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Intestinal mucosa permeability following oral insulin delivery using core shell corona nanolipoparticles

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ABSTRACT

Chitosan nanoparticles (NC) have excellent capacity for protein entrapment, favorable epithelial permeability, and are regarded as promising nanocarriers for oral protein delivery. Herein, we designed and evaluated a class of core shell corona nanolipoparticles (CSC) to further improve the absorption through enhanced intestinal mucus penetration. CSC contains chitosan nanoparticles as a core component and pluronic F127-lipid vesicles as a shell with hydrophilic chain and polyethylene oxide PEO as a corona. These particles were developed by hydration of a dry pluronic F127-lipid film with NC suspensions followed by extrusion. Insulin nested inside CSC was well protected from enzymatic degradation. Compared with NC, CSC exhibited significantly higher efficiency of mucosal penetration and, consequently, higher cellular internalization of insulin in mucus secreting E12 cells. The cellular level of insulin after CSC treatment was 36-fold higher compared to treatment with free insulin, and 10-fold higher compared to NC. CSC significantly facilitated the permeation of insulin across the ileum epithelia, as demonstrated in an *ex vivo* study and an *in vivo* absorption study. CSC pharmacological studies in diabetic rats showed that the hypoglycemic effects of orally administrated CSC were 2.5-fold higher compared to NC. In conclusion, CSC is a promising oral protein delivery system to enhance the stability, intestinal mucosal permeability, and oral absorption of insulin.

1. Introduction

Despite rapid progress made in the development of modern drug delivery technologies, an efficient oral delivery of therapeutic proteins and peptides remains to be achieved. Oral delivery of protein drugs faces several barriers, including pre-systemic degradation, limited mucosal diffusion, and poor intestinal epithelial membrane permeability [1–3]. A variety of innovative approaches have been developed to tackle these challenges, including the use of small molecule permeation enhancers, enzyme inhibitors, and the encapsulation of protein drugs into microspheres or nanoparticles [4,5]. The development of nanoparticles with biodegradable and non-toxic polymers has become one of the major focal areas in this field due to their ability to protect proteins from degradation in harsh pH environment, by enzymes in the gastrointestinal (GI) tract [6–9], and their ability to modulate physicochemical characteristics, drug release, and biological behavior [10,11].

Among those polymeric nanoparticles, chitosan and chitosan derivative based nanocarriers (NCs), appear to be particularly promising [12]. They can significantly enhance the oral absorption of peptides [13] and have excellent capacity for protein entrapment and low toxicity [14]. They enhance the oral absorption of proteins by several mechanisms, such as bioadhesion with the negative charged cell membrane, transient widening of tight junctions and increasing cell permeability by affecting paracellular and intracellular pathways without changing junctional morphology [15]. However, they cannot effectively transport therapeutic proteins across the mucus barrier due to the electrostatic interaction between the anionic mucus gel and cationic nanoparticles [16]. It also has been reported that the binding of NCs to the surfaces of epithelial cells and subsequent absorption-enhancing effects are significantly reduced by mucus [16–18]. Although the adhesion of

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NCs to mucus can slow particle transit time through the GI tract, and thus enhance drug absorption, the absorption enhancement is limited by mucus turnover [19].

Recent studies have revealed that nanoparticles coated with hydrophilic polymers exhibit decreased adhesion to mucus components [19]. Our previous work also shows that Pluronic F127 modified lipid vesicles have superior mucus penetration, compared to unmodified lipid vesicles [20]. To further improve the mucus penetration of chitosan nanoparticles, we attempted to design a core shell corona nanolipoparticles (CSC), using chitosan nanoparticles as a core component and F127-lipid vesicles as a shell with polyethylene oxide PEO chains as the corona. By enveloping the F127-lipid shell, the positively charged chitosan nanoparticles (NC) would be shielded, ensuring free diffusion of NC in mucus. Additionally, protein nested in the CSC core would be better protected from enzymatic degradation, and therefore the efficacy of drug delivery could be further enhanced. To the best of our knowledge, the CSC particles, which were designed to feature advantages of both NC and Pluronic F127-lipid vesicles, are the newly designed nano-size lipoparticles for oral protein delivery with significantly enhanced mucus penetration.

