PEG-PGA ENVELOPED OCTAARGININE-PEPTIDE NANOCOMPLEXES

A novel protein-based drug delivery strategy to overcome biological barriers

NANOCOMPLEJOS OCTAARGININA-PEPTIDO RECUBIERTOS CON PEG-PGA

Una nueva estrategia de liberación de fármacos proteicos para superar barreras biológicas

NANOCOMPLEJOS OCTAARGININA-PEPTIDO REVESTIDOS CON PEG-PGA

Unha nova estratexia de entrega de fármacos proteicos para superar as barreiras biolóxicas

Máster en Biotecnología Avanzada

Nikolaos Kalamidas

Supervisor: Prof. Maria José Alonso

Center of Research in Molecular Medicine and Chronic Diseases (CIMUS)

February 2018

La Prof. **MARIA JOSÉ ALONSO**, catedrática del Departamento de Farmacología, Farmacia y Tecnología Farmacéutica de la Universidad de Santiago de Compostela e investigadora principal en el instituto CIMUS, en calidad de tutora informa que:

El presente trabajo de fin de Máster con título:

PEG-PGA Enveloped Octaarginine-Peptide Nanocomplexes-A novel protein-based drug delivery strategy to overcome biological barriers

Nanocomplejos Octaarginina-Péptido recubiertos de PEG-PGA-Una nueva estrategia de liberación de fármacos proteicos para superar barreras biológicas

Nanocomplejos Octaarginina-Péptido revestidos de PEG-PGA–Unha nova stratexia de entrega de fármacos proteicos para superar as barreiras biolóxicas

se ha realizado en el Centro de Medicina Molecular y Enfermedades Crónicas (CIMUS) por **NIKOLAOS KALAMIDAS** bajo su dirección y autoriza su presentación en la Facultad de Ciencias de la Universidad de A Coruña.

Santiago de Compostela, 29 de enero de 2018

ALONSO FERNANDEZ MARIA JOSE - 09724164V

Firmado digitalmente por ALONSO FERNANDEZ MARIA JOSE - 09724164V
Nombre de reconocimiento (DN): c=ES, serialNumber=09724164V, sn=ALONSO FERNANDEZ, givenName=MARIA JOSE, cn=ALONSO FERNANDEZ MARIA JOSE - 09724164V
Fecha: 2018.02.01 09:05:52 +01'00'

Fdo. **MARIA JOSÉ ALONSO**

Table of contents

Resumen

Hoy en día, el número de fármacos que se utilizan de naturaleza proteica ha incrementado debido a su alta eficacia y selectividad. Sin embargo, su utilidad se ha visto limitada por las dificultades que presentan en atravesar barreras biológicas, siendo la vía parenteral su administración más común. Para solucionar este desafío, la nanotecnología y en concreto la nanomedicina ha aportado nanosistemas capaces de transportar compuestos bioactivos directamente a la diana terapéutica e incluso pudiendo ser administrados por rutas más amigables para los pacientes.

El objetivo de este trabajo fin de Máster es desarrollar nanocomplejos entre péptidos terapéuticos (insulina y péptido antiobesidad) y un péptido de permeación celular (octaarginina modificada) y su envolvimiento con copolímeros de poli(etilenglicol)-ácido poli(glutámico) (PEG-PGA). Énfasis se ha dado a la administración oral y de nariz-cerebro.

En un primer lugar, se diseñaron nanocomplejos de octaarginina modificada e insulina recubiertos con una serie de copolímeros (PEG-PGA) de estructuras diversas con el futuro propósito de usarlos por vía oral. Los nanosistemas tienen un tamaño entre 180-250 nm, una estrecha distribución de tamaños, una carga neutral, una morfología esférica y una excelente eficiencia de asociación a la insulina. Se realizó un estudio comparativo entre diversos copolímeros estructuralmente diferentes para aumentar la densidad de poli(etilenglicol) (PEG) en la superficie del nanosistema y se concluyó que se aumentaba entre 7 veces la densidad cuando se empleaba un copolímero ramificado con el mayor grado de sustitución de PEG y la cadena más larga de ácido poli(glutámico) (PGA) en comparación con el copolímero en dibloque con la cadena de PGA más pequeña. El sistema presentó una buena estabilidad en condiciones de almacenamiento.

Por otra parte, se adaptó el nanosistema empleado con anterioridad a la administración nariz-cerebro con un péptido antiobesidad. Los nanosistemas tienen un tamaño entre 100-120 nm, una estrecha distribución de tamaños, carga neutral, una alta carga de péptido y una excelente eficiencia de asociación. El sistema se pudo obtener liofilizado con trehalosa al 2% como lioprotector.

En resumen, este trabajo ha aportado información relevante a cerca de estos novedosos nanosistemas y además, se espera continuar realizando estudios más exhaustivos.

Abstract

Nowadays, the number for protein-type drugs has increased due to their high efficiency and selectivity. Nevertheless, their use has been limited by the difficulties they present in crossing biological barriers, being the parenteral route their most common administration. To solve this challenge, nanotechnology and specifically nanomedicine has provided nanosystems able to transport bioactive compounds right away to the therapeutic target and even to being administrated through friendlier routes for patients.

The aim of this Master's Thesis project relied on the formation of nanocomplexes between therapeutic peptides (insulin and antiobesity peptide) and cell permeating peptides (CPPs) and their further envelopment with protective polymers (PEG-PGA). Emphasis was given to oral and nose-to-brain administration.

First, modified octaarginine-insulin nanocomplexes coated with structurally diverse copolymers (PEG-PGA) were designed with the aim of using them for the oral administration. The enveloped nanocomplexes (ENCPs) had a particle size ranges between 180 and 250 nm, narrow size distribution, neutral charge, spherical morphology and an excellent association efficiency of insulin. A comparative/screening study between structurally diverse copolymers was made in order to increase the poly(ethylene glycol) (PEG) density in the surface of ENCPs and it was concluded that, when a ramified copolymer with the highest substitution degree of PEG and the longer PGA chain is used, the density increases up to 7 times, comparing to the diblock copolymer with a shorter PGA chain. The system displayed a good stability in storage conditions.

Besides, the previously used nanosystem was adapted to a nose-to-brain administration with an antiobesity peptide. These nanosystems had a particle size ranges between 100 and 120 nm, narrow size distribution, neutral charge, high loading peptide and an excellent association efficiency. The system could be obtained lyophilized with trehalose 2% as lyoprotectant.

In summary, this project has provided valuable information about these novel nanosystems and, besides, it is expected to continue carrying out more exhaustive studies.

Acknowledgements

First of all, I would like to thank my supervisor Prof. Alonso for giving me the opportunity to do this Master Thesis in her research lab.

I would also like to express my most sincere gratitude to Ph.D Samaridou Eleni for being a great guide and teacher, for supporting me in all the research aspects and connected me to this work. Without her patient and encourage, this Master Thesis could not be possible.

Thanks to everyone in the lab who made a fantastic and positive environment.

Special thanks to my family and friends for your unconditional support during my internship.

Finally, I would like to thank Alba for her loving support and this Master Thesis is dedicated to her.

CHAPTER 1

Introduction

1. Introduction

Over the past decades, the therapeutic use of proteins has gained significant popularity within the pharmaceutical industry, due to their high potency, specificity and selectivity, and their wide range of applications in medicine and biotechnology. Nevertheless, the challenge of developing therapeutic peptides and proteins has exercised the minds of pharmaceutical scientists over the last decades, owing to their difficult access to the therapeutic target. Up to date, the most common administration route for peptide drugs is the parenteral route, a route however associated with patient discomfort and, eventually, to lack of compliance and failure of treatment. For this reason, the scientific interest is turned to more patient-friendly routes. Thus, administration routes which were of minor importance as parts of drug delivery in the past, such as nasal, ophthalmic, buccal, rectal, intrauterine, vaginal, transdermal, intranasal and pulmonary, have assumed added importance in protein and peptide delivery (Jitendra *et al*, 2011). However, upon the non-parenteral administration of these biomolecules, they face a high risk of enzymatic degradation, poor permeability across biological barriers and elimination during first-pass hepatic clearance. The oral, nasal and pulmonary routes were the first non-invasive routes of protein delivery which have been investigated in this field despite the observed low bioavailability of the peptides and proteins through these routes. Throughout this thesis emphasis will be given in the oral and nose-to-brain (N-to-B) administration routes.

1.1 Non-parenteral routes of peptide delivery

Oral peptide drug delivery

The oral route is one of the most conventional and safest modalities of drug administration. Delivering however therapeutically active peptides by the oral route has been a challenge and a goal for many decades. The oral route is considered unsuitable for the systemic delivery of therapeutic peptides and proteins because of their potential degradation by the strongly acid environment in the stomach and the proteolytic enzymes in the intestinal tract, as well as pre-systemic elimination in the liver. The physiological and anatomical barriers to bioactive absorption via the gastrointestinal (GI) tract are primarily chemical, enzymatic and permeability related (i.e., through the mucus layer and/or intestinal epithelium).

Nose-to-brain (N-to-B) peptide drug delivery

The N-to-B administration is another non-invasive route that enables the direct transport of drugs from the nose to the central nervous system (CNS), while avoiding the blood-brain barrier (BBB). This is possible because of the direct connections from the nose to the brain through the olfactory and/or the trigeminal nerves (Alex *et al*, 2016; Samaridou and Alonso, 2017). Moreover, this route offers benefits such as: (i) easy administration and a self-medication, (ii) rapid absorption and (iii) avoidance of liver and gut first-pass metabolism (Clementino *et al*, 2016). The potential of this patient-friendly route, however, may be affected by the physicochemical properties of the administered biomolecules such as size, lipophilicity and degree of ionization. At the same time, the presence of metabolic enzymes in the mammalian olfactory mucosa and the localization of the olfactory epithelium in the roof of the nasal cavity make it difficult for drugs to gain access to the targeted region, leading eventually to bioavailabilities less than 1% (Samaridou and Alonso, 2017).

The contribution of Nanotechnology

One of the strategies to overcome these challenges and achieve efficient protein drug delivery is employing nanostructured delivery carriers or nanocarriers. Nanocarriers (such as liposomes, solid lipids nanoparticles, dendrimers, polymers, hydrogel nanoparticles and carbon nanotube materials among others) are structures of sizes ranging from 1 to 100 nm in at least one dimension (Figure 1). Nanocarrier-mediated drug delivery systems can protect the therapeutic peptide from enzymatic degradation while improving its absorption through the mucosa, either by increasing the membrane permeability of the drug and/or the carrier or by inhibiting drug efflux transporters (Zhang et al, 2013). The application of these systems as delivery carriers for bioactive compounds allows moreover the reduction in the toxicity of the free drug to non-target organs (Wilczewska *et al*, 2012, De Jong and Borm, 2008). Thus, the use of innovative nano-sized systems may provide improved drug concentrations and prolonged half-lives leading to an improved therapeutic effect (Samaridou and Alonso, 2017).

