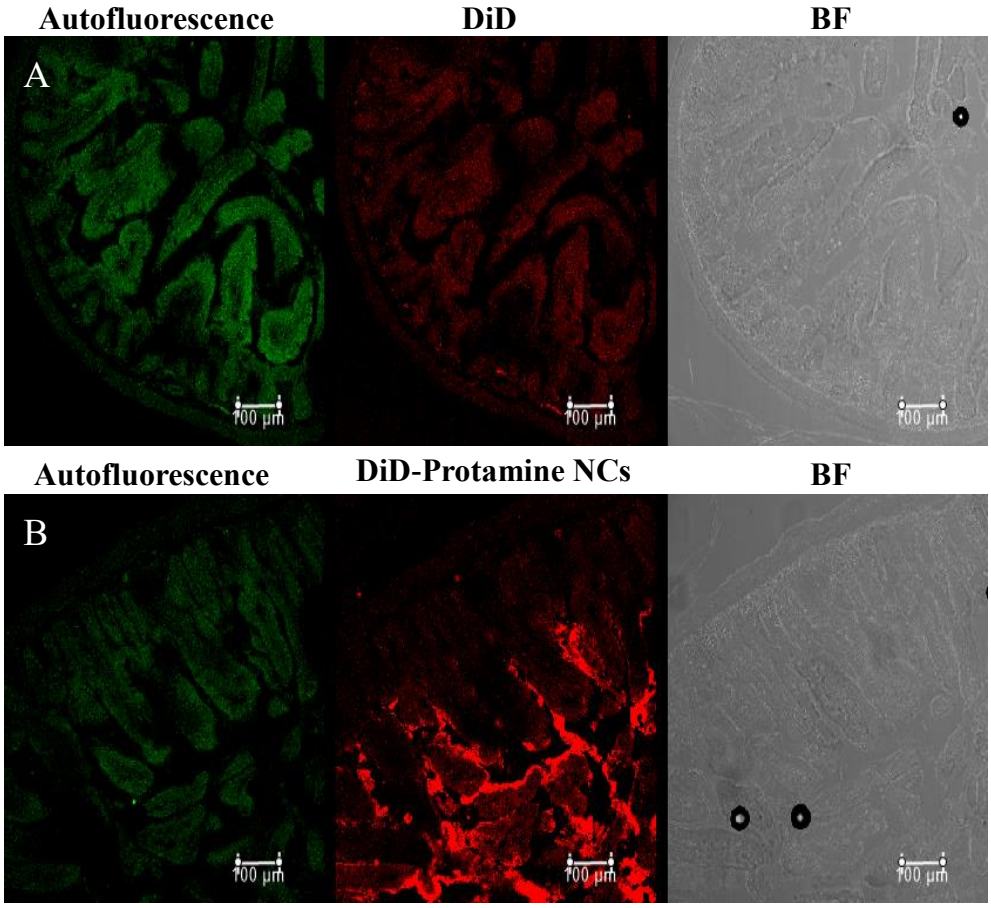


**Figure 4C:** Confocal images showing the interaction of (A) free DiD (control) and (B) DiD-protamine NCs with mouse intestinal tissue (duodenum) 1h after oral administration. (20X magnification with air objectives).

### *3.3.2. In vivo* biological activity of insulin loaded protamine nanocapsules

To determine the bioactivity of the encapsulated insulin, insulin-loaded protamine nanocapsules were injected subcutaneously into fasted healthy rats. The insulin bioactivity was measured by monitoring blood glucose levels and comparing the response to an equivalent dose of free insulin solution (1 IU/Kg). As shown in **Figure 5**, after the s.c. injection of the insulin solution, the glucose level rapidly decreased by 40% at 0.5 h and this effect lasted for 2 h, followed by the recovery of the glucose level back to ≈100%. On the contrary, the administration of insulin-loaded protamine

nanocapsules led to a moderate and constant reduction of the glucose level, from 10% up to 30% reduction within the first hour and this effect was maintained for 3h. The relative bioavailability of the insulin-loaded protamine NCs was found to be 78.9±3 when compared to the free insulin control (*p<0.05) (Table 2), suggesting that not all the insulin is released from the NCs within the first 8 hours.

From this study it was evident that the insulin maintained its bioactivity and that encapsulating insulin in protamine nanocapsules generated a unique profile compared to the free insulin i.e. a delayed and more controlled release of the insulin leading to a moderate response, which lasts for a longer duration than that of the free insulin (control). These results correlate with the observations we previously made in our *in vitro* release studies in simulated intestinal media (Chapter 2), where insulin release gradually increases up to ≈50% at 1h which is then followed by a constant and controlled release up to ≈70% after 6h. Hence, it is appropriate to assert that the method employed for the preparation of insulin loaded protamine nanocapsules preserves insulin and that this nanosystem is suitable for the delivery of peptides as it controls the release and does not compromise the bioactivity.

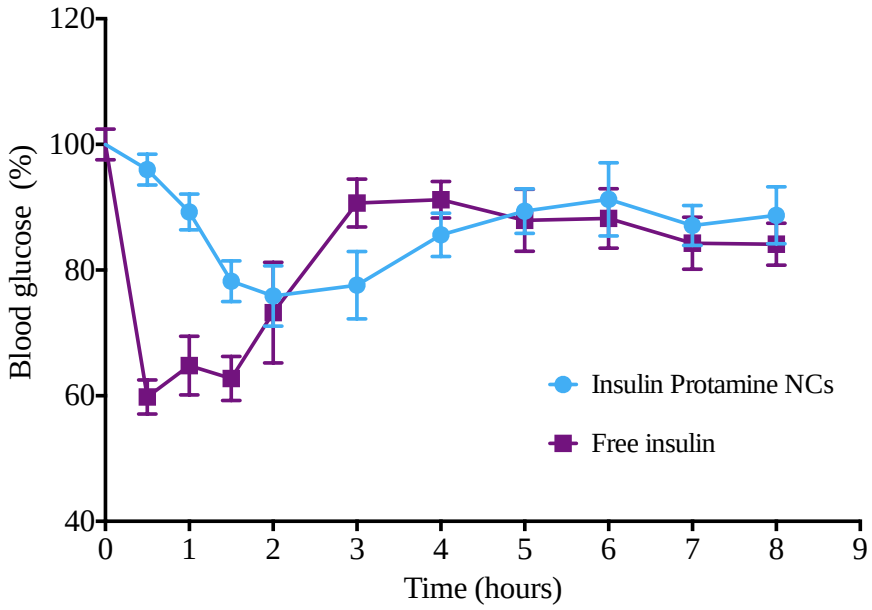


**Figure 5:** Standardized hypoglycemic effect following subcutaneous injection of insulin loaded protamine nanocapsules and free insulin saline solution (control) at 1IU/kg to healthy rats. Expressed in blood glucose % of initial. (Mean ± SEM, n=13).

### *3.3.4. In vivo* intra-intestinal administration of insulin loaded protamine nanocapsules

Based on these favourable preliminary observations, insulin loaded nanocapsules were administered intra-duodenally and intra-jejunally to non-diabetic rats. Intra-duodenal administration of insulin-loaded protamine nanocapsules (50 IU/kg), led to a moderate reduction of glucose levels (≈20%) after 1 h, which was maintained throughout the duration of the experiment (8 h). This effect was significantly different to the one observed for the blank protamine nanocapsules (placebo control) and the free insulin (control), where no response was observed at all (**Figure 6**). As shown in **Table 2**, the relative pharmacological bioavailability (compared with that of free insulin administered subcutaneously) of insulin was 1.5% and 1% when administered intraduodenally in the form of protamine and PSA-coated protamine NCs, respectively. However, no response was observed for free insulin solution after intraduodenal administration. These results suggested that although a low bioavailability is observed, the protamine-based NCs still protect a fraction of insulin from degradation and enhance its intestinal absorption as compared to free insulin. No significant difference ($p>0.05$) was observed at each time point between intra-duodenal and intra-jejunal administration (results not shown).