In the present study, insulin was chosen as a model biomolecule for testing the CSC system. Insulin has currently attracted great interest in developing protein delivery approach, due to its wide clinical use, but limited routes of administration. Herein, CSC was prepared by hydration of a polymer-lipid film with NC suspension to form core–shell structure and they were then characterized in terms of particle size, zeta potential, and morphology. Their ability to protect the encapsulated insulin from enzymatic degradation, mucus penetration property, and cell uptake efficiency and permeability were evaluated in HT29-MTX-E12 (E12) cells. Pharmacological and pharmacokinetic studies were conducted in streptozotocin (STZ) induced diabetic Sprague-Dawley rats.

2. Materials and methods

2.1. Materials

Protasan UP CL113 was purchased from Nova Matrix (Drammen, Norway). Egg phosphatidylcholine (EPC) was purchased from Q.P. Corp (Tokyo, Japan). Pluronic F127 was donated by BASF (Ludwigshafen, Germany). RPMI1640 medium, Alexa Fluor-555-labeled wheat germ agglutinin and 0.25% trypsin-0.53 mmol/L EDTA were purchased from Invitrogen (Ontario, Canada). Paraformaldehyde was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). All the other reagents were of analytical grade. Alexa 488 labeled insulin was synthesized by Novo Nordisk A/S (Copenhagen, Denmark). HT29-MTX-E12 (E12) cells (52nd–56th passages) cultured for 14–18 days were supplied by the ADME Department of Novo Nordisk A/S. Caco-2 cell lines were obtained from the American Type culture collection (ATCC, Manassas, VA, USA). Cells from the 40th –43rd passages were used in the present study.

2.2. Preparation and characterization of nanocarriers

2.2.1. Preparation and characterization of chitosan nanoparticles

Chitosan nanoparticles (NC) were prepared using a previously reported procedure [21], based on the ionotropic gelation of chitosan with sodium tripolyphosphate (TPP) where the positively charged amino groups of chitosan interact with the negative charged TPP. Briefly, TPP (1.0 mg/mL) was added to chitosan solution (1.0 mg/mL) under magnetic stirring at room temperature to produce NC at a final chitosan/TPP weight ratio of 6:1. The insulin-loaded NC were obtained by mixing the insulin solution in 0.01 M NaOH (3.75 mg/mL) with NC solution at a theoretical content of 30% (w/w) of insulin. Self-assembled NC were collected and washed thrice with distilled water by centrifugation at 16,000 g on a 10-μL glycerol bed for 30 min. The centrifuged NC were then re-dispersed in distilled water and stored at 4 °C until use.

The association efficiency and loading capacity of insulin in NC were determined by subtracting the amounts of free insulin in supernatants quantified using high performance liquid chromatography (HPLC) from the amounts added, using the following equation:

$$\text{Association efficiency} = \frac{\text{total amount of insulin added} - \text{free insulin}}{\text{total amount of insulin added}} \quad (1)$$

$$\text{Loading capacity} = \frac{\text{total amount of insulin added} - \text{free insulin}}{\text{weight of nanoparticles}} \quad (2)$$

2.2.2. Preparation of core shell nanolipoparticles

The homogeneous F127-lipid film was prepared by drying a chloroform solution containing egg phosphatidylcholine with F127 (4:1, mol/mol) and then hydrating the film with NC suspensions (lipids/NP = 6:1, w/w) for 30 min at room temperature, followed by 6 rounds of extrusion through a polycarbonate membrane with 200-nm pores (Avestin Inc., Canada). As a control, core shell nanolipoparticles (CS) without hydrophilic corona was prepared by encapsulating NC in a pure lipid vesicle without F127.