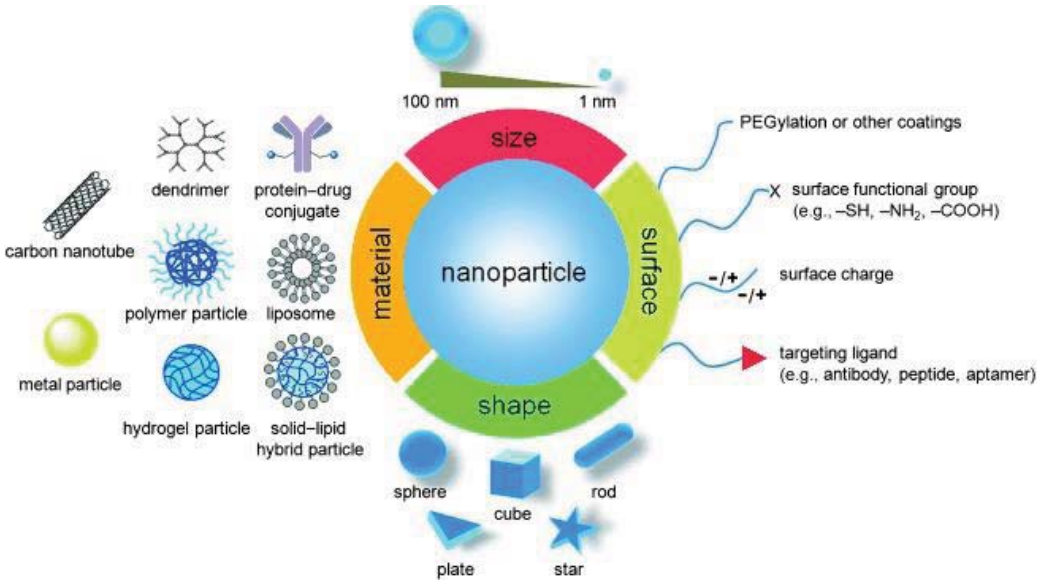


Figure 1: Types of different nano-sized systems that have been explored as carriers for drug delivery, along with an illustration of the basic physicochemical properties (Adapted from Sun *et al*, 2014)

Cell permeating peptides

Cell permeating peptides (CPPs) are positive-charged, short-chain peptides, that can interact electrostatically with the negatively charged proteoglycans and phospholipids present in cell membranes, allowing them to transport molecules or exogenous particles into the cells in different pathways (Walsh *et al*, 2011). The ability of CPPs to interact with membranes is influenced by their physicochemical properties (e, g. their charge, the hydrogen bonds that could be formed between these molecules and the cell surface), as well as their size and structure. For that reason, CPPs constitute a promising tool for their ability to interact with biological membranes and enable the efficient cellular uptake of therapeutic peptide and protein as well as across biological barriers via different pathways (endocytosis or direct cytoplasmatic translocation) (Kristensen *et al*, 2016).

Recent research has pointed out a particular category of CPPs, which contains arginine-rich peptides, such as oligoarginines and polyarginines, because of its excellent cell permeation properties. Owing to the presence of guanidinium groups inside the poly/oligo-arginine side chains, there is greater internalization based on the ionic strength and also the formation of hydrogen bonds between these groups and the anionic phosphate, sulfate and carboxylate groups located on the cell surface. Specifically, Octaarginine (r8) is one of the most common and powerful CPP, which high cellular uptake is due to its special structure and number of arginine (Futaki *et al*, 2006).

Pegylation

Another approach frequently used for improving the efficiency of nanoparticle-based drug delivery, by enhancing their mucodiffusion and tissue targeting, is decorating the surface of the nanocarriers with polyethylene glycol (PEG). PEG is the most extensively studied polyether in drug delivery as it is approved by the Food and Drug Administration (FDA), it is biocompatible, non-immunogenic, non-antigenic and highly soluble in water (Zhao *et al*, 2016). Hanes and co-workers have demonstrated through several studies in the past that the densely coating of the surface of nanoparticles with low molecular weight polyethylene glycol, reduces hydrophobic interactions, hydrogen bonding and interpenetrating polymer network, led to their rapid diffusion through human mucus (Ensign *et al*, 2012; Xu *et al*, 2015).

Overall, this information shows that the combination of CPPs with PEGylated protecting copolymers may represent a useful strategy for making feasible nanocarriers.

CHAPTER 2

Aim of the Master Thesis

2. Aim of the Master Thesis

The main objective of this study was the rational design and the development of a novel nanocarrier intended to help therapeutic peptides to overcome the biological barriers, associated with the different non-parenteral administration routes. Emphasis was given to oral and nose-to-brain peptide delivery.

Our approach here relied on the formation of nanocomplexes between therapeutic peptides and cell permeating peptides (CPPs) and their further envelopment with protective polymers. To that purpose, insulin and an anti-obesity peptide were employed as model peptides.

CHAPTER 3

PEG-PGA Enveloped CPP-Peptide Nanocomplexes for the Oral Delivery of Insulin

3. PEG-PGA Enveloped CPP-Peptide Nanocomplexes for the Oral Delivery of Insulin

3.1 Introduction

Diabetes mellitus is one of the most problematic and chronic diseases, that affects more than 400 million people worldwide, according to the World Health Organization (WHO) (Global Report on Diabetes, 2016). Insulin is a 51-amino acid peptide, discovered in 1921, and nowadays is used parenterally as recombinant insulin to treat diabetes. Several efforts had been made to deliver insulin orally, with no clinical or commercial success so far.

As already mentioned in the introduction section, nanotechnology has much to contribute in this field. Following the studies of Morishita and colleagues, who have shown the ability of CPPs to promote intestinal absorption of insulin (Morishita *et al*, 2007) and the pioneer contribution of Couvreur and co-workers that shows the possibility of administering insulin in rats using polyacrylate nanocapsules (Damgé *et al* 1990), a whole series of nanocarriers have been designed such as liposomes (Takendri *et al* 2003), chitosan nanoparticles (Prego *et al*, 2006), poly (lactic/glycolic) (PLGA) nanoparticles (Santander-Ortega et al. 2009) and solid lipids (Yuan *et* al, 2009) among others.

This work focuses on a nanosystem previously developed by the group of Prof. Alonso, as a continuation of this work. As a model peptide, insulin was chosen and associated through electrostatic and hydrophobic forces to a hydrophobic (lauric acid, C12) derivative of octaarginine (C12-r8) (Figure 2). Subsequently, these nanocomplexes were enveloped by the copolymer PEG-polyglutamic acid, intended to protect them against the harsh intestinal compartment (Vila *et al*, 2004) and enhance their mucodiffusion (Xu *et al*, 2015). These rationally designed nanostructures are hereby called enveloped nanocomplexes (ENCPs).

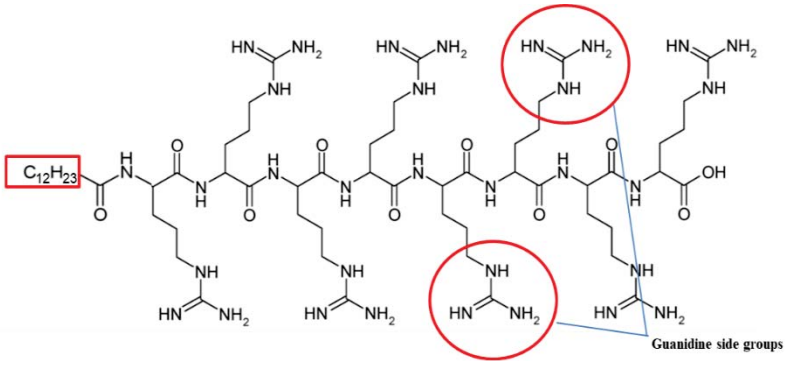


Figure 2: The chemical structure of C12-r8 (CPP) showing the guanidine side groups

According to previous studies performed by the group of Prof. Alonso, this nanosystem exhibited a number of important features, like desired physicochemical properties, high peptide association efficiency, the ability remain stable in simulated intestinal media while protecting the associated peptide from enzymatic degradation and most importantly the highest *in vitro* cellular uptake of insulin ever reported. However, upon testing its ability to move through the intestinal mucus, this carrier resulted in a moderate mucodiffusion. For that reason, the objective here was to enhance the diffusion of this system through mucus by increasing the PEG density on its surface, following a screening between structurally diverse PEG-PGA coating materials. Our hypothesis was based on the conclusion of Xu *et al* that nanoparticles with a high PEG surface density rapidly diffuse through the open spaces of mucus, as opposed, to nanoparticles with a low PEG density which are immobilized in mucus (Figure 3). The ENCPs resulting from this study were characterized in terms of (i) their physicochemical properties, (ii) their morphology, (iii) their PEG surface density and (iv) their stability upon storage.

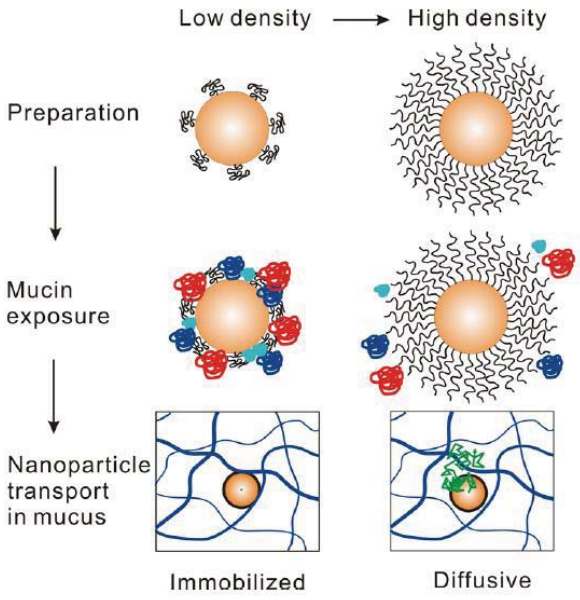


Figure 3: Schematic illustration of the impact of PEG surface on a mucus *ex vivo* transportation (Adapted from Xu *et al*, 2015)

3.2 Materials and Methods

3.2.1 Materials

The recombinant human insulin monomer (Apidra®, MW=5823 Da) was kindly provided by Sanofi (Paris, France). Lauryl acid coupled to the *N*-terminal of octaarginine (D-type)

(C12-r8, MW=1449.85 Da) was purchased from China Peptides (Shangai, China). The copolymers: (i) diblock PEG5k-b-PGA(10) (113 units of PEG (MW of 5000 Da) and 10 units of PGA, MW=6880 Da), (ii) diblock PEG5k-b-PGA(30) (113 units of PEG (MW of 5000 Da) and 30 units of PGA, MW=9983 Da), (iii) branched PEG5k(10)-g-PGA(10) (113 units of PEG (MW of 5000 Da) with a 10% substitution degree, 10 units of PGA, MW=10413 Da), (iv) branched PEG5k(10)-g-PGA(30) (113 units of PEG (MW of 5000 Da) with a 10% substitution degree, 30 units of PGA, MW=2900 Da), (v) branched PEG5k(30)-g-PGA(10) (113 units of PEG (MW of 5000 Da) with a 30% substitution degree, 10 units of PGA, MW=24910 Da) and (vi) branched PEG5k(30)-g-PGA(30) (113 units of PEG (MW of 5000 Da) with a 30% substitution degree, 30 units of PGA, MW=55210 Da) were purchased from Polypeptide Therapeutic Solutions (PTS, Valencia, Spain). Sodium hydroxide (NaOH) was purchased from Scharlau distributed by Cienytech (Santiago de Compostela, Spain). Deuterium oxide (D_2O, MW=20.03 g/mol) was purchased from Sigma Aldrich (St. Luis USA). Ultrapurified water was obtained from Millipore Milli-Q Plus water purification system (Darmstadt, Germany). All other chemicals were of analytical grade.