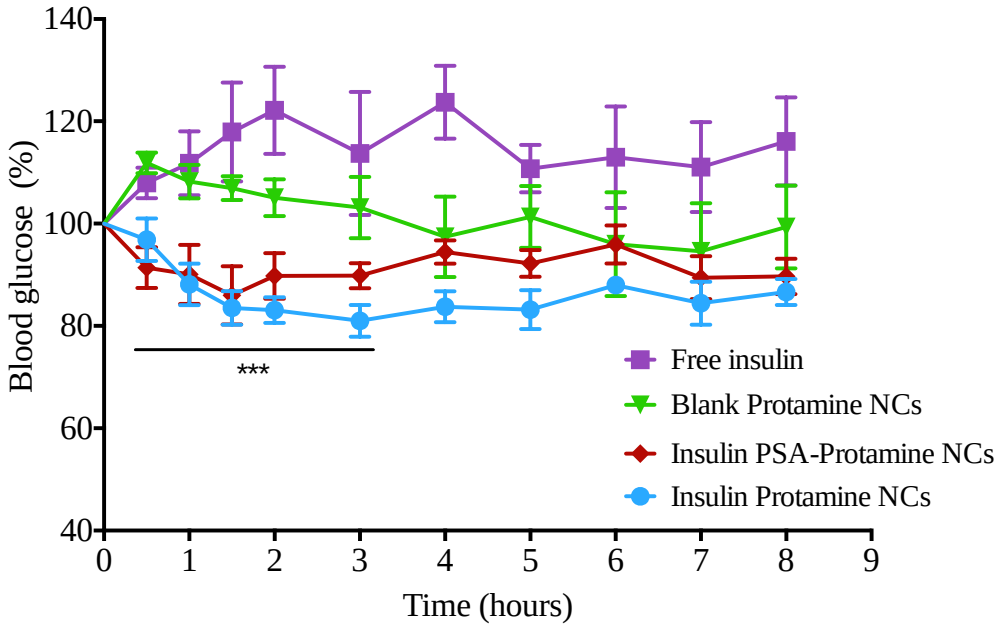


**Figure 6:** Standardized hypoglycemic effect following intra-duodenal (ID) administration of insulin loaded protamine (n=11) and PSA-coated NCs (n=15), at 50IU/kg, blank protamine NCs (placebo, n=12), and free insulin saline solution (n=16)

at 50IU/kg to healthy rats. Expressed as blood glucose % of initial. (Mean ± SEM, significant difference ***$p < 0.05$ compared to placebo control).

**Table 2:** Parameters for plasma glucose levels and relative pharmacological bioavailability for protamine NCs (n=11) and PSA-coated NCs (n=15), following intraduodenal administration at 50IU/kg, compared to free insulin (n=13) by S.C at 1 IU/kg. Data represents the mean ± SE, *$p<0.05$ significant difference between protamine NCs by s.c and free insulin by s.c).

| Formulations | Insulin dose (IU/kg) | $C_{min}$ (%) | $T_{min}$ (h) | AAC | PA% |
|---|---|---|---|---|---|
| Insulin S.C. | 1 | 59 ±3 | 0.5 | 101.1 ±12 | - |
| Protamine S.C. | 1 | 75 ±5 | 2 | *79.8 ±6 | 78.9 ±3 |
| Protamine NCs I.D. | 50 | 80 ±3 | 3 | 83.3 ±7 | 1.65 ±0.5 |
| PSA-Protamine NCs I.D | 50 | 85 ±6 | 1.5 | 51.3 ±9 | 1.01 ±0.8 |

*Cmin,* minimum plasma glucose concentration (% of initial); *Tmin,* time to *Cmin*; *AAC,* area above the plasma glucose levels time curves; *PA%,* relative pharmacological bioavailability (based on AAC for subcutaneous administration (SC)).

The glycaemic response in healthy rats observed in this study is relatively low (modest) when compared to other reported glycaemic responses in diabetic rats [12]. This difference is due to physiological differences of the two models i.e. the autoregulation process triggered by exogenous insulin in the blood supresses the endogenous secretion of insulin by β-cells in healthy rats whereas this autoregulation phenomenon does not exist in diabetic rats [33]. However, in this study we preferred to work with non-diabetic rats in order to reduce the variability in the data associated to the induction of diabetes using streptozotocin (STZ), which can lead to varying degrees of hyperglycaemia amongst rats due to beta cell deficiency/failure. Moreover, because of the beta cell deficiency the animals become prone to hypoglycaemia and very sensitive to insulin. Which implies that even a minor amount of insulin may produce a drastic reduction of glucose. Hence, we hypothesized that even a minor response in healthy rats would be much more admissible in terms of clinical relevance as compared to a diabetic rat model. Not surprising, low responses and even no

response at all have been reported for healthy rats following intra-gastric administration of insulin loaded nanoparticles [33, 34]. Nonetheless, some authors have reported good responses in non-diabetic rats. On this regard some authors found that a glycaemic response could only be observed when the healthy rats were given a glucose challenge, confirming that the insulin loaded in NCs is indeed absorbed into the blood circulation but the effect could be suppressed by autoregulation [8, 33].

Other authors using the same model (non-diabetic rats) have some differences in their experimental protocols compared to ours; for instance, they administer the insulin carriers intra-ileally [35]. The ileum is associated with less enzymatic activity and a high amount of M-cells when compared to the duodenum and jejunum hence higher absorption occurs in this region. Moreover, in most cases, we found that the rats were subjected to longer fasting periods e.g. 24 to 48 h [15, 34, 36, 37]

Although, these differences in the experimental procedures used could justify the differences in the data reported we also had a number of hypothesis that could explain the limited *in vivo* performance of the NCs, namely (i) The positive *in vitro* stability and insulin release data of the NCs might be slightly different in the *in vivo* situation; (ii) The limited transport of the NCs across the intestinal epithelium. In fact, even though fluorescence associated to the nanocapsules was found to cross the monolayers (Caco-2 and Caco-2/Raji), this transport was not particularly high and the amount of insulin transported remains to be elucidated; (iii) The formulation administered *in vivo* exhibited a high viscosity that could impede its miscibility with the intestinal fluids and hampered the adequate diffusion of the NCs across the mucus.

Finally, we should emphasize the fact that the experimental protocol and animal model used in this study is not the one typically used to assess the efficacy of insulin nano-formulations. Determining the concentration of insulin available in the blood, to ascertain if the glucose levels are indeed a reflection of the amount of insulin absorbed into the blood circulation is also one of the next steps of this work and it is expected to shed light on the absorption of insulin. Ultimately, the final aim is that the formulation will be incorporated into a final dosage form such as protective microbeads or enteric-coated capsules to improve the performance of the system in terms of controlled or targeted release and higher insulin absorption.

## 4. Conclusions

In this work protamine nanocapsules were evaluated for their potential as nanocarriers for oral peptide delivery. Protamine nanocapsules did not induce maturation of DCs implying that the oral administration of this formulation would not induce an undesirable immunological response. The results obtained following subcutaneous injection of insulin-loaded protamine nanocapsules in healthy rats confirmed that the bioactivity of the insulin is preserved. On the other hand, following intra-jejunal and intra-duodenal administration of the formulation, a moderate reduction of the glucose levels was observed and this effect was maintained for at least 8 h. While these data provide a preliminary efficacy proof of concept of oral insulin delivery using protamine nanocapsules, they also raise concerns about the most appropriate *in vitro* and *in vivo* model and protocols to be adopted in order to deduce meaningful data that could be easily extrapolated to and predictive for clinical relevance.

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# Overall discussion

# OVERALL DISCUSSION

## Introduction

The oral modality of administration is the most desirable route of delivery, especially in the case of drugs used for chronic treatments, i.e. diabetes. Unfortunately, this modality of administration has phenomenal barriers, which hamper the absorption of peptide from the GIT. The use of peptide nanocarriers that can prevent enzymatic degradation, control the release and favour the interaction of the peptide with the small intestine is nowadays considered a technological strategy worthwhile to explore [1].

