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Facilitated nanoscale delivery of insulin across intestinal membrane models

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ABSTRACT

The effect of nanoparticulate delivery system on enhancing insulin permeation through intestinal membrane was evaluated in different intestinal epithelial models using cell cultures and excised intestinal tissues. Multilayered nanoparticles were formulated by encapsulating insulin within a core consisting of alginate and dextran sulfate nucleating around calcium and binding to poloxamer, stabilized by chitosan, and subsequently coated with albumin. Insulin permeation through Caco-2 cell monolayer was enhanced 2.1-fold, facilitated by the nanoparticles compared with insulin alone, 3.7-fold through a mucus-secreting Caco-2/HT29 co-culture, and 3.9-fold through excised intestinal mucosa of Wistar rats. Correlation of Caco-2/HT29 co-culture cells with the animal-model intestinal membrane demonstrates that the mucus layer plays a significant role in determining the effectiveness of oral nanoformulations in delivering poorly absorbed drugs. Albumin was applied to the nanoparticles as outermost coat to protect insulin through shielding from proteolytic degradation. The effect of the albumin layering on insulin permeation was compared with albumin-free nanoparticles that mimic the result of albumin being enzymatically removed during gastric and intestinal transport. Results showed that albumin layering is important toward improving insulin transport across the intestinal membrane, possibly by stabilizing insulin in the intestinal conditions. Transcellular permeation was evidenced by internalization of independently labeled insulin and nanoparticles into enterocytes, in which insulin appeared to remain associated with the nanoparticles. Transcellular transport of insulin through rat intestinal mucosa may represent the predominant mechanism by which nanoparticles facilitate insulin permeation. Nanoformulations demonstrated biocompatibility with rat intestinal mucosa through determination of cell viability via monitoring of mitochondrial dehydrogenases. Insulin permeation facilitated by the biocompatible nanoparticles suggests a potential carrier system in delivering protein-based drugs by the oral route.

1. Introduction

Therapeutic proteins and peptide drugs are of interest in the treatment of several metabolic diseases (Peppas and Carr, 2009). Oral administration is of particular interest as for example, oral insulin is considered to provide greater blood glucose control in type 1 diabetes mellitus than subcutaneous insulin (Raj and Sharma, 2003; Damgé et al., 2007). However, oral delivery is limited by proteolytic degradation of insulin if not shielded, and by poor absorption through the intestinal mucosa if not chemically or physically facilitated (Hamman et al., 2005; Morishita and Peppas, 2006). Strategies for clinical delivery of insulin by the oral route include administration with absorption enhancers or enzyme inhibitors (Mesiha and Sidhom, 1995; Radwan and Aboul-Enein, 2002), chemical modification (Asada et al., 1995) and design of delivery systems (Marschutz and Bernkop-Schnurch, 2000). Nanoparticulate delivery systems are designed to carry and deliver insulin to the site of absorption and facilitate permeation through the intestinal membrane to achieve a pharmacological effect (Thanou et al., 2001).

In the present study, multicomponent nanoparticles encapsulating insulin were prepared by ionotropic gelation of dilute alginate and dextran sulfate through the addition of calcium ions, forming a stable colloidal dispersion by the presence of poloxamer and chitosan, and subsequent coating with albumin. The pH-sensitive alginate network forms an impermeable structure in acidic gastric conditions (Kamiya and Klibanov, 2003; George

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and Abraham, 2006), preventing premature insulin release in combination with dextran sulfate, increasing insulin protection in combination with albumin coating applied as a sacrificial target to gastrointestinal proteases (Reis et al., 2007). Alginate and chitosan, as mucoadhesive polymers are designed to increase the residence time of nanoencapsulated insulin at the site of absorption, facilitating the uptake, permeation and internalization of insulin by enterocytes (George and Abraham, 2006; Chickering and Mathiowitz, 1995; Sarmento et al., 2007). In addition, chitosan transiently opens intercellular tight junctions to improve the paracellular transport (Artursson et al., 1994; Bernkop-Schnurch, 2000).

In vitro permeability studies using cell culture preparations or excised tissue have been used to evaluate the potential effect of delivery systems on enhancing drug permeation. In addition, *in vitro* intestinal models enable the determination of an anatomical site as a route for drug delivery by isolation of the interaction of nanoencapsulated drug with the intestinal membrane independently of other factors such as transit time. Cells from human colon adenocarcinoma (Caco-2 cell monolayers) have been used as an intestinal model to evaluate the effect of delivery systems on drug permeation via transcellular and paracellular transport (Artursson and Karlsson, 1991; Artursson et al., 2001; Shah et al., 2006). Caco-2 cell monolayers are similar to the small intestinal epithelial layer by differentiation into columnar absorptive cells, including tight junctions, brush border, enzymes and carrier-mediated transport systems (Delie and Rubas, 1997; Hilgendorf et al., 2000; Behrens and Kissel, 2003). However, goblet, mucus-secreting and M-cells are absent, thus HT29 subclones have been used in co-culture with Caco-2 cells to mimic the small intestinal epithelial layer by containing both mucus and the columnar absorptive cells (Walter et al., 1996; Lesuffleur et al., 1990; Karlsson et al., 1993; Wikman-Larhed and Artursson, 1995). Tight junctions of cellular models are similar to that presented in the colon, and have been shown to underestimate drug permeability via paracellular transport (Walter et al., 1996; Ichikawa and Peppas, 2003; Schilling and Mitra, 1990). Experiments performed with cell monolayers provide results with lower variability than animal tissues, however cell monolayers lack three-dimensional macrostructure and lack cells of varying degrees of differentiation, as are found in animal tissues (Grass, 1997). Therefore, excised rat intestinal mucosa mounted in Ussing chambers have also been used as an intestinal model to evaluate the permeation enhancing effect of delivery systems, presenting mucus and epithelial layers, and the enzymatic barrier of the human small intestinal mucosa (Aoki et al., 2005; Bravo-Osuna et al., 2007; Ungell et al., 1998).