3.2.2 Preparation of C12-r8-Insulin Nanocomplexes (NCPs)

C12-r8-Insulin NCPs were prepared taking advantage of both hydrophobic and ionic interactions. Briefly, C12-r8 was dissolved in Milli-Q water at a concentration of 1 mg/mL (Solution A). Insulin (Apidra®) on the other hand was dissolved at a concentration of 1 mg/mL in basic media pH 12 (0.01 N NaOH), rendering it negatively charged (Solution B). The complexes were formed instantly upon adding the solution B to solution A under a magnetic stirring at C12-r8:Insulin molar ratio equal to 8:1. The pH of all NCPs was finally adjusted to 7 with HCl.

3.2.3 Preparation of Enveloped C12-r8-Insulin Nanocomplexes (ENCPs)

The NCPs that resulted from the aforementioned complexation process had a positive charge and were then enveloped by structurally different polymeric materials, i.e. negatively charged diblock and branched types of PEG-PGA at different Insulin:PEG-PGA mass ratios to allow the proper comparison of the different polymeric coatings. The different copolymers as well as the different Insulin:PEG-PGA mass ratios used are summarized in Table 1. According to the film hydration method, PEG-PGA copolymers were dissolved in Milli-Q water at a concentration of 1 mg/mL. Then, the water phase was added in a round flask and evaporated under reduced pressure at 37 ºC, leading to the

formation of a thin film. The previously produced NCPs (without adjusting their pH) were then transferred to the same flask and maintained for 10 min under rotation at room temperature and atmospheric pressure allowing the hydration of the polymeric film. After the NCPs were enveloped with the PGA-PEG film, the pH of the final ENCPs suspension was adjusted to 7 with HCl. Figure 4 presents a schematic illustration of the enveloping protocol.

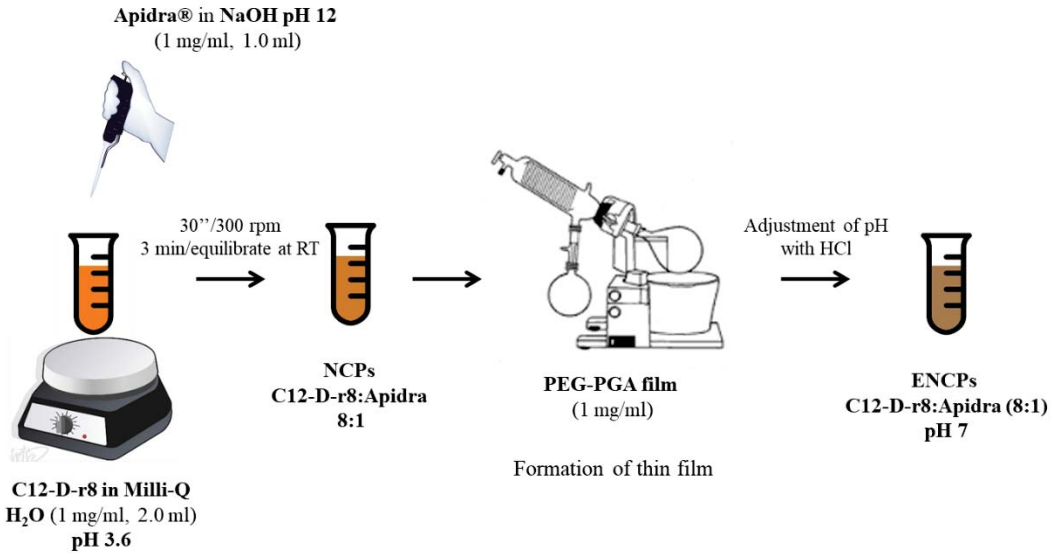


Figure 4: Schematic representation of the protocol to follow for the formation of insulin ENCPs

Table 1: Summary of the different copolymers and Insulin:PEG-PGA mass ratio tested

Copolymers	Insulin:PEG-PGA mass ratio
Diblock PEG5k-PGA(10)	**1:0.7**
Diblock PEG5k-PGA(30)	**1:0.36**
Branched PEG5k(10)-g-PGA(10) A	**1:0.62**
Branched PEG5k(10)-g-PGA(10) B	**1:3**
Branched PEG5k(10)-g-PGA(30)	**1:1**
Branched PEG5k(30)-g-PGA(10)	**1:2.34**
Branched PEG5k(30)-g-PGA(30)	**1:1.92**

3.2.4 Physicochemical characterization and morphology of the nanocomplexes

Particle size and polydispersity index (PdI) were determined by Dynamic Light Scattering (DLS) using a Malvern Zeta-Sizer (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) fitted with a red laser light beam (λ=632.8 nm). The Z-potential was calculated from the mean electrophoretic mobility values determined by Laser Doppler Anemometry (LDA) using the same device. For the measurement of particle size and PdI, 50 µL of the formulations were transferred directly into a particle size cuvette without any dilution. In case of the Z-potential measurements, 400 µL of the sample was diluted with 400 µL of Milli-Q water. The morphological analysis of the NCPs and ENCPs were carried out using a field emission scanning electron microscope (FE-SEM, ultra plus, Zeiss, Germany), where 10 µL of the sample was placed on a metallic rounded platform with the silicon wafer and carbon adhesive tabs and left to dry overnight. The samples were subjected to sputter coating of 10 nm of iridium with Quorum iridium sputter coater (Q150T, Quorum technologies, U.K.) prior to observation.

3.2.5 Determination of association efficiency and loading of Insulin

The association efficiency (AE) of insulin to NCPs and ENCPs was determined by an indirect method following separation of the insulin complexes from the aqueous media and measuring the free insulin in the suspension media. To that end, 500 µL of the formulation were isolated by centrifugation (Eppendorf Centrifuge, 5430R, Germany) at 15,000 g for 15 min at 15 °C. The aqueous supernatant was collected and the amount of free insulin in the supernatant was determined by reverse phase HPLC (Agilent, 1100 Series, USA), using a C18 column (Superspher® RP-18 end-capped). A buffer of phosphoric acid and sodium perchlorate was mixed with acetonitrile (at ratio 93:7 as phase A and 43:57 as phase B, both at pH 2.3). The AE of insulin in the formulation was calculated considering the total insulin amount involved in the formulation and the free insulin found in the supernatant. The final loading was calculated dividing the amount of insulin associated (AE x total insulin in the formulation) by the theoretical amount of all the materials involved in the formulation.

3.2.6 Storage stability of Enveloped Nanocomplexes (ENCPs)

For the determination of the stability of ENCPs upon storage, the ENCPs were stored at different temperatures: (i) 4°C, (ii) room temperature (25°C) and (iii) 37°C for up to 4

weeks and the measurements of the particle size were carried out by DLS as described above.

3.2.7 Quantification of PEG surface density of the Enveloped Nanocomplexes (ENCPs)

With the purpose of quantifying the density of PEG on the surface of the resultant ENCPs, we first isolated the previously prepared ENCPs by centrifugation at 15,000 g, 15 min and 15 ℃, resuspended the precipitate in D_2O and analysed them by proton nuclear magnetic resonance spectroscopy (^{1}H NMR: proton frequency of 500 MHz). The quantification of the PEG was performed by integrating the characteristic peak of the PEG at δ=3.6 ppm (singlet), corresponding to the CH_2-group of ethylene glycol, in reference to a known amount external standard (Trimethylsilylpropanoic acid). Spectra were processed and analyzed using MestReNova V11. A known mass of PEG--PGA polymers was serially diluted in D_2O for generation of a calibration curve for the PEG signal in ^{1}H NMR. In addition, solutions of known concentrations of C12-r8 and insulin (Apidra®), as well as non-complexed mixtures of all the components of the system were analysed to exclude the risk of peak overlapping. PEG-PGA copolymer controls of known amount diluted in D_2O were also performed and analysed by ^{1}H NMR. As an additional control, an equal volume of the ENCPs were lyophilized and weighed, following their isolation with centrifugation.

The surface PEG density was calculated as the number of PEG molecules per 100 nm^2 surface area on nanoparticles, assuming all surface PEG chains were full length. According to Xu *et al*, the PEG density ([Γ]), representing the number of PEG molecules on the nanoparticle surface per 100 nm^2 was calculated by dividing the total PEG content (MPEG, in mole) detected by ^{1} H NMR by the total surface area of all nanoparticles.

$$[\Gamma] = [(MPEG \times 6.02 \times 10^{23})/ (W_{NP}/d_{NP}/ 4/3\ \pi\ (D/2)^3)] /[4\pi\ (D/2)^2]$$

Where W_{NP} is the total mass of nanoparticles, d_{NP} is the density of nanoparticle, and D is the diameter of nanoparticles as measured by dynamic light scattering.

According to the report by Auguste and co-workers, a full surface mushroom coverage [Γ*] represents the number of unconstrained PEG molecules per 100 nm^2. To determine [Γ*], the surface area occupied by one PEG chain was estimated assuming random-walk statistics and that the PEG chain occupies an area at the interface given by a sphere of diameter ξ.

$$\xi = 0.76m^{0.5}\ [Å]$$

where m is the molecular weight of PEG chain

The surface area occupied by one PEG molecule can be determined from $(\xi/2)^2$, so in this case, $[\Gamma^*] = 100/(\xi/2)^2$

Based on the ratio $[\Gamma]/[\Gamma^*]$ we can conclude how densely the PEG is packed on the nanoparticle surface; a ratio <1 indicates low density and PEG molecules are in mushroom conformation; a ratio >2 indicates high density and the PEG molecules are in a dense brush conformation.

In addition, to confirm the calculations and get $[\Gamma]$ value, we also used equations (1–5), where R_F is the Flory Radius of the PEG graft, D is the distance between PEG grafts, A is the area occupied by each PEG chain and L is the length/thickness of the PEG layer:

$$(1)\quad R_F = aN^{3/5}$$

where a is the monomer length and N is the number of monomers per polymer chain

$$(2)\quad D = 2(A/\pi)^{1/2}$$

$$(3)\quad A = 6M_{PEG}/dN_Afq$$

where M_{PEG} is the molecular weight of the PEG chains, d is the average diameter of the nanoparticles, N_A is the Avogadro number, f is the mass fraction of PEG in the PEG-PGA enveloped nanocomplexes and q is the density of the ENCPs.

$$(4)\quad L = Na^{5/3}/D^{2/3}$$

$$(5)\quad [\Gamma] = 1/A$$

3.3 Results and discussion

The search for the most suitable characteristics of the PEG-PGA coated nanocomplexes with respect to their use as nanocarriers for oral delivery led us to investigate the effect of PEG surface density on their transport across the intestinal mucosa. The idea here was to screen structurally different PEG-PGA copolymers as coating materials for the previously developed C12-r8-insulin nanocomplexes, aiming at increasing their PEG surface density. The novelty of this approach relies on the comparison of different PGA chain lengths (10

units versus 30 units of PGA), different architectures of the copolymer (diblock versus branched) (Figure 5) and different PEG substitution degrees of the PGA chain (10% versus 30%) in the case of the branched polymers, in terms of the resulting number of PEG molecules residing in the surface of the resulting nanocomplexes.