In this work protamine-based NCs were rationally developed for the oral delivery of peptides, using one of the most challenging peptides, insulin, as a model drug. The natural polyaminoacid, protamine was selected as the corona of the NCs because of its ability to increase cellular internalization and its mucointeraction property which can enhance mucosal delivery of peptides [2]. The inner lipidic core was mainly composed of Miglyol, a medium chain caprylic capric tryglyceride, well known for it ś penetration enhancing property and for protecting peptides from degradation [3, 4]. Whilst, sodium glychocolate is a remarkable bile acid salt surfactant that prevents proteolytic degradation of peptides and promotes their absorption [5]. Another surfactant molecule, PEGst-40 (PEG, 2 KDa), was added to be located at the oil/water interphase, as it was found essential for the colloidal stability of the nanocarrier and was supposed to improve its mucodiffusion properties [6]. Optionally, the protamine NCs were provided with an additional coating layer of polysialic acid (PSA), a low molecular weight polysaccharide that was intended to form a shield around the NCs to prevent the adsorption of enzymes (**Figure 1**) [7].

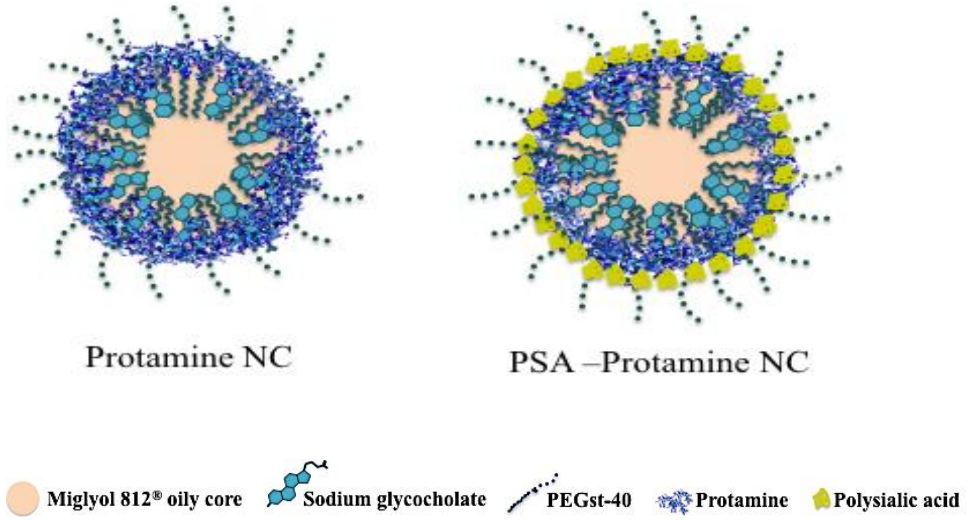


**Figure 1:** Schematic representation of protamine NCs and PSA coated protamine NCs**.**

**Physicochemical and pharmaceutical characterization of insulin-loaded nanocapsules**

Using these biomaterials, protamine NCs were prepared using the solvent displacement method, a simple and fast method that is easy to scale-up [8]. PSA was added as an external coating layer through ionic interaction with protamine and its presence around the shell was confirmed by the charge inversion of the NCs. The NCs obtained were characterized according to size, zeta potential, and insulin loading capacity (**Table 1**). The nanometric size (<500 nm) and nearly neutral surface charge of the NCs was found to be adequate for the oral delivery of insulin, since these properties could promote their penetration across the intestinal mucosa. Moreover, the introduction of the protamine shell around led to formulations with an acceptable insulin association efficiency (AE%), a fact that underlines the importance of the polymeric core in preventing the diffusion of insulin out of the nanosystem into the external phase.

**Table1:** Physicochemical properties, insulin association efficiency (AE%) and loading capacity (LC%), including stability properties of protamine-based NCs as compared to the control, the nanoemulsion (NE) (mean ± SD, n=3).

| Formulation | Size (nm) | ζ-pot (mV) | AE % | Stability (6 h) | Release (6 h) | Proteolysis (% reduction, 1 h) |
|---|---|---|---|---|---|---|
| **NE** | 345 ±13 | -21 ±1 | 35 ±11 | No stable | - | - |
| **Protamine NCs** | 382 ±69 | +6 ±3 | 62 ±16 | Stable in FaSSIF/FeSSIF | 70 ±1% | 72 ±3 |
| **PSA-Protamine NCs** | 301 ±84 | -4 ±1 | 51 ±9 | Stable in FaSSIF/FeSSIF | 60 ±2% | 60 ±4 |

After optimizing the physicochemical properties of the protamine and PSA-coated protamine NCs it was critical to assess their stability and insulin release profile in different simulated intestinal media; SIF, FaSSIF-V2 and FeSSIF-V2. The studies revealed that the NCs are stable in these media, especially when they were provided with an extra coating layer of PSA. During this 6 h-incubation time in the FaSSIF medium, comparable to the fasted state *in vivo*, a biphasic insulin release pattern was observed (**Figure 2**). The peptide was gradually released within the first hour, followed by a constant release rate up to ≈70% of the encapsulated insulin at (6h) for both formulations. The stability and controlled release profile of the formulations were attributed to the core-shell structure of the NCs, where the presence of the polymeric shell provides a steric protection and prevents a rapid diffusion of insulin to the external media [9, 10].

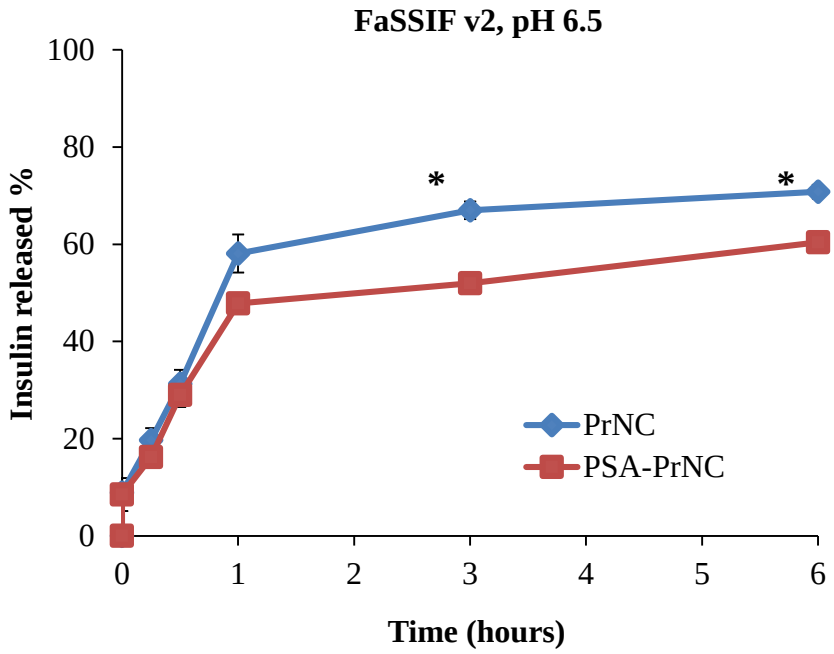


**Figure 2:** *In vitro* release profiles of insulin from protamine NCs and PSA-protamine NCs incubated in FaSSIF-V2 (B), (mean ± SD, n=3, *p<0.05).

Proteolysis studies were then performed to elucidate the ability of the formulations to protect the associated insulin from enzymatic degradation. For this, insulin-loaded protamine NCs and PSA-protamine NCs and free insulin solution (control) were incubated in simulated intestinal fluid containing enzymes (FeSSIF-V2). It was evident that the presence of the extra coating layer of PSA reinforced the stability of the NCs (**Figure 3**). Moreover, the bile salt surfactant, SGC well known for inhibiting protease enzyme activity, also provided improved stability to the nanosystems, and significantly contributed towards preserving insulin from enzymatic degradation [11]. In contrast, for free insulin, a drastically rapid decrease of the insulin amount was observed, with approximately all the insulin degraded in the first 15 minutes. This observation highlighted the importance of encapsulating insulin as a stability reinforcement strategy.

