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The interaction of protamine nanocapsules with the intestinal epithelium: A mechanistic approach

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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, capable of translocating protein cargos through cell membranes, while PSA is a low molecular weight polysaccharide used to enhance the stability of macromolecules and nanocarriers. The aim of this work was to study *in vitro* the mechanism of interaction of these NCs with different intestinal cell models (Caco-2, Caco-2/Raji mimicking follicle associated epithelium and Caco-2/HT29-MTX to study the effect of mucus). For this, a fluorescent marker, TAMRA was covalently linked to protamine. The interaction and transport of the NCs with the Caco-2 cells 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. The formulations, administered intra-jejunally to healthy rats (4 h fasting) resulted in a moderate reduction of the glucose levels (20% reduction), which lasted for up to 4 h. This work raises prospects that protamine-based nanocapsules may have the potential as oral peptide delivery nanocarriers.

1. Introduction

The intestinal mucosa is a large interface that consists of a monolayer of epithelial cells connected by tight junctions (TJs) and protected by a thick mucus layer. Although this mucosa is highly specialized in the absorption of nutrients, e.g. amino acids, 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 e.g. *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 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 (NCs) [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

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protamine and double layer polysialic acid (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 improved stability and 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, *in vivo* studies were performed in healthy rats in order to elucidate the pharmacological activity of insulin loaded protamine based nanocapsules after intra-jejunal administration.

2. Materials and methods

2.1. Materials and animals

Protamine sulfate of low molecular weight (5 kDa) was purchased from Yuki Gosei Kogyo, Ltd. (Tokyo, Japan). The stabilizing surfactants, polyoxyethylene 40 monostearate, and sodium glycocholate (SGC) were bought from Croda Europe Ltd. (Snaith, UK) and Dextra (Reading, UK) respectively. Caprylic/capric triglyceride (Miglyol® 812) was purchased from Cremer, Oleo Division (Witten, Germany) and colominic acid sodium salt (polysialic acid, PSA) from 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, partially purified mucin type III from porcine stomach and Alcian blue were purchased from Sigma-Aldrich (Leuven, Belgium). All cell culture media and reagents were purchased from Invitrogen (Merelbeke, Belgium). Anhydrous monobasic sodium phosphate and sodium hydroxide were purchased from Scharlab (Barcelona, Spain). Pancreatin (8% USP specification) was purchased from Biozym (Hamburg, Germany). Organic solvents used were of HPLC grade and all other products used were of high purity or reagent grade.

Male Sprague–Dawley rats (250–300 g) were obtained from the Central Animal House, University of Santiago de Compostela (Spain). They were kept under 12 h light/12 h dark cycles and fed a standard laboratory rodent diet (Panlab A04, Panlab laboratories). All animal experiments were reviewed and approved by the ethics committee of the University of Santiago de Compostela (ref. 1500AE/12/FUN01/FIS02/CDG3) 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.

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® 812 (59 mg) 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 0.01 M HCl. This organic phase was immediately poured over 10 mL of an aqueous phase containing 0.15% w/v protamine under magnetic stirring at 300 rpm. 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 82,656*g* for 1 h (at 15 °C).

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 mild shaking (300 rpm) for 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, 300 rpm) 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 Section 2.2.1.

2.2.3. Physicochemical and morphological properties of protamine NCs

The mean size and polydispersity index (PDI) of the protamine NCs were measured after dilution (50×) 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 1 mM KCl (Zetasizer®, NanoZS, Malvern Instruments, Malvern, UK). The transmission electron microscopy (TEM, Joel 2010, 80 kV, 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) phosphotungstic acid solution, allowed to dry and then viewed under transmission electron microscopy (TEM).

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 by ultracentrifugation at 82,656*g* for 1 h at 15 °C (Avanti® J-E, Ultracentrifuge, Beckman Coulter, USA). 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 isolated NCs suspension (concentration: 18.6 mg/mL) were mixed with acetonitrile (0.1 mL), 0.1 M phosphate buffer (0.75 mL) and Triton™ X-100 (0.05 mL). The mixture was vortexed at a high speed to obtain a clear solution.

The amount of insulin loaded into the NCs was quantified by an HPLC (Agilent model 1100 series LC and a diode-array detector set at 214 nm) method in an isocratic mode. 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 phases, eluted at 1 mL/min at 44:56 v/v, were a mixture of phosphate buffer (0.1 M, pH 2.3) and acetonitrile (phase A 93:7 and phase B 43:57 v/v). The column was set at 35 °C and the injection volume was 10 μL. Calibration