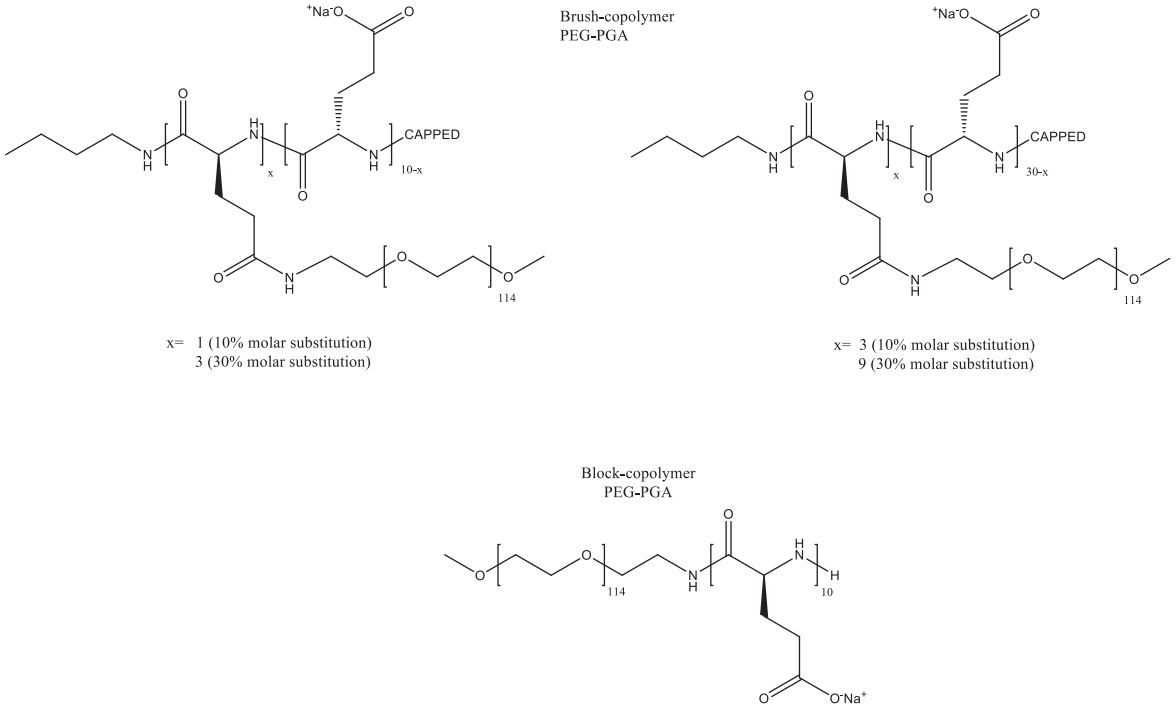


Figure 5: The chemical structure of different PEG-PGA copolymers

3.3.1 Development and physicochemical characterization of C12-r8-Insulin Nanocomplexes (NCPs)

As a first step C12-r8:Insulin nanocomplexes were formulated at a molar ration of C12-r8 to insulin equal to 8:1, following the method as described above, in order to be later coated with different PEG-PGA copolymers. The NCPs had an average diameter 209 ± 14 nm, low PdI and a positive zeta potential value +20.2 ± 0.45 mV (Table 2). As shown in table 2, the drug loading of insulin was 32.9% ± 0.07 and its association efficiency was as high as 98.7% ± 0.20. According to the FE-SEM analysis, the NCPs appeared monodispersed with a spherical shape, as shown in Figure 6.

Table 2: Physicochemical properties, drug loading and association efficiency of non-coated C12-r8-Insulin Nanocomplexes (mean ± SD, n=3)

NCPs C12-r8-Insulin (8:1)				
Size (nm)	**PdI**	**Z-pot (mV)**	**Loading (% wt)**	**A.E. (%)**
209 ± 14	0.15	+20.2 ± 0.45	32.9 ± 0.07	98.7 ± 0.20

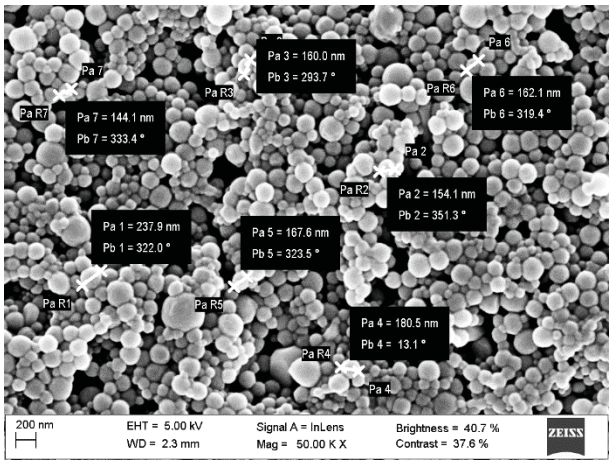


Figure 6: FE-SEM photograph of non-coated C12-r8:Insulin Nanocomplexes

3.3.2 Development and physicochemical characterization of Enveloped C12-r8-Insulin Nanocomplexes (ENCPs)

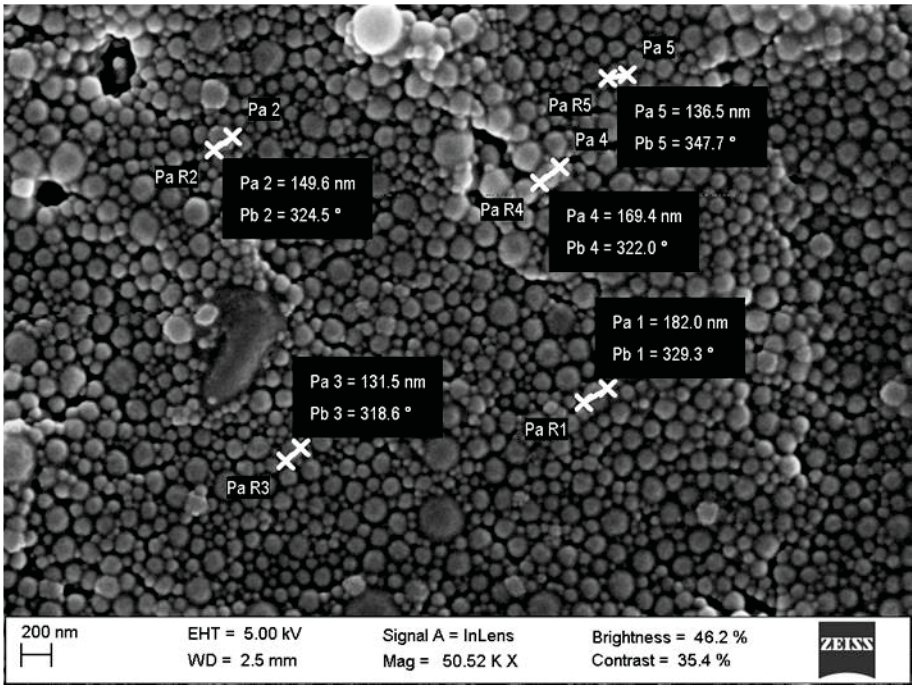
According to previous studies, the film hydration method allowed the efficient accommodation of PEG-PGA onto the surface of the cationic NCPs without impairing the association efficiency of insulin. During this coating process, the surface charge of the NCPs changed from positive to neutral values due to the attachment of the diblock or branched PGA-PEG. This could be attributed to the fact that the film hydration method allows for a gradual and more effective envelopment of the NCPs with the PGA-PEG molecules.

Table 3 summarises the characteristics of the enveloped nanocomplexes prepared according to above-described techniques. In general, the size oscillates within the range 180-250 nm, depending on the structure of the PEG-PGA copolymer used. As expected, the zeta potential was close to neutral in all cases, owing to the presence of long PEG chains in the surface of the system. However, in the case of the PEG(30)-g-PGA(10)

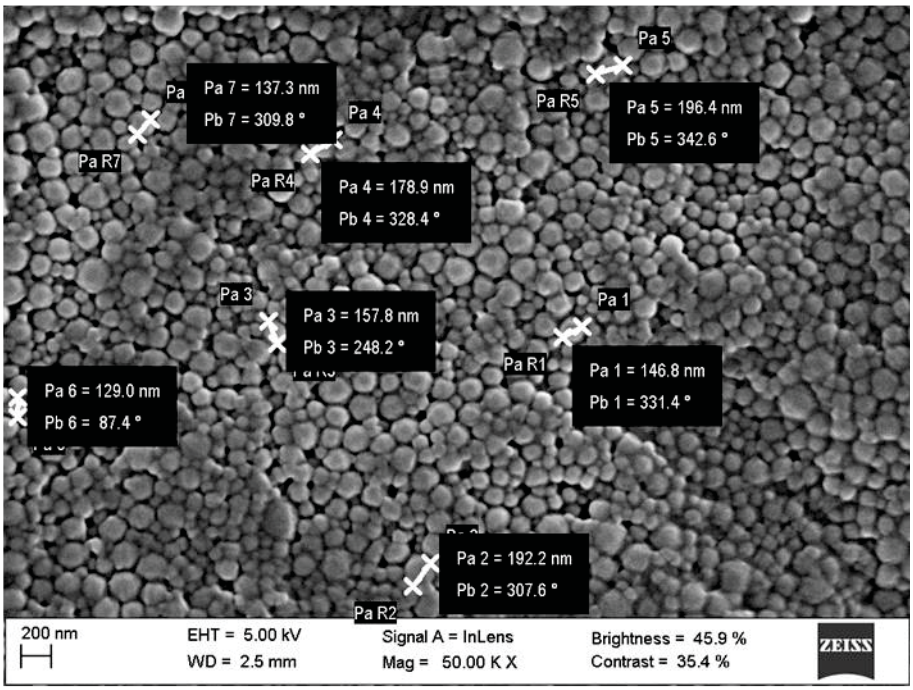
copolymer, no turn of zeta potential was observed, as the z-potential of the resulting system remained around +22 mV. This could have been related to the lack of interactions between the positive charge of NCPs and the short chain of PGA (10 units) in the particular structure, probably due to the obstruction caused by the high PEG substitution degree (30%). Nevertheless, the ENCPs exhibited high insulin association efficiency and loading in all cases (Table 3).

The morphology of the enveloped nanocomplexes was observed by field emission scanning electron microscopy (FE-SEM). From a morphological point of view, the images obtained by FE-SEM from the different ENCPs are very similar, except for the nanocomplexes coated with PEG5k(30)-g-PGA(10), in agreement with the data obtained by DLS. As expected from the estimated surface charge of these later nanocomplexes, the difference is clear (Figure 7), since the formulation with the PEG5k(30)-g-PGA(10) copolymer exhibits morphological similarities to the non-coated nanocomplexes.