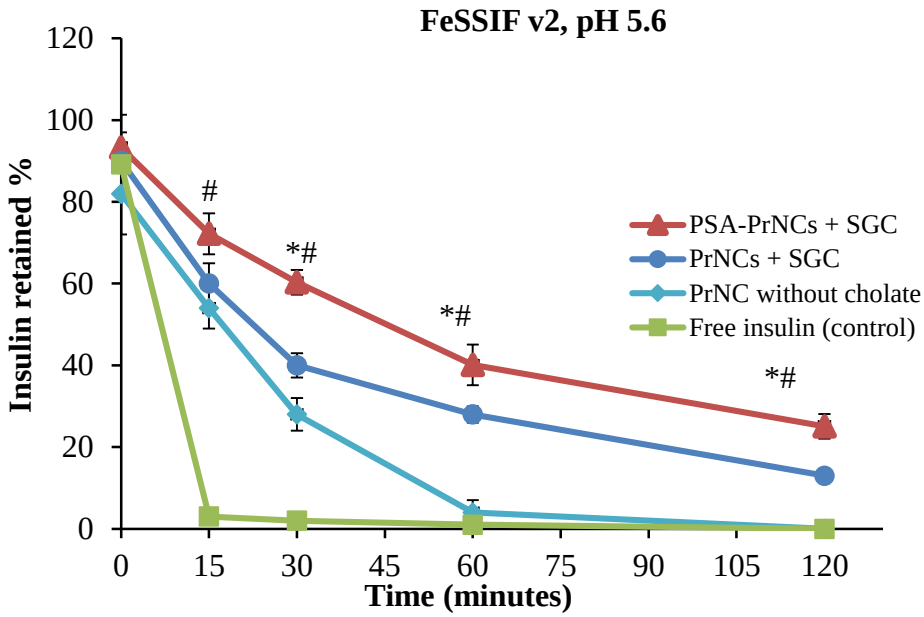


**Figure 3:** Stability profiles of the associated insulin after incubation of NCs in FeSSIF-V2 containing pancreatic enzymes (mean ± SD, n=3), significant difference ($p<0.05$) for PSA-PrNCs+SGC compared to *PSA-PrNCs+SC and #PrNC without cholate/free insulin.

**Mechanism of interaction of the nanocapsules with the Caco-2 monolayers and isolated human intestinal tissue**

Both formulations were nontoxic to Caco-2 cells at a NC concentration range of 0.5 to 8 mg/ml for 2h and 6h incubation time. This confirmed the safety profile of the nanosystems for oral administration. Subsequently, the formulations were evaluated for their ability to promote paracellular transport by monitoring the TEER values of the cell cultures, which correlates to the modulation of the tight junctions (TJ). TEER values of the Caco-2 cell monolayers were not modified after 1 and 2 h-exposure to the NCs, however a significant reduction was only observed after 4 h incubation time. This behavior is in relation with one reported for other polycations, such as chitosan and polyarginine[12]. In the case of chitosan, it has been postulated that if interfres with the calcium transfer, thereby disrupting the TJs [14]. In the case of polyarginine, it has been described that the TJs opening is due to their transient internalization in cell–cell junctions via clathrin-mediated endocytosis [15]. In the case of protamine, although the mechanism by which it opens TJs has not been fully elucidated, it is presumed that it disrupts the microfilaments of the TJ proteins [13]. Importantly, the results obtained in this study indicate that the initial TEER was completely recovered within an 1h after the removal of the NCs [16]. This recovery process is an important

feature since complete disruption of the TJs is undesirable as this may lead to the entry of pathogens and toxins.

To further elucidate the mechanism through which protamine and PSA/protamine NCs might facilitate the absorption of peptide drugs, transport studies of the fluorescent nanocapsules (TAMRA covalently linked to the protamine shell) were performed in the presence of inhibitors. The results in terms of amount of fluorescence internalized by the cells suggested that nanocapsules might have been internalized into the enterocytes using both, caveolae (predominant) and clathrin pathways (**Figure 4**). Caveolae are extremely abundant at the surface of epithelial cells have been reported to be the predominant entry for particles above 200 nm, although multiple pathways are normally involved in this internalization process[18, 19]. This mechanism was supposed to be favoured by the ability of protamine to enhance membrane translocation through its high content of arginines, which carry the guanidine group essential for cell membrane translocation [20].

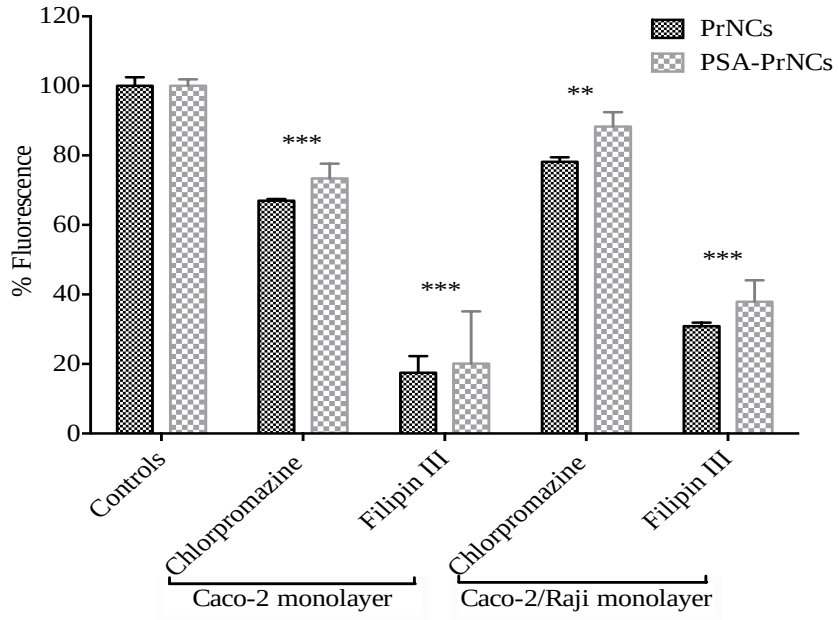


**Figure 4:** Comparison of transport under clathrin and caveolae inhibitors, (chlorpromazine and Filipin III respectively), A-Intracellular uptake of NCs and B-Transport in Caco-2 and FAE monolayers after 2h incubation with inhibitors. Untreated cells used as controls and fluorescence (%) represents the amount of fluorescence transported to the basolateral side in comparison to that in the basolateral side of the respective control, (mean ± SD, n=3, N=2; ***$p<0.05$).

Since enterocytes (90%) and goblet cells (10%) represent the two most abundant cells in the intestinal epithelium, the co-culture of Caco-2 cells and mucus-producing goblet cells HT29-MTX was used as a transport model incorporating the mucus barrier. Caco-2:HT29-MTX cells seeded at a 3:1 proportion exhibited a homogenous mucus layer, which was found not affect the permeation enhancing property of the nanocarriers. The absence of a visible effect of the mucus on the interaction and transport of the fluorescence nanocapsules (**Figure 5**) was attributed to their neutral surface charge which was expected to facilitate the muco-penetration of eth nanocapsules [21].