The aim of this study was to evaluate the effect of multilayered nanoparticles on enhancing insulin permeation through the intestinal membrane, comprising the effect of albumin layer and the poloxamer additive, and evaluating the use of Caco-2 and Caco-2/HT29 cell monolayers, and rat intestinal mucosa as intestinal membrane models. Biocompatibility of nanoparticles was evaluated via cell viability measurements, and internalization of nanoencapsulated insulin was determined in rat small intestinal tissue through confocal microscopy using insulin and nanoparticles, which were independently labeled with fluorescent probes.

2. Materials and methods

2.1. Materials

Alginic acid sodium salt, low molecular weight chitosan, bovine serum albumin (BSA), streptozotocin (STZ), fluorescein isothiocyanate-insulin (FITC-insulin) labeled from bovine pancreas, rhodamine B isothiocyanate (RBITC), FITC-dextran 40 (FD40), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), N-(2-hydroxyethyl)piperazine-N 0-(2-ethane-sulfonic acid) (HEPES) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma–Aldrich Chemie (France). Dextran sulfate sodium salt was purchased from Fluka (Switzerland). Polyvinylpyrrolidone K 30 and poloxamer 188 (Lutrol F68) were kindly supplied by BASF (Germany). Calcium chloride was purchased from Riedel-de-Haën (Germany). Lactic acid was purchased from VWR BDH Prolabo (France). Actrapid® INN-insulin human (rDNA) (Novo nordisk A/S, Denmark) was kindly supplied by Hospitais da Universidade de Coimbra. All other chemicals used were of highest grade commercially available. The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC37-HTB) and used between passage number 53 and 83. The HT29 cell line was obtained from the American Type Culture Collection (ATCC HTB-38) and used between passage number 12 and 25. Small intestinal tissues of Wistar rats for permeation studies were provided by the Center for Pharmaceutical Studies at University of Coimbra. Penicillin, streptomycin, non-essential amino acids, fetal bovine serum, trypsin-EDTA, L-glutamine, and Hank's buffered salt solution (HBSS) were purchase from Invitrogen (Spain). SnakeSkin Pleated Dialysis Tubing 10 K MWCO was purchased from Thermo Fisher Scientific Inc., USA, and SpectralPor molecular porous membrane tubing 8000–10,000 MWCO was purchased from Spectrum Laboratories Inc., USA. Epithelial voltohmmeter EVOM was purchased from World Precision Instruments, USA.

2.2. Methods

2.2.1. Nanoparticle preparation

Nanoparticles with mean diameter of 350–450 nm and polydisperse size distribution were prepared by ionotropic gelation and polyelectrolyte complexation as previously described (Woitiski et al., 2009a). Nanoparticles were prepared by dropwise addition of 7.5 mL of 0.20% calcium chloride in 117.5 mL of pH 4.9 0.06% (w/v) alginic sodium salt, 0.04% (w/v) dextran sulfate, 0.04% (w/v) poloxamer 188 and 0.006% (w/v) insulin solution under stirring. Further stabilization was obtained by dropwise addition of 25 mL of 0.04% chitosan dissolved in 0.04% lactic acid at pH 4.6. Finally, nanoparticles were coated by dropwise addition of 25 mL of 0.46% bovine serum albumin solution at pH 5.1.

Albumin-free nanoparticles were formulated by the same method, as were nanoparticles containing FITC-insulin and RBITC-alginate. Multilayered nanoparticles were concentrated by dialysis using regenerated cellulose membrane tubing with 10 K MWCO in 10% polyvinylpyrrolidone K 30 as dialysis medium for 48 h at 4 °C.

2.2.2. Conjugation of insulin with FITC, and alginate with RBITC

FITC was covalently bound to insulin as previously described (Clausen and Bernkop-Schnurch, 2000). Briefly, 0.2% FITC in dimethylsulfoxide was gradually added in volumes of 25 μL to 40 mg of insulin dissolved in 20 mL of 0.1 M Na_2CO_3. NH_4Cl was added to a final concentration of 50 mM after the solution was incubated for 8 h at 4 °C, then incubated for another 2 h at 4 °C before dialysis and lyophilization. RBITC was attached to alginate as previously described (Strand et al., 2003). Briefly, 25 μL of 0.1% RBITC dissolved in dimethylsufoxide were added to 2% alginate solution at pH of 8.0, and incubated at 40 °C during 1 h, followed by the addition of NH_4Cl to stop the reaction. Alginate and insulin solutions were dialyzed separately against distilled water for 72 h to separate unbound label. The dialysis media were changed every 12 h.

2.2.3. Scanning electron microscopy (SEM)

Multilayered nanoparticle suspension was directly deposited on a polished aluminum sampler holder, dried under vacuum,