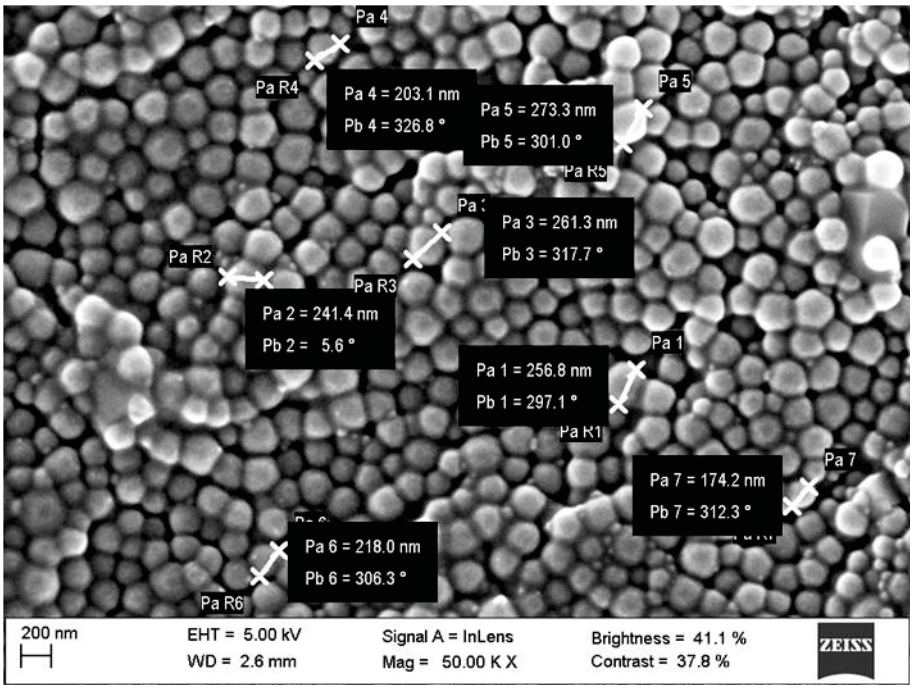
A



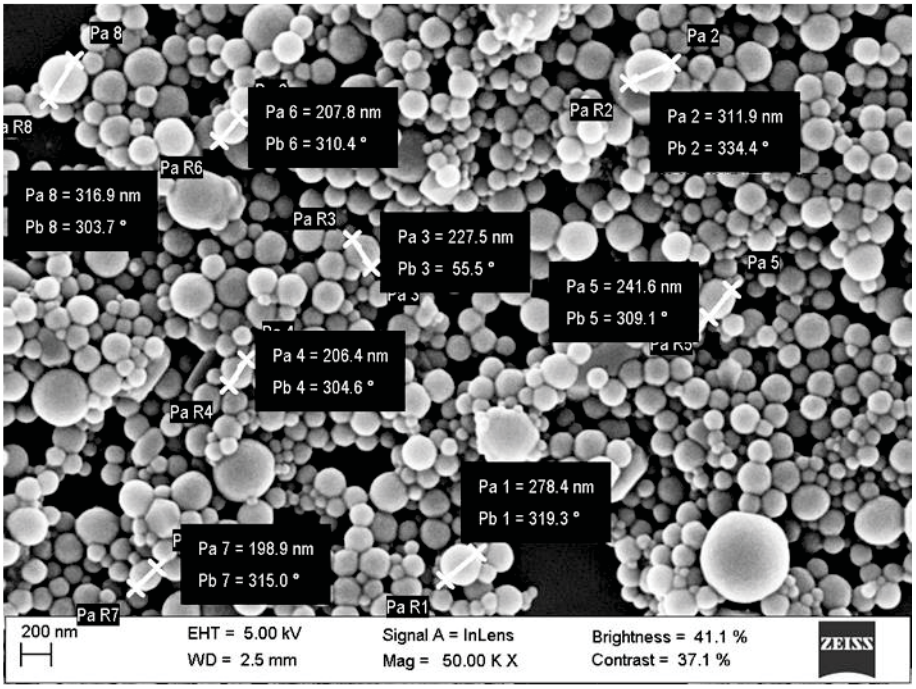
B


Pa 7 = 137.3 nm
Pb 7 = 309.8 °
Pa 4
Pa R7
Pa R4
Pa 4 = 178.9 nm
Pb 4 = 328.4 °
Pa 5
Pa R5
Pa 5 = 196.4 nm
Pb 5 = 342.6 °
Pa 3
Pa 3 = 157.8 nm
Pb 3 = 248.2 °
Pa 1
Pa R1
Pa 1 = 146.8 nm
Pb 1 = 331.4 °
Pa 6 = 129.0 nm
Pb 6 = 87.4 °
Pa 2 = 192.2 nm
Pb 2 = 307.6 °
Pa R2
200 nm
EHT = 5.00 kV
WD = 2.5 mm
Signal A = InLens
Mag = 50.00 K X
Brightness = 45.9 %
Contrast = 35.4 %
ZEISS

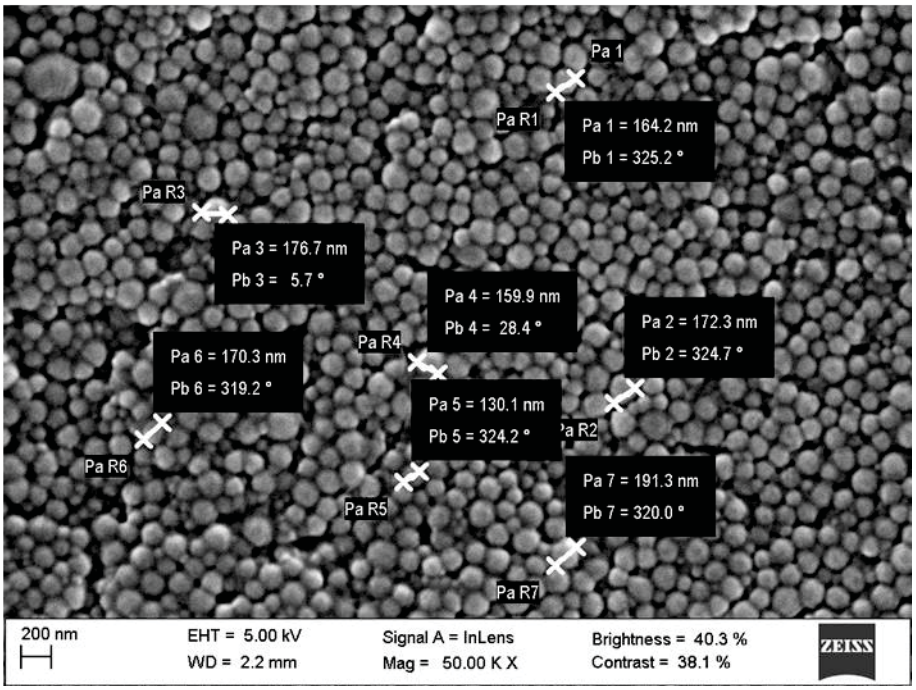
C


Pa 4
Pa R4
Pa 4 = 203.1 nm
Pb 4 = 326.8 °
Pa 5
Pa 5 = 273.3 nm
Pb 5 = 301.0 °
Pa R5
Pa 3 = 261.3 nm
Pb 3 = 317.7 °
Pa R2
Pa R3
Pa 2 = 241.4 nm
Pb 2 = 5.6 °
Pa 1
Pa 1 = 256.8 nm
Pb 1 = 297.1 °
Pa R1
Pa 7
Pa 7 = 174.2 nm
Pb 7 = 312.3 °
Pa 6 = 218.0 nm
Pb 6 = 306.3 °
Pa R6
200 nm
EHT = 5.00 kV
WD = 2.6 mm
Signal A = InLens
Mag = 50.00 K X
Brightness = 41.1 %
Contrast = 37.8 %
ZEISS

D


Pa 8
Pa 6 = 207.8 nm
Pb 6 = 310.4 °
Pa 2
Pa R2
Pa 2 = 311.9 nm
Pb 2 = 334.4 °
Pa R6
Pa 8 = 316.9 nm
Pb 8 = 303.7 °
Pa R3
Pa 3 = 227.5 nm
Pb 3 = 55.5 °
Pa 5
Pa 5 = 241.6 nm
Pb 5 = 309.1 °
Pa 4 = 206.4 nm
Pb 4 = 304.6 °
Pa R4
Pa 1 = 278.4 nm
Pb 1 = 319.3 °
Pa 7 = 198.9 nm
Pb 7 = 315.0 °
Pa R7
Pa R1
200 nm
EHT = 5.00 kV
WD = 2.5 mm
Signal A = InLens
Mag = 50.00 K X
Brightness = 41.1 %
Contrast = 37.1 %
ZEISS

E


Pa 1
Pa R1
Pa 1 = 164.2 nm
Pb 1 = 325.2 °
Pa R3
Pa 3 = 176.7 nm
Pb 3 = 5.7 °
Pa 4 = 159.9 nm
Pb 4 = 28.4 °
Pa 2 = 172.3 nm
Pb 2 = 324.7 °
Pa R4
Pa 6 = 170.3 nm
Pb 6 = 319.2 °
Pa 5 = 130.1 nm
Pb 5 = 324.2 °
Pa R2
Pa R6
Pa 7 = 191.3 nm
Pb 7 = 320.0 °
Pa R5
Pa R7
200 nm
EHT = 5.00 kV
WD = 2.2 mm
Signal A = InLens
Mag = 50.00 K X
Brightness = 40.3 %
Contrast = 38.1 %
ZEISS