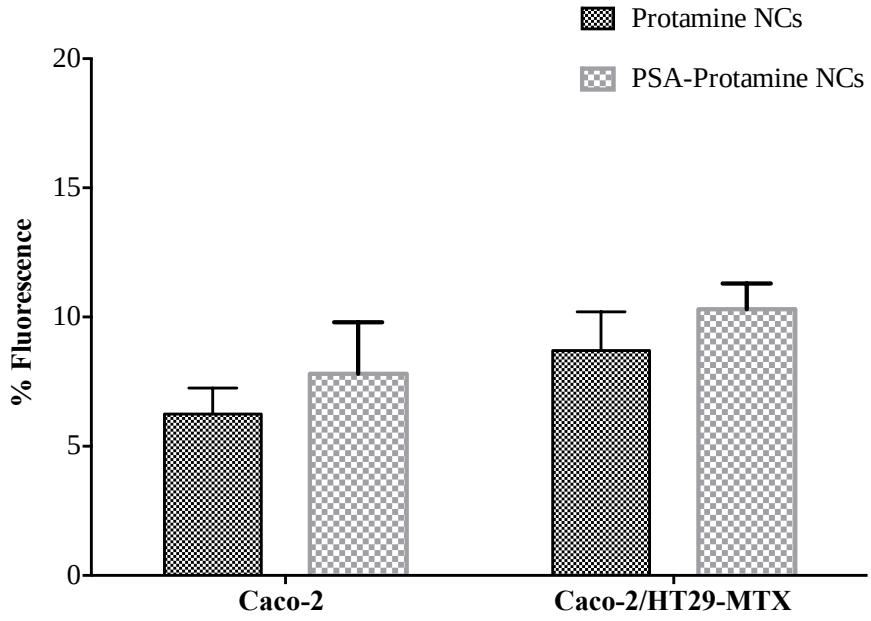


**Figure 5:** Caco-2 monolayer (A) and Caco-2/HT29-MTX (B) stained with Alcian blue, demonstrating the presence of mucus (magnification 400x) and Transport of coated and uncoated protamine NCs in Caco-2 and Caco-2/HT29-MTX monolayers after 2h incubation (C) (mean ± SD, n=3, N=3).

The permeability enhancing property of the NCs was further explored in human intestinal tissue. Confocal images showed that TAMRA-labelled protamine and PSA-protamine NCs interact with the intestinal epithelium, as fluorescence could be visualized within the epithelia **(Figure 6)**. Most importantly, no toxic effects were found on the intestinal tissues after being in contact with both formulations (5 mg/ml NC concentration, for 2h).

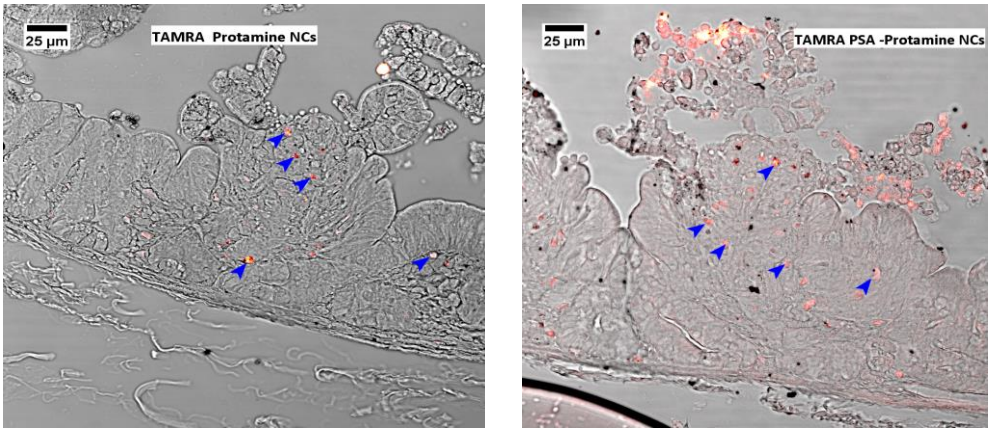


**Figure 6:** Confocal images showing the transport of Protamine NCs and PSA-Protamine NCs in human intestinal epithelial tissue.

**Immunotoxicity of the nanocapsules**

Based on the positive response observed in the Caco-2 monolayers and in the isolated human tissue further *in vitro* immune-toxicity were performed in murine bone marrow-derived dendritic cells (BM-DCs). The results showed that both formulations are non-toxic to DCs. However, the presence of PSA showed the tendency to induce DC maturation, which may imply that this formulation could cause inflammation of the GIT. Surprisingly, this data contradict the fact that PSA has been reported to alleviate both the immunogenicity and antigenicity of injected peptides and proteins [22]. Moreover, this LPS effect which could be associated with the presence of PSA in the NCs might be overestimated in this *in vitro* cell model in comparison to the *in vivo* situation, hence correlating this data to *in vivo* data would shed more light. This is an important aspect in the development of therapeutics for the treatment of chronic diseases as it is crucial to ascertain that their repeated use would not provoke inflammatory diseases by activating dendritic cells.

***In vivo* performance of the nanocapsules**

The *in vitro* results clearly underlined the value of the rational design. Indeed the developed nanocapsule formulations fulfilled a number of criteria, as indicated in the target product profile (**Table 1**). Based on these promising properties, we studied the *in vivo* interaction of the nanocapsules with the intestinal epithelium of mice using IVIS and confocal microscopy of intestine section. The results showed that the nanocapsules adhere to the intestinal epithelium, although no clear evidence of their internalization was obtained.

Thereafter, *in vivo* efficacy experiments were performed in normal rats in order to assess the glucose response after insulin administration. In the first set of experiments. the bioactivity of the insulin-loaded NCs was compared to that of free insulin (control) following subcutaneous administration (Insulin dose: 1 IU/kg). The results showed that insulin maintained it's bioactivity after its association into both PSA coated and non-coated protamine NCs. However, the absorption rate was different from the NCs formulations as compared to the one of the insulin in solution. Indeed, because of their controlled release properties, the NCs led to a moderate response within the first hour, which gradually increased and lasted for a longer duration than that of the free insulin [23]. These results correlated with the observations made previously on *in vitro* release studies in simulated intestinal media (FaSSIF-v2), where 50% of the encapsulated insulin was only released after an hour, followed by a controlled (constant) release over a period of 6h. These data were also corroborated by circular dichroism (CD) studies, which confirmed that no conformational change occurred to the insulin structure after loading in NCs [24].

Finally, the *in vivo* efficacy of the formulations was evaluated by intraduodenal and intrajejunal administration (50 IU/kg), to non-diabetic rats after 4 h fasting [25–27]. In both cases, a moderate decrease of blood glucose levels (20% reduction) was observed and this effect was maintained for the duration of the study. The modest response observed in this study (**Figure 7**), in comparison to reported studies in literature was attributed to several reasons including the type of animal model used (non-diabetic rats), the short fasting time (4 h) and the viscosity of the formulation which may hamper the miscibility with intestinal fluids and absorption of the NCs [23, 28]. Most importantly it appears that most *in vivo* reports are based on diabetic rats and in these studies drastic glucose reduction is observed. Indeed this phenomenon is to be expected from a physiological point of view as the pancreatic islets is defective in this model and the blood insulin levels rise gradually together with the input of extraneous insulin due to lack of self–modulation function which is present in non-diabetic rats. [29]. However, recently there has been a concern on the high variability of *in vivo* data and, in our study, we presumed that using non-diabetic rats would reduce this variability as we exclude the effects of inducing diabetes to the animals.