2.2.3. Characterization of nanocarriers

The mean particle sizes and zeta potential values of different nanocarriers were measured using a Malvern Zetasizer NanoZS (Malvern Instruments, London, U.K.). Each measurement was made in triplicate. A transmission electron microscope (TEM, CM-200, Philips, Netherlands) was used to observe their morphology. The samples were stained with an aqueous solution of phosphotungstic acid (1%, w/v) before observation and the images were taken at 160 kV. The stability of these nanocarriers in stimulated gastric fluid (SGF) and stimulated intestinal fluid (SIF) were tested by measuring the changes in the particle size and polydispersity index (PDI) after a 2-h incubation.

2.3. In vitro enzymatic degradation assay

The stability of nanocarriers against enzymatic degradation was carried out using a procedure described previously [22]. In brief, trypsin (2500 IU/mg) and chymotrypsin (800 IU/mg) were dissolved in Tris buffer (pH = 8.0) containing 1 mM $CaCl_2$ at final concentrations of 250 IU/mL and 50 IU/mL, respectively. Each enzyme solution (50 μL) was separately incubated with 950 μL of test formulations (NC and CSC) containing 5 IU/mL of insulin. These formulations were pre-incubated at 37 °C for 15 min. 50 μL of each sample was taken at pre-determined intervals and the enzyme activity was terminated by addition of 50 μL ice cold acetonitrile solution containing 0.1% trifluoroacetic acid. Samples were subsequently treated with Triton X-100 to remove the CSC and CS lipid shell to release insulin. The samples were then analyzed using HPLC to determine the amount of insulin. Plain insulin solution and insulin-loaded NC suspensions were used as control and treated samples under the same experimental conditions.

2.4. Cell-based assays

2.4.1. Cytotoxicity of nanocarriers

Cytotoxicity of various nanocarrier suspensions in Caco-2 cells was evaluated using the MTT assay. Briefly, Caco-2 cells were seeded onto 96-well plates at a density of 1.0×10^4 cells per well. After a 48-h culture, nanocarrier suspensions were added to the culture media. After a 2-h incubation at 37 °C, the suspensions were replaced by 100 μL of MTT solution (0.5 mg/mL in HBSS, pH = 7.4) and incubated for an additional 3 h at 37 °C. Subsequently, the MTT medium was removed and 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance of the resultant solutions was measured at 490 nm using a microplate reader (Bio-Rad, USA). Each sample was analyzed in five replicates.

2.4.2. Transport study in E12 cell monolayers

The transport study was conducted following a previously reported procedure [23]. The E12 cell monolayers were washed thrice with pre-warmed 1× Dulbecco's phosphate buffered saline (PBS, pH 7.4) and equilibrated in the 1× HBSS (with Ca^{2+} and Mg^{2+}, 25 mM D-glucose) at 37 °C and 95% relative humidity for 30 min. Alexa 488 insulin stock solution (0.5 mg/mL) and the Alexa 488 insulin loaded nanocarrier suspensions were diluted with PBS. On the day of the experiment, the donor (apical) solutions were prepared by diluting an aliquot of these solutions into HBSS to make a final Alexa 488 insulin concentration of 40 μg/mL. For all the transport experiments, a total of 200 μL of each acceptor (basolateral) sample was removed at 0, 15, 30, 45, 60, or 90 min after drug administration. For each acceptor sample taken, 200 μL of fresh HBSS was added to the mixture to maintain a constant volume. The samples were transferred onto a 96-well titer plate and Alexa 488 insulin was detected using a microplate fluorometer (Model 680, Bio-Rad, USA) at an excitation filter of 485 nm and an emission filter of 530 nm. To eliminate the effects of mucus on the transport of free Alexa 488 insulin, the E12 cells were incubated with 10 mM N-acetyl cysteine (NAC) in HBSS for 30 min to remove the mucus prior to the transport study. Apparent permeability coefficient (P_{app}) and enhancement ratio (R) were calculated using the following equations.

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \quad (3)$$