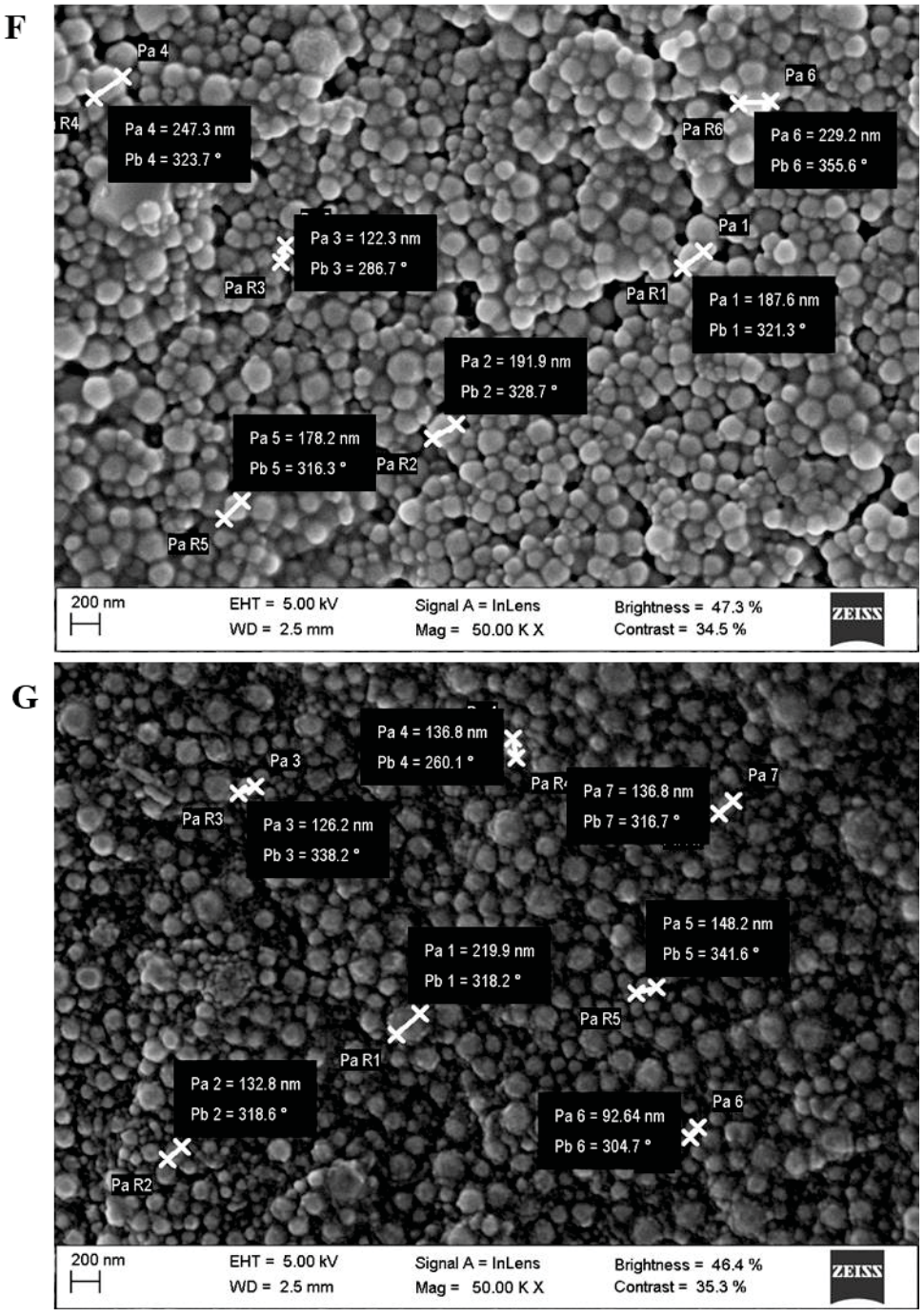


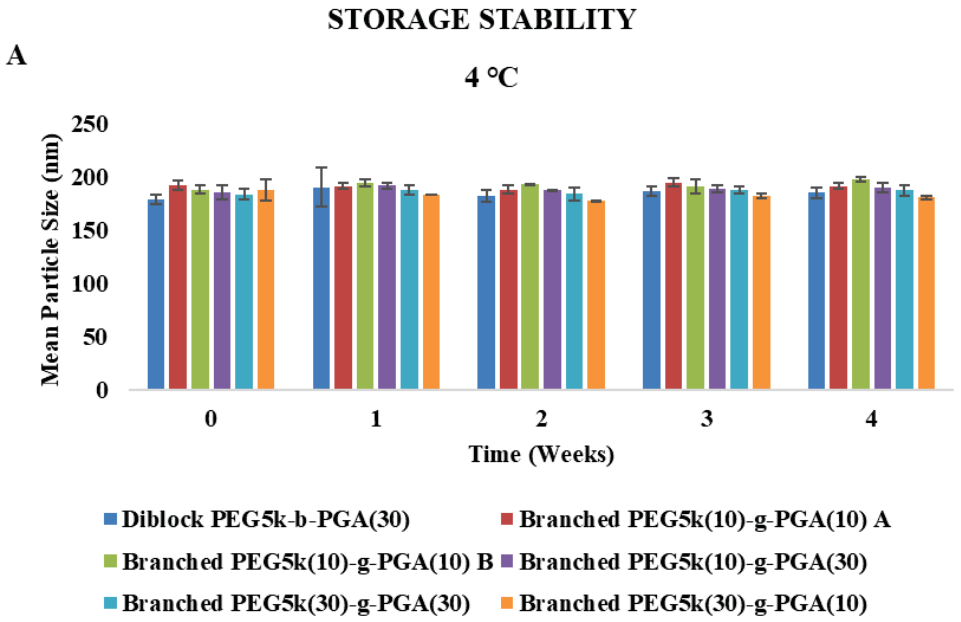
Figure 7: FE-SEM photographs of enveloped nanocomplexes. **(A)**: PEG5k(10)-g-PGA(10) A coated C12-r8:Insulin nanocomplex. **(B)**: PEG5k(10)-g-PGA(10) B coated C12-r8:Insulin nanocomplex. **(C)**: PEG5k(10)-g-PGA(30) coated C12-r8:Insulin nanocomplex. **(D)**: PEG5k(30)-g-PGA(10) coated C12-r8:Insulin nanocomplex. **(E)**: PEG5k(30)-g-PGA(30) coated C12-r8:Insulin nanocomplex. **(F)**: PEG5k-b-PGA(10) coated C12-r8:Insulin nanocomplex and **(G)**: PEG5k-b-PGA(30) coated C12-r8:Insulin nanocomplex

Table 3: Physicochemical properties of coated C12-r8:Insulin Nanocomplexes (mean ± SD, n=3)

Copolymer	Size (nm)	PdI	Z-pot (mV)	Loading (% wt)	A.E. (%)
PEG5k-b-PGA(10)	203 ± 10	0.07	+7.7 ± 0.4	26.8 ± 0.0	99.6 ± 0.0
PEG5k-b-PGA(30)	220 ± 5	0.05	+5.6 ± 0.3	29.6 ± 0.03	99.5 ± 0.1
PEG5k(10)-g-PGA(10) A	235 ± 9	0.07	+6.7 ± 0.1	27.5 ± 0.06	99.6 ± 0.1
PEG5k(10)-g-PGA(10) B	237 ± 9	0.1	+6.7 ± 0.1	16.5 ± 0.01	99.2 ± 0.05
PEG5k(10)-g-PGA(30)	250 ± 23	0.1	+6.7 ± 0.1	25.0 ± 0.0	99.7 ± 0.0
PEG5k(30)-g-PGA(10)	188 ± 10	0.07	+22.7 ± 1.4	26.9 ± 0.0	99.6 ± 0.0
PEG5k(30)-g-PGA(30)	224 ± 2.3	0.05	+5.4 ± 0.4	20.2 ± 0.01	99.4 ± 0.08

3.3.3 Storage stability study of Enveloped Nanocomplexes (ENCPs)

The particle size of the coated C12-r8-insulin nanocomplexes were analysed upon storage at 4 ºC, at room temperature, and at 37 ºC for up to 1 month. ENCPs were able to retain their physicochemical characteristics at all cases after being stored for 4 weeks under the conditions mentioned above, as shown in Figure 8.



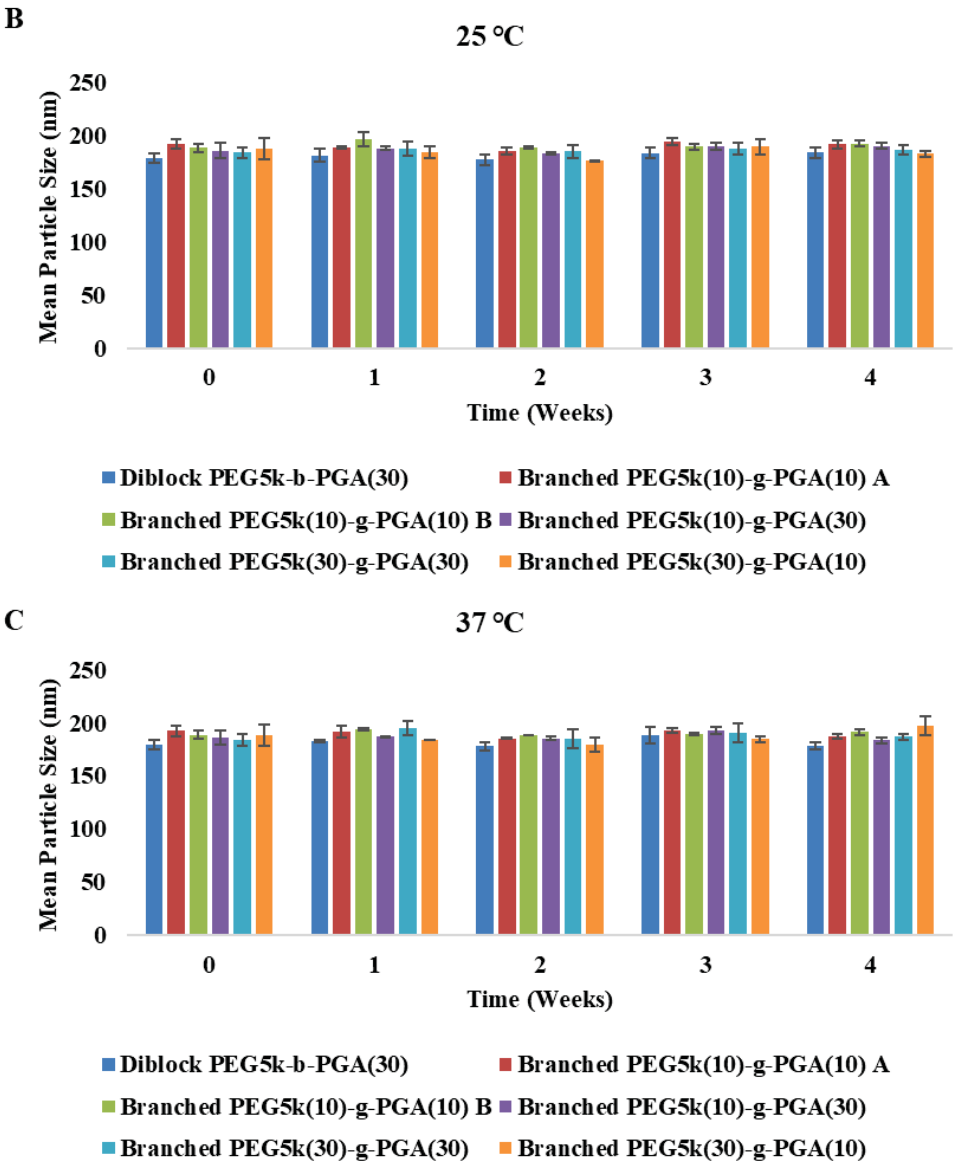


Figure 8: Graphics representing the evolution of particle size with time upon storage at **(A)** 4 °C, **(B)** 25 °C and **(C)** 37 °C

3.3.4 Quantification of PEG surface density of Enveloped Nanocomplexes (ENCPs)

As mentioned, our idea here was to compare structurally different PEG-PGA copolymers aiming at increasing the PEG content in the surface of ENCPs. Due to the aggregation problem in the ENCP with copolymer PEG5k(30)-g-PGA(10) detected visually and also by DLS, we have not tested this formulation. Table 4 summarizes the results of this screening. As resulted from the aforementioned analysis, the highest PEG surface density was achieved in the case of branched copolymer with the higher PEG substitution degree and the larger PGA chain (PEG5k(30)-g-PGA(30)), leading to a final increase of the PEG

density in the surface of the carrier up to ~7 times in comparison to the diblock copolymer with smaller PGA chain (PEG5k-b-PGA(10)).