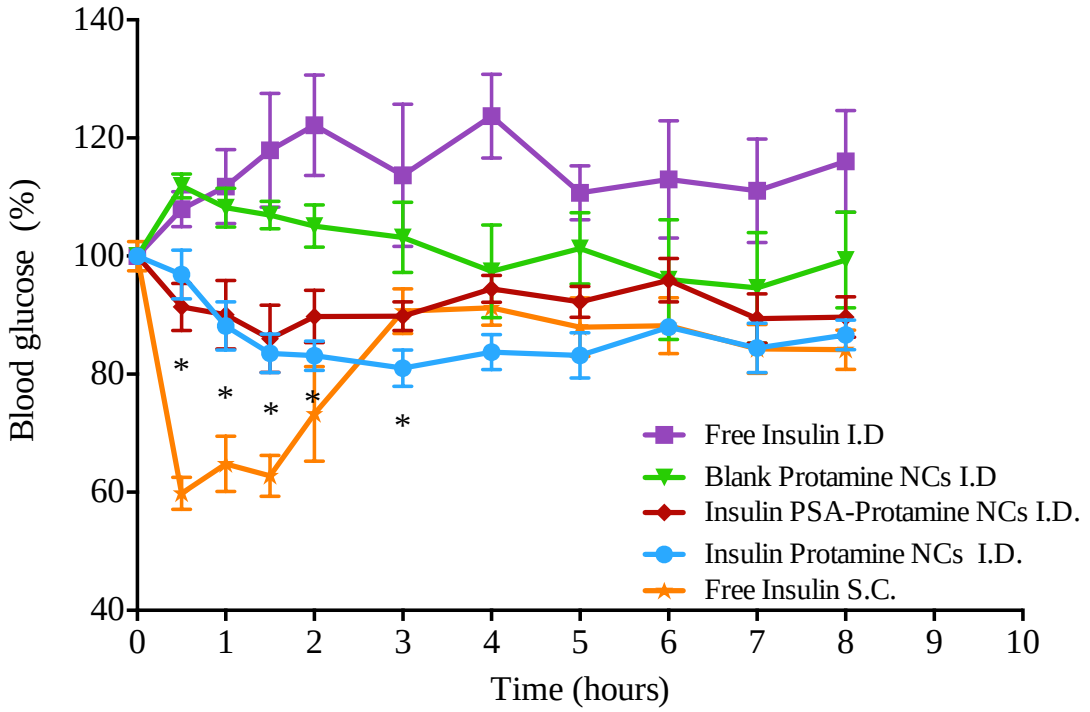


**Figure 7:** Standardized hypoglycemic effect following intra-duodenal administration of insulin loaded protamine NCs (50IU/kg, n=11), PSA-coated protamine NCs (50IU/kg, n=15), blank protamine NCs (placebo) (n=12) at the same NC concentration and insulin saline solution (50IU/kg, intraduodenally n=16 and subcutaneously 1IU/kg) in non-diabetic rats. (Mean ± SEM, *$p < 0.05$ significant difference for protamine NCs and PSA/Protamine NCs compared to the placebo control.

In summary, this work highlights the flexibility of the nanocapsules delivery platform in terms of accommodating a number of ingredients based on a rational design. The results obtained also underline the relative value of the *in vitro* studies in terms of predicting the *in vivo* performance of oral peptide nanoformulations. The nanocapsules developed in this study meet several prerequisites for the oral delivery of peptides, such as their nanometric size, a neutral surface charge, an adequate colloidal stability and the capacity to control the release and prevent the degradation of insulin. What remains unclear is whether or not the apparent capacity of the nanocapsules to enter the intestinal mucosa can be extrapolated from *in vitro* to *in vivo*. Maybe an adequate final dosage form could help the nanocapsules to accomplish their goal. In the meantime, the effective adhesion or internalization of the nanocapsules and the intracellular release of insulin remains to be addressed.

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# Conclusions

# CONCLUSIONS

This thesis project was focused on the design and development of an oily-core shell type, protamine-based nanosystem for the efficient oral delivery of insulin (and other similar macromolecules). The results obtained from the experimental work led to the following conclusions:

1. Nanocapsules (NCs) with a single protamine or double protamine and polysialic (PSA) layer were successfully prepared using a simple solvent displacement method. These nanocapsules had the capacity to encapsulate insulin (62 and 51 %), with a nanometric size (382 and 301 nm) for protamine and PSA/protamine NCs, respectively and were both characterised with spherical in shape a surface charge close to neutral.

2. Protamine and PSA/protamine NCs were stable in simulated intestinal media and protected the loaded insulin from enzymatic degradation. The extra layer of PSA and the presence of the bile salt surfactant sodium glycocholate (SGC), reinforced the stability of nanosystem and minimized the enzymatic degradation of the loaded insulin.

3. *In vitro* cell studies carried out in different cell culture models simulating the intestinal epithelium, showed that protamine and PSA/protamine NCs exhibit a very low toxicity. Moreover, these studies indicated that both types of NCs interact with the monolayers involving two different mechanisms, which maybe responsible for either a transcellular or paracellular transport of the associated peptide.

4. *In vivo* studies performed in non-diabetic rats confirmed that the NCs are able to preserve the loaded insulin, maintain its bioactivity and minimizing its degradation by enzymes. Both formulations were able to promote the absorption of insulin and gave a modest decrease (20 %) of the initial glucose levels following intra-intestinal administration.

5. The nanocarriers developed in this study were converted into powder (freeze-dried form), stable during storage at room temperature (25 ℃) and preserves the associated insulin for at least 6 months.

Overall, the work presented here portrays protamine-based NCs as a potential nanocarrier candidate for oral peptide delivery. The extensive *in vitro* studies on these NCs highlights their stability in intestinal fluids and even during storage as an important property for the use of this nanocarrier in oral peptide delivery. Moreover, it was evident that the NCs could be modulated and adapted to improve the stability and peptide-release profile. The safety profile of the NCs both, *in vitro* and in human intestinal tissue, together with the penetration enhancing effect are also interesting properties for this nanocarrier. Although the *in vivo* efficacy studies showed a modest glucose response, the data presented here are still encouraging that adjustments in the animal model and protocol of the experimental procedure used could give a more appreciable response.

# ANNEX 1

## Development and characterization of solvent-free protamine nanocapsules as carriers for mucosal delivery of therapeutics

# Abstract

Local delivery of drugs and biopharmaceuticals for the treatment of inflammatory bowel diseases remains a challenge. Innovative nanomedicines with appropriate properties raise the possibility of efficient drug targeting. Hence, the overall aim of this study was to develop and characterize a protamine-based nanosystem for topical delivery of therapeutics to inflamed intestinal mucosa.

Protamine NCs with a new composition were prepared by the self-emulsifying process without the use of any organic solvent. A model lipophilic anti-inflammatory peptide, cyclosporin A, was encapsulated in the oily core, which was then post-coated with the protamine polymer. The loaded formulation was isolated from the free drug by gel filtration and the entrapment efficiency was determined using reverse phase high-performance liquid chromatography. Positively charged protamine NCs of approximately 160-180 nm with a high entrapment efficiency (95%) and drug loading capacity (5%), exhibiting a good stability in simulated intestinal media were obtained. The results demonstrated herein indicate the feasibility of the developed nanosystem to serve as a potential carrier for mucosal administration of poorly water-soluble therapeutics.

## 1. Introduction

The encapsulation of highly hydrophobic compounds in polymeric lipid NCs, constituted by an oily core and a polymeric shell, improves their solubility, protects them against the harsh gastrointestinal (GIT) environment and enzymatic degradation, while enhancing permeability across the biological membranes [1]. Moreover, nanomedicines administered by the oral route are expected to accumulate in the inflamed mucosal tissues, thereby allowing specific drug targeting. Hence, nanocarriers are useful tools for the formulation of therapeutics intended for transmucosal modalities of administration, for both local and systemic effect [2].

Polymeric structures, depending on their surface composition and charge, are believed to adhere to the negatively charged mucosal compartments or positively charged inflammatory proteins. The nanometric size may, in turn, promote the penetration and impact their epithelial uptake as well as the uptake by macrophages or dendritic cells [3]. For example, Beloqui *et al.*, proposed nanostructured lipid carriers as a targeted drug delivery system for the treatment of colitis [4]. Negatively charged budesonide-loaded particles of 200 nm reduced the secretion of TNF-α by activated macrophages *in vitro* and prolonged residence time in the colon, decreasing the levels of pro-inflammatory cytokines and TNF-α when tested *in vivo* in a murine model of dextran sulfate-induced colitis. Another parallelly developed prototype coated with cationic trimethylchitosan (TMC) showed enhanced mucoadhesion and the ability to open the tight junctions and thus increase the paracellular permeability in inflamed Caco-2 monolayers. The main benefits of local therapy include the reduction of systemic adverse effects and increased drug concentration at the site of action. However, in order to be able to reach the intestinal mucosa, the nanocarriers must withstand harsh conditions of the gastrointestinal tract and enzymatic digestion of intestinal excretes [5].

Protamine is a well-known cationic polypeptide with an arginine rich character and cell-penetrating properties that may have the potential for use in the design of systems for transporting drugs to the mucous membrane surfaces [6]. Hence, the main objective of this study was to develop and characterize protamine-coated lipid-core NCs prepared using the solvent-free method and evaluate the feasibility of these

nanocarriers to serve as a platform for topical delivery of pharmaceutical compounds to inflamed intestinal mucosa.

## 2. Materials and methods

### 2.1. Materials

Glycerol monolinoleate (Maisine$^{TM}$ 35-1) used in this work was a kind gift from Gattefossé (France). Polysorbate 80 (Tween® 80), sodium deoxycholate (SDC), pancreatin 4 x USP and trehalose were purchased from Sigma-Aldrich (Spain). Polyoxyethylene 40 monostearate (PEGst-40) (Simulsol® M52) was obtained from Seppic (France). Protamine sulfate was purchased from Yuki Gosei Kogyo (Japan). Cyclosporin A was purchased from Fagron Iberica (Spain). All other products and chemicals used during the experiments and the chromatographic analysis were of high purity or reagent grade.

### 2.2. Preparation of protamine NCs

The formulation components were selected rationally to provide a nanocarrier with solubilizing and mucoadhesive properties. A new technique for the preparation of protamine NCs was developed. First, an oil-in-water nanoemulsion was prepared by blending the oil and surfactant in 2:3 ratio and dispersing them in the aqueous phase. Afterwards, the obtained nanoemulsion was post-coated with the polymer to form blank NCs. Briefly, the optimized nanosystem was prepared as follows: 0.10 g of Maisine 35-1 and 0.15 g of Tween 80 were mixed together by vortexing at 1200 rpm for 2 minutes. The aqueous phase was prepared by dissolving 0.03 g of PEGst-40 and 0.05 g of SDC in 4.00 ml of ultrapure water, and then poured over the oil-surfactant blend while vortexing at 1200 rpm for 5 minutes. One ml of the prepared nanoemulsion was added to 1 ml of protamine solution (5.00 mg/ml) whilst stirring to obtain a NC formulation with a volume of 2 ml. The solution was gently stirred at 300 rpm for 30 min and then incubated at room temperature for 2 hours.

### 2.3. Encapsulation of cyclosporin A

A concentrated solution of cyclosporin A (CsA) in ethanol (20 mg/ml) was mixed with Maisine 35-1 and the drug-loaded system was prepared following the previously described procedure (see 2.1). Cyclosporin A-loaded protamine NCs, containing 0.5 mg/ml of CsA, were characterized as defined in section 2.3. Then, the prepared nanosystem was isolated by size exclusion chromatography (SEC) using CentriPure P10 hydrated gel filtration columns (Zetadex-25, emp Biotech). The gel filtration method was validated for blank nanocarriers and for the solution of free cyclosporin A in ethanol in order to be able to separate the cyclosporin A-loaded NCs from the unencapsulated drug. In brief, the column was first equilibrated with 15 ml of ultrapure water (elution buffer). One ml of the formulation was then transferred to the column and allowed to enter the gel bed completely. The void volume was discarded and the cleaned sample was eluted with 1.6 ml of ultrapure water, recovering 1.2 ml of the formulation. Entrapment efficiency (EE%) was determined using the direct method by comparing the amount of drug in the final formulation of NCs to the initial amount added during preparation (Equation 1) using reverse phase high-performance liquid chromatography (RP-HPLC). First, the isolated NCs were digested in acetonitrile in order to extract CsA from the formulation and then centrifuged at 14000 x g for 5 minutes to obtain a clear aliquot. This aliquot was injected in the C18 HPLC column followed by isocratic elution (60% acetonitrile, 30% isopropanol, 10% water) at 60 ℃ and UV detection at 205 nm (in-house method developed by Cuesta and Reim ández Troiti ño). The theoretical drug loading was calculated with regard to the mass of cyclosporin A and other components added during the preparation of the NCs (Equation 2). Finally, the drug loading capacity and yield were calculated using Equation 3 and Equation 4 respectively

### 2.4. Determination of size and zeta potential

The average diameter and polydispersity index (PDI) of protamine lipid NCs were characterized by dynamic light scattering after diluting the samples 1:100 with ultrapure water. Zeta potential was determined by laser-Doppler anemometry after 1:100 sample dilution with potassium chloride 1 mM. The measurements were performed on a Zetasizer® (NanoZS, Malvern Instruments, UK).

### 2.5. Stability studies of protamine nanocapsules in biological fluids

Simulated intestinal fluid (SIF) pH 6.8 was prepared as described in European Pharmacopoeia (2011). Fasted state upper small intestine fluid (FaSSIF-V2) pH 6.5 and fed state upper small intestine fluid (FeSSIF-V2) pH 5.8 were prepared according to Jantratid *et al.* (2008). Protamine NCs were incubated under moderate shaking at 37 ℃ in SIF, FaSSIF-V2 and FeSSIF-V2 in the presence of digestive enzymes from porcine pancreas. Samples were collected and measurements performed at time points 0, 0.5, 1, 2 and 4 h. The samples incubated in FeSSIF-V2 were centrifuged prior to measurements at 13600 x g for 5 minutes at 20 ℃ to eliminate aggregates of pancreatin. Size distribution, polydispersity index (PDI) and count rate of the NCs were monitored by dynamic light scattering.

### 2.6. Stability studies during storage

Colloidal stability of blank NCs in storage was followed during a period of 1 week at 4 ℃, 25 ℃ or 37 ℃. Samples of three different batches were withdrawn at predetermined time intervals, determining particle size and zeta potential (as 2.3) and describing the suspension appearance to ensure continued viability.

### 2.7. Freeze-drying studies

Blank and CsA-loaded NCs were freeze-dried in order to evaluate their ability to be transformed into a dry powder formulation. Three cryoprotectant solutions (glucose, sucrose and trehalose) at two different concentrations (5% and 10%) were studied. The influence of freezing temperature on the lyophilization process was also investigated. Optimized lyophilization of the blank and drug-loaded nanosystem was performed as follows: samples of NCs suspension (0.5 ml) containing the cryoprotectant trehalose (10%) were frozen overnight at -80 ℃ and then transferred to the lyophilizer. The initial drying step was performed for 24 h at -35 ℃ and 2-10 millitorr, followed by secondary drying for another 24 h at 0 ℃ and finally a third step for 16 hours at 20 ℃ at the same pressure. After the freeze-drying, the particles

were resuspended with ultrapure water by vortexing for 5 min and then characterized for size, zeta potential and polydispersity index.

### 2.8. Statistical analysis

All the experiments and measurements were performed in triplicates (n=3) and the results are reported as the mean values ± standard deviation (SD).

## 3. Results and discussion

### 3.1. Preparation of protamine NCs

Monodisperse protamine NCs (PDI=0.2) with the average size ranging from 160-180 nm were prepared by a low-energy emulsification method without the use of any organic solvent or heat. The NCs had a positive zeta potential of +14 mV, indicating the presence of protamine around the oil-filled core, when compared to the corresponding nanoemulsion formulation. A long-chain monoglyceride/diglyceride mixture, i.e. Maisine 35-1, that works as an oil phase, enabled fine droplet formation in the presence of the non-ionic surfactant Tween 80 (HLB=15) upon dilution with water. The attachment of protamine to the system was possible after incorporation of an anionic surfactant, SDC, into the droplet surface. SDC led to a decreased zeta potential of the nanoemulsion and thus allowed the coupling of the positively charged polymer through electrostatic attractions. Thereby, the polymeric shell was formed around the lipid droplets. Protamine NCs were sterically stabilized by introducing polyethylene glycol chains (PEGst-40 stearate) on the NC surface. Steric stabilization of the colloidal system with a layer of adsorbed polymer was necessary to enhance the repulsive interaction forces and thus minimize particle aggregation. The developed nanosystem is illustrated in **Figure 1**.