Table 4: PEG surface density, configuration, PEG efficiency % and amount of PEG (mg) per mg of formulations (mean ± SD, n=3)

	PGA-PEG copolymer	mg PEG/mg formulation	%Efficiency	PEG density (PEG chain/100 nm^2)	Configuration
A	**PEG5k-b-PGA(10)**	**0.014 ± 0.007**	**24%**	**0.08 ± 0.04**	**BRUSH**
B	**PEG5k-b-PGA(30)**	**0.009 ± 0.06**	**28%**	**0.07 ± 0.04**	**BRUSH**
C	**PEG5k(10)-g-PGA(10)**	**0.020 ± 0.02**	**32%**	**0.18 ± 0.14**	**BRUSH**
C (x3)	**PEG5k(10)-g-PGA(10) (x3)**	**0.047 ± 0.01**	**9%**	**0.23 ± 0.07**	**BRUSH**
D	**PEG5k(30)-g-PGA(10)**	**-**	**-**	**-**	**-**
E	**PEG5k(10)-g-PGA(30)**	**0.027 ± 0.003**	**16%**	**0.17 ± 0.01**	**BRUSH**
F	**PEG5k(30)-g-PGA(30)**	**0.088 ± 0.013**	**22%**	**0.49 ± 0.08**	**BRUSH**

The conclusions made from this comparative study can be summarized in 3 different points;

(1) Regarding the architecture of the copolymer: the branched architecture slightly increased the PEG surface density (Table 4, [A] versus [C])

(2) Regarding the PEG substitution degree: It appears that a higher substitution degree indeed leads to higher PEG density (Table 4, [E] versus [F]).

(3) Regarding the length of PGA & the flexibility of the molecule: Smaller molecules attach in greater extent to the surface of the nanocomplexes than larger molecules with the same degree of PEG substitution, leading to higher PEG content (Table 4, [A] versus [B] & [3C] versus [E]).

3.4 Conclusion & Future perspectives

The work presented in this chapter show the successful development of a nanosystem intended for oral drug delivery. The results obtained from the experimental work led to the following conclusions:

- C12-r8-insulin nanocomplexes were successfully prepared with a particle size around 200 nm, low particle distribution (PdI=0.1), positive surface charge (Z-potential around +20 mV) and spherical shape. The NCPs exhibited an excellent insulin association efficiency (~98%) with high insulin loading (~32 % wt).
- The enveloped C12-r8-insulin nanocomplexes (ENCPs) were prepared using the film hydration method, exhibited particle sizes between 180-250 nm, narrow size distribution and spherical shape. The Z-potential change from positive to neutral charge except for the ENCP with PEG(30)-g-PGA(10) that remain positive. All the ENCPs exhibited a high insulin association efficiency (~100%) with a high insulin loading.
- The results of screening analysis for increase the PEG density in the surface of ENCPs exhibited that the highest PEG surface density was achieved in the case of branched copolymer with the higher PEG substitution degree (30%) and the larger PGA chain (30 units) (PEG5k(30)-g-PGA(30)), leading to a final increase of the PEG density in the surface of the carrier up to ~7 times in comparison to the diblock copolymer with smaller PGA chain (10 units) (PEG5k-b-PGA(10)).
- During the storage stability at 4 °C, 25 °C and 37 °C, the ENCPs were able to retain their physicochemical properties up to 1 month.

One of the next step for this study is to compare the results with an *ex vivo* mucodiffusion study to a better understanding of a relation between mucus and PEG density surface.

CHAPTER 4

PEG-PGA Enveloped CPP-Peptide Nanocomplexes for the Nose-to-Brain Delivery of an anti-obesity peptide

4. PEG-PGA Enveloped CPP-Peptide Nanocomplexes for the Nose-to-Brain Delivery of an anti-obesity peptide

4.1 Introduction

Obesity is one of the most important known risk factor for several common chronic conditions including cardiovascular disease and diabetes mellitus. According to WHO, 13% of adults in the world are obese (WHO, 2017).

Even though the treatment for obesity are diet, exercise and behavioral modification, the research for pharmacology treatment is booming. Several studies have suggested that the nose-to-brain (N-to-B) route is a promising approach for the treatment of brain-related diseases. Particularly, glucagon-like peptide-1 (GLP-1) receptors located in the brain can be stimulated by agonist GLP-1, which have effectivity for weight reduction. Thus, N-to-B route may be an alternative administration for antiobesity treatment.

However, N-to-B drug delivery is also limited by many factors such as: (i) the administration dose/volume, (ii) the barrier of the nasal epithelium, (iii) the nasal metabolic activity and (iv) the presence of a protective mucus layer. In recent years, scientific efforts have been focused on the design of nanocarriers capable of enhancing nose-to-brain drug delivery, including polymer- and lipid-based nanoparticles (NPs) (Mistry *et al*, 2010; Samaridou and Alonso, 2017).

In this work we adapted the nanosystem described in the previous chapter for the N-to-B delivery of an antiobesity peptide (AOP), keeping in mind the requirements of this novel administration route. For that reason, ENCPs designed to transport a therapeutic peptide from the nose to the brain owed to have adequate physicochemical properties (particle size ≤ 100 nm (Ahmad *et al*, 2017)), high peptide loading, provide controlled drug release and be able to be uptaken and travel through the olfactory pathways to the different brain areas. The ENCPs resulting from this study were characterized in terms of (i) their physicochemical properties and (ii) their lyophilization capacity.

4.2 Materials and Methods

4.2.1 Materials

The antiobesity peptide was kindly provided by NovoNordisk (Bagsværd, Denmark). Lauryl acid coupled to the *N*-terminal of octaarginine (D-type) (C12-r8, MW=1449.85 Da) was purchased from China Peptides (Shangai, China). The copolymer diblock (m[PEG]$_{455}$-b-[PGA]$_{10}$, methoxy poly(ethylene glycol)-block-poly(L-glutamic acid sodium salt) MW=22000 Da, 20000 Da of PEG and 2000 Da of PGA) was purchased from Alamanda Polymers (Huntsville, USA). Trehalose was purchased from Sigma-Aldrich (Madrid, Spain). Sodium hydroxide (NaOH) was purchased from Scharlau distributed by Cienytech (Santiago de Compostela, Spain). Ultrapurified water was obtained from Millipore Milli-Q Plus water purification system (Darmstadt, Germany). All other chemicals were of analytical grade.

4.2.2 Preparation of C12-r8-AOP Nanocomplexes (NCPs)

C12-Octaargine-AOP NCPs were prepared according to the previously described method with slight modifications, as for the N-to-B route is important to achieve smaller particle sizes. Briefly, C12-r8 was dissolved in Milli-Q water at a concentration of 0.78 mg/mL (Solution A), whereas the therapeutic peptide was dissolved at a concentration of 1 mg/mL in basic media pH 11 (0.001 N NaOH) (Solution B).The complexes were formed instantly upon adding the solution B to solution A under a magnetic stirring corresponding to a molar ration of C12-r8:AOP equal to 4:1, which was chosen as optimal from a previous screening.

4.2.3 Preparation of Enveloped C12-r8-AOP Nanocomplexes (ENCPs)

The NCPs that resulted from the aforementioned complexation process had a positive charge and were enveloped by negatively charged diblock type PEG-PGA (m[PEG]455-b-[PGA]10, methoxy-poly(ethylene glycol) - block - poly(L-glutamic acid sodium salt) MW=22000 Da, 20000 Da PEG and 2000 Da PGA, Alamanda Polymers, USA), at AOB:PEG-PGA mass ratio 1:0.35, leading to the formation of ENCPs. Similar to the protocol described above, PEG-PGA copolymer was dissolved in Milli-Q water at a concentration of 1 mg/mL. Then, the water phase was evaporated in a round flask under reduced pressure at 37 ºC, leading to the formation of a thin film. Thus, the NCPs (without adjusting their pH) were transferred to the same flask and maintained for 10 min under

rotation at room temperature and atmospheric pressure. Figure 9 summarized a schematic illustration of the protocol.

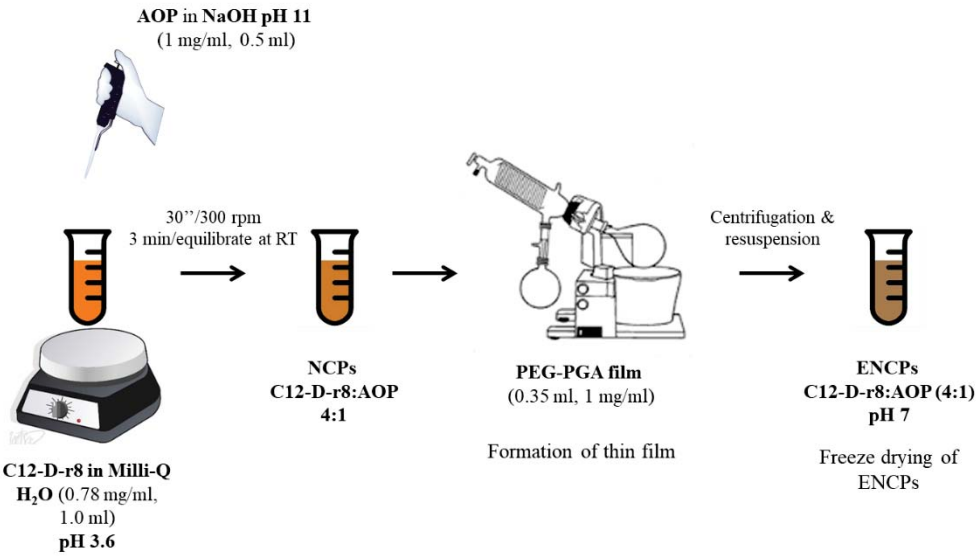


Figure 9: Schematic representation of the protocol followed for the formation of ENCPs

4.2.4 Physicochemical characterization of Nanocomplexes

As described above, particle size and PdI were determined by Dynamic Light Scattering (DLS) using a Malvern Zeta-Sizer (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) fitted with a red laser light beam (λ=632.8 nm). The Z-potential was calculated from the mean electrophoretic mobility values determined by Laser Doppler Anemometry (LDA) using the same device.

4.2.5 Determination of association efficiency and loading of the AOP

The association efficiency (AE) of the antiobesity peptide to the ENCPs was determined by an indirect method following separation of the peptide complexes from the aqueous media and measuring the free peptide in the suspension media. To that end, 500 µL of the formulation were isolated by ultrafiltration using Amicon® Ultra filters (100 kDa cut-off) by centrifugation (Eppendorf Centrifuge, 5430R, Germany) at 14,000 g for 10 min at 15 °C. The aqueous supernatant was collected and the amount of free peptide in the supernatant was determined by reverse phase HPLC (Agilent, 1100 Series, USA), using a SunFire C18 column (4.6x250 nm) 5 nm (Waters®). The mobile phase was acetonitrile mixed with trifluoroacetic acid (TFA) 0.1% (as phase C) and Milli-Q water mixed with TFA 0.1% (as phase D). The AE of the AOP in the formulation was calculated considering the total peptide amount involved in the formulation and the free peptide found in the

supernatant. The final loading was calculated dividing the amount of the AOP associated (AE x total peptide in the formulation) by the theoretical amount of all the materials involved in the formulation.

4.2.6 Lyophilization study of the Enveloped Nanocomplexes (ENCPs)

In order to improve the storage stability of the ENCPs, a lyophilization study was done to assess the possibility of processing the nanoparticles suspension as a powder. To that end, freshly prepared nanocomplexes were lyophilized in the presence of 2% (w/v) trehalose, used as a lyoprotectant. Freeze drying was performed using the Genesis VirTis 25EL Pilot Lyophilizer (SP Scientific, USA). The dried samples were resuspended in water and characterized by dynamic light scattering as described above.

4.3 Results and discussion

In this chapter, the idea was to develop C12-r8:AOP nanocomplexes coated with PEG-PGA copolymer exhibiting suitable physicochemical properties for a nanosystem that in the future studies could be administrated via N-to-B.

4.3.1 Development and physicochemical characterization of C12-r8-AOP Nanocomplexes (NCPs)

The nanocomplexes, prepared by the method described previously (Figure 9), exhibited a diameter smaller than 100 nm (Table 5), in accordance to the requirements for an efficient nose-to-brain drug delivery, whereas their surface charge was within the range of +26 mV.

Table 5: Physicochemical properties of C12-r8:AOP nanocomplexes (mean ± SD, n>3)

NCP C12-r8:AOP (4:1)		
Size (nm)	**PdI**	**Z-pot (mV)**
71.9 ± 6.7	0.11	+25.6 ± 1.4

4.3.2 Development and physicochemical characterization of Enveloped C12-r8-AOP Nanocomplexes (ENCPs)

In the next step, the C12-r8-AOP nanocomplexes were enveloped with diblock PEG-PGA copolymer (m[PEG]455-b-[PGA]10) in order to obtain ENCPs. Based on the calculated zeta potential values (Table 6), the reversion of the z-potential of the NCPs from positive

to almost neutral made evident the success of the enveloping process. Table 6 summarizes the physicochemical properties as well as the drug loading of peptide and the association efficiency. Briefly, the size oscillates within the range 100-120 nm. As expected, the zeta potential was around +11 mV and the PdI was low. Most importantly, the ENCPs exhibited a high peptide association efficiency (~100%) with a really high peptide loading (~31 % wt).

Table 6: Physicochemical properties of enveloped C12-r8:AOP nanocomplexes (mean ± SD, n>3)

ENCP PEG20k-b-PGA(10) C12-r8:AOP (4:1)				
Size (nm)	**PdI**	**Z-pot (mV)**	**Loading (% wt)**	**A.E. (%)**
113.3 ± 5.6	0.08	+11.4 ± 0.88	30.67 ± 0.0	100 ± 0.0

4.3.3 Lyophilization study of Enveloped Nanocomplexes (ENCPs)

As resulted from this study, the formulations could be freeze-dried in the presence of 2% trehalose and be reconstituted successfully, while retaining their particle size (Figure 10).

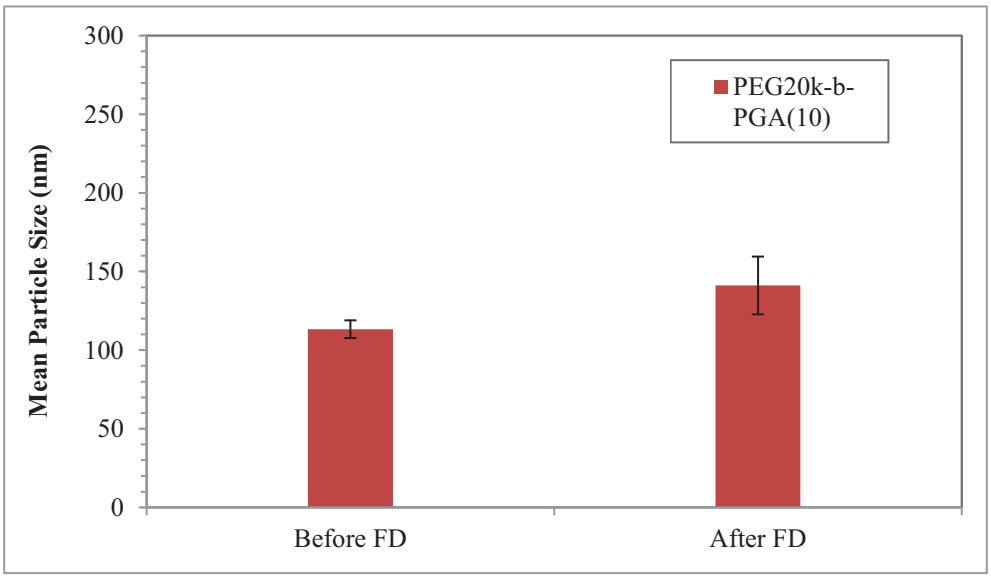


Figure 10: Mean particle size of PGA-PEG coated NCPs before (113 nm ± 5.6) and after (141 nm ± 18.3) freeze-drying in the presence of trehalose (mean ± SD; n>3)

4.4 Conclusion & Future perspectives

The work presented here portray a successfully developed nanosystem intended for nose-to-brain drug delivery. The results obtained from the experimental work led to the following conclusions:

- C12-r8:AOB nanocomplexes were successfully prepared with a small particle size (around 70 nm), low particle distribution (PdI=0.1) and a positive surface charge (Z-potential around +26 mV).
- The resultant nanosystem (ENCP) from enveloped the previous nanocomplexes with PEG-PGA copolymer, exhibited a small particle size (around 100-120 nm), narrow size distribution (PdI=0.08) and neutral Z-potential (around +11 mV). In addition, the ENCPs exhibited a high peptide association efficiency (~100%) with a really high peptide loading (~31 % wt).
- The ENCPs were converted into powder form in the presence of trehalose (%2 w/w) as a lyoprotectant.

Overall, these results open the way for several future studies such as (i) *in vitro* release studies, (ii) *in vitro* mucodiffusion studies and (iii) *in vivo* N-to-B biodistribution studies among others.

References

Ahmad, E., Feng, Y., Qi, J., Fan, W., Ma, Y., He, H., … Wu, W. Ahmad, E., Feng, Y., Qi, J., Fan, W., Ma, Y., He, H., Xia, F., Dong, X., Zhao, W., Lu, Yi., Wu, W. (2017). Evidence of nose-to-brain delivery of nanoemulsions: cargoes but not vehicles. Nanoscale. 9(3): 1174–1183.

Alex, A. T., Joseph, A., Shavi, G., Rao, J. V., Udupa, N. (2016). Development and evaluation of carboplatin-loaded PCL nanoparticles for intranasal delivery. Drug Deliv. 23(7): 2144–2153.

Clementino, A., Batger, M., Garrastazu, G., Pozzoli, M., Del Favero, E., Rondelli, V., Gutfilen, B., Barboza, T., Sukkar, M. B., Souza, S A. L., Cantù, L., Sonvico, F. (2016). The nasal delivery of nanoencapsulated statins -An approach for brain delivery. Int J Nanomedicine. 11: 6575–6590.

Damgé, C., Michel, C., Aprahamian, M., Couvreur, P., Devissaguet, J. P. (1990). Nanocapsules as carriers for oral peptide delivery. J Control Release. 13(2–3): 233–239.

De Jong, W. H., Borm, P. J. a. (2008). Drug delivery and nanoparticles:applications and hazards. Int J Nanomedicine. 3(2): 133–149.

Ensign, L. M., Cone, R., Hanes, J. (2012). Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. Adv Drug Deliv Rev. 64(6): 557–570.

Futaki, S., Ohashi, W., Suzuki, T., Niwa, M., Tanaka, S., Ueda, K., Harashima, H., Sugiura, Y. (2001). Stearylated arginine-rich peptides: A new class of transfection systems. Bioconjug Chem. 12(6): 1005–1011.

Jitendra, Sharma, P. K., Bansal, S., Banik, A. (2011). Noninvasive routes of proteins and peptides drug delivery. Indian J Pharm Sci. 73(4): 367–75.

Kamei, N., Morishita, M., Ehara, J., Takayama, K. (2008). Permeation characteristics of oligoarginine through intestinal epithelium and its usefulness for intestinal peptide drug delivery. J Control Release. 131(2): 94–99.

Kristensen, M., Birch, D., & Mørck Nielsen, H. (2016). Applications and Challenges for Use of Cell-Penetrating Peptides as Delivery Vectors for Peptide and Protein Cargos. International Journal of Molecular Sciences, 17(2), 185.

Lai, S. K., Wang, Y.Y., Hida, K., Cone, R., Hanes, J. (2010). Nanoparticles reveal that human cervicovaginal mucus is riddled with pores larger than viruses. Proc Natl Acad Sci. 107(2): 598–603.

Obesity and overweight. (2017, October). Retrieved December 15, 2017, from http://www.who.int/mediacentre/factsheets/fs311/en/

Perry, J. L., Reuter, K. G., Kai, M. P., Herlihy, K. P., Jones, S. W., Luft, J. C., Desimone, J. M., Napier, M., Bear, J. E., Desimone, J. M. (2012). PEGylated PRINT nanoparticles: The impact of PEG density on protein binding, macrophage association, biodistribution, and pharmacokinetics. Nano Lett. 12(10): 5304–5310.

Prego, C., Fabre, M., Torres, D., Alonso, M. J. (2006). Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery. Pharm Research. 23(3): 549–556.

Samaridou, E., Alonso, M. J. (2017). Nose-to-brain peptide delivery - The potential of nanotechnology. Bioorg Med Chem.

Santander-Ortega, M. J., Bastos-González, D., Ortega-Vinuesa, J. L., Alonso, M. J. (2009). Insulin-loaded PLGA nanoparticles for oral administration: An in vitro physico-chemical characterization. J Biomed Nanotechnol. 5(1): 45–53.

Sun, T., Zhang, Y. S., Pang, B., Hyun, D. C., Yang, M., Xia, Y. (2014). Engineered nanoparticles for drug delivery in cancer therapy. Angew Chemie Int Ed. 53(46):12320-12364.

Takeuchi, H., Matsui, Y., Yamamoto, H., Kawashima, Y. (2003). Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. J Control Release. 86(2–3): 235–242.

Vila, A., Gill, H., McCallion, O., Alonso, M. J. (2004). Transport of PLA-PEG particles across the nasal mucosa: Effect of particle size and PEG coating density. J Control Release. 98(2): 231–244.

Walsh, E. G., Adamczyk, B. E., Chalasani, K. B., Maher, S., O'Toole, E. B., Fox, J. S., Brayden, D. J., Leonard, T. W., Brayden, D. J. (2011). Oral delivery of macromolecules: rationale underpinning Gastrointestinal Permeation Enhancement Technology (GIPET). Ther Deliv. 2(12): 1595–1610.

Wilczewska, A. Z., Niemirowicz, K., Markiewicz, K. H., Car, H. (2012). Nanoparticles as drug delivery systems. Pharmacol Rep. 64(5): 1020–1037.

World Health Organization. (2016). Global Report on Diabetes. Isbn, 978, 88. https://doi.org/ISBN 978 92 4 156525 7.

Xu, Q., Ensign, L. M., Boylan, N. J., Schön, A., Gong, X., Yang, J.-C, Lamb, N. W., Cai, S., Yu, T., Freire, E., Hanes, J. (2015). Impact of Surface Polyethylene Glycol (PEG) Density on Biodegradable Nanoparticle Transport in Mucus ex vivo and Distribution in vivo. ACS Nano. 9 (9): 9217–9227.

Yuan, H., Chen, C. Y., Chai, G. H., Du, Y. Z., Hu, F. Q. (2013). Improved transport and absorption through gastrointestinal tract by pegylated solid lipid nanoparticles. Mol Pharm. 10(5): 1865–1873.

Zhang, L., Wang, S., Zhang, M., Sun, J. (2013). Nanocarriers for oral drug delivery. J Drug Target. 21(6): 515–527.

Zhao, H., Lin, Z. Y., Yildirimer, L., Dhinakar, A., Zhao, X., Wu, J. (2016). Polymer-based nanoparticles for protein delivery: design, strategies and applications. J. Mater. Chem. B. 4(23): 4060–4071.