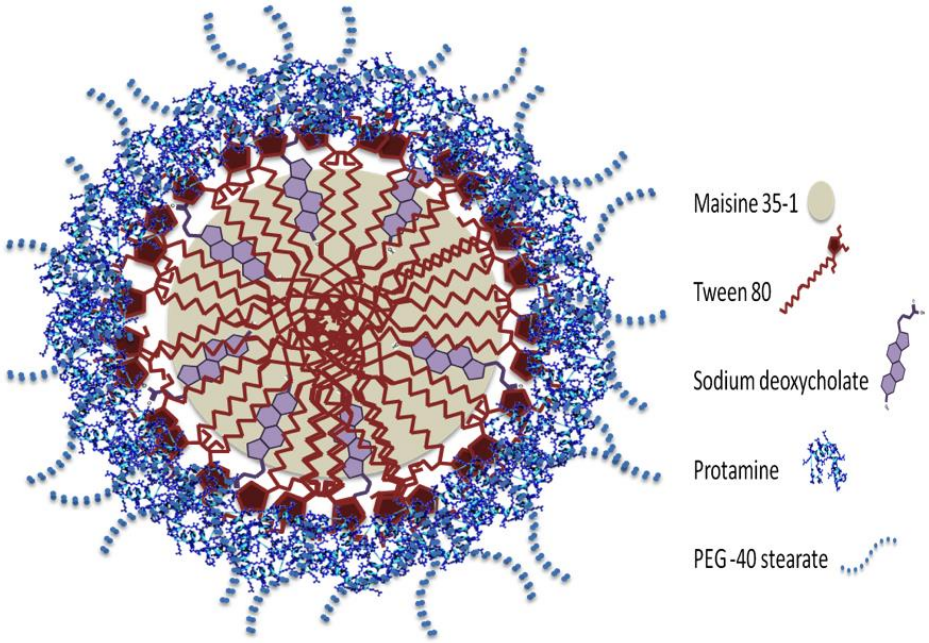


**Figure 1:** A schematic illustration of core-shell protamine NCs prepared by the solvent-free method.

### 3.2. Encapsulation efficiency and drug loading capacity

A model highly hydrophobic and anti-inflammatory drug, cyclosporin A, was encapsulated in the oily core of the nanocarriers. As indicated in **Table 1**, the entrapped drug did not influence the physicochemical properties of protamine NCs. The loaded system was isolated from the free drug by size exclusion chromatography and the concentration of cyclosporin A extracted from the digested formulation was calculated after HPLC analysis (direct method).

**Table 1:** Physicochemical characteristics of blank and drug-loaded protamine NCs

| Protamine NCs | Size (nm) | PDI | ζ-potential (mV) | EE (%) | LC (%) |
|---|---|---|---|---|---|
| **Blank** | 163 ± 8 | 0.2 | +14 ± 2 | N/A | N/A |
| **CsA-loaded** | 153 ± 12 | 0.2 | +15 ± 1 | 95 | 5 |

Protamine nanocapsules exhibited high encapsulation efficiency of cyclosporin A equal to 95%. The loaded formulation was freeze-dried, without any cryoprotectant, and the obtained powder was weighed in order to determine the yield of the nanocapsules and the drug loading capacity. These parameters were calculated to be equal to 87% and 5%, respectively. The latter corresponds to the theoretical drug loading value, i.e. 4.6%.

### 3.3. Stability of CsA-loaded NCs in simulated intestinal fluids

Similarly to the blank system, the cyclosporin A-loaded NCs were stable when incubated in SIF, FaSSIF-V2 and FeSSIF-V2 with pancreatic enzymes. As shown in **Figure 2 and 3** below, the size and count rate of the particles remained relatively constant throughout the studies. Further, the incubation with biorelevant media did not affect the polydispersity index of the colloidal nanosystem.

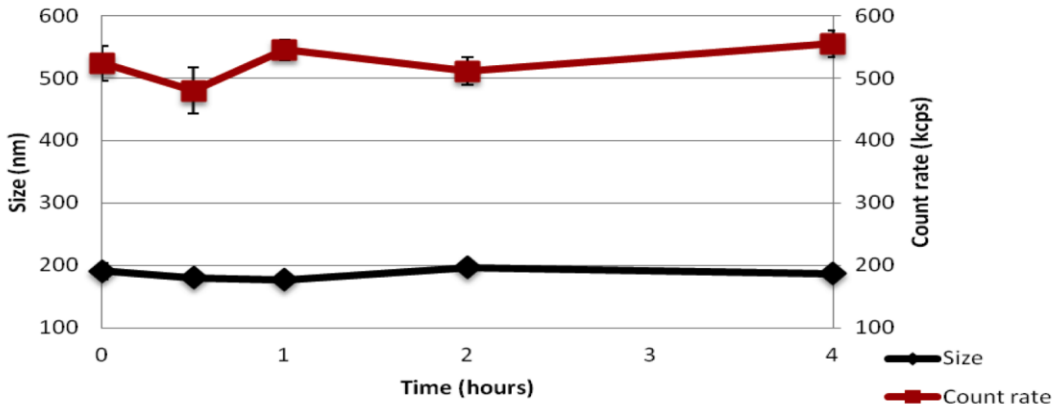


**Figure 2:** Colloidal stability of CsA-loaded protamine NCs in FaSSIF-V2.

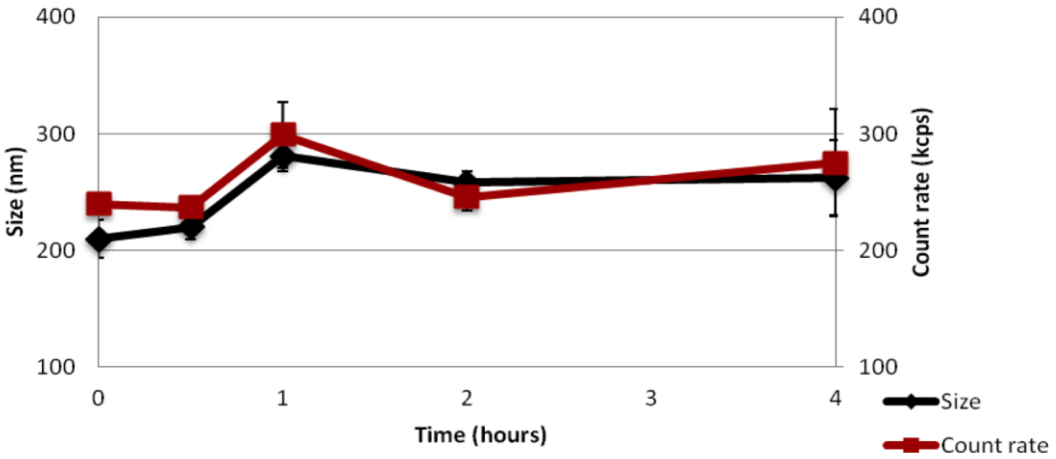


**Figure 3:** Colloidal stability of CsA-loaded protamine NCs in FeSSIF-V2

### 3.4. Freeze-drying of protamine NCs

Initial freeze-drying performed without any cryoprotectant resulted in a collapsed cake that was not fully resuspendable. After the screening of different sugars, 10% trehalose was selected to protect protamine NCs from freezing damage. Polydispersity index of the NCs suspension frozen at -80 ℃ was lower compared to the freezing at -

20 ℃. As indicated in **Table 2**, blank and drug-loaded NCs lyophilized under previously defined optimal conditions, could easily be resuspended with water retaining in principle their initial physiochemical properties.

**Table 2:** Physicochemical properties of blank and CsA-loaded protamine NCs after freeze-drying.

| Nanosystem / conditions | **Size (nm)** | **PDI** | **ζ-potential (mV)** |
|---|---|---|---|
| **Blank / before freeze-drying** | 179 ± 3 | 0.2 | +15 ± 1 |
| **Blank / after resuspension** | 196 ± 7 | 0.2 | +19 ± 1 |
| **CsA-loaded / before freeze-drying** | 198 ± 12 | 0.2 | +15 ± 1 |
| **CsA-loaded / after resuspension** | 176 ± 8 | 0.3 | +10 ± 3 |

## 4. Conclusions

Overall, a nanosized carrier with physicochemical characteristics appropriate for mucosal administration of therapeutics was developed using a new and low-cost preparation method that allowed efficient entrapment of a lipophilic drug. In this study, protamine nanocapsules exhibited high encapsulation efficiency and drug loading capacity of cyclosporine A, stable in simulated intestinal fluids. From these successful preliminary results for the presented prototype the next steps include more comprehensive *in vitro* and *in vivo* efficacy experiments.

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