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Goblet cell targeting nanoparticle containing drug-loaded micelle cores for oral delivery of insulin

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ABSTRACT

Oral administration of insulin remains a challenge due to its poor enzymatic stability and inefficient permeation across epithelium. We herein developed a novel self-assembled polyelectrolyte complex nanoparticles by coating insulin-loaded dodecylamine-graft-γ-polyglutamic acid micelles with trimethyl chitosan (TMC). The TMC material was also conjugated with a goblet cell-targeting peptide to enhance the affinity of nanoparticles with epithelium. The developed nanoparticle possessed significantly enhanced colloid stability, drug protection ability and ameliorated drug release profile compared with graft copolymer micelles or ionic crosslinked TMC nanoparticles. For in vitro evaluation, Caco-2/HT29-MTX-E12 cell co-cultures, which composed of not only enterocyte-like cells but also mucus-secreting cells and secreted mucus layer, were applied to mimic the epithelium. Intracellular uptake and transcellular permeation of encapsulated drug were greatly enhanced for NPs as compared with free insulin or micelles. Goblet cell-targeting modification further increased the affinity of NPs with epithelium with changed cellular internalization mechanism. The influence of mucus on the cell uptake was also investigated. Ex vivo performed with rat mucosal tissue demonstrated that the nanoparticle could facilitate the permeation of encapsulated insulin across the intestinal epithelium. In vivo study preformed on diabetic rats showed that the orally administered nanoparticles elicited a prolonged hypoglycemic response with relative bioavailability of 7.05%.

1. Introduction

Recent advances in biotechnology and genetic engineering have made the mass production of therapeutic biomacromolecules feasible, such as protein and peptide drugs. Oral administration is undoubtedly the most preferred route for drugs that needs frequent dosing in terms of safety and patient compliance issue. However, oral delivery of biomacromolecules remains a challenge due to their inherent low permeability though the intestinal epithelium and poor enzymatic stability (Frokjaer and Otzen, 2005; Gupta et al., 2013). Thus, novel drug delivery system, which could protect the biomolecules from the harsh environment in gastrointestinal tract and enhance their permeability into the systemic circulation, is desirable for their oral application. Nanocarriers are considered to be promising vehicles for oral delivery of biomolecular therapeutics by enhancing the stability and mucosal permeation of the encapsulated drug (Chen et al., 2011).

Self-assembled micelles formed by amphiphilic graft copolymers were increasingly studied for biomolecules delivery due to their unique properties (Francis et al., 2003; Zhang et al., 2013). Small nanostructures could be easily formed in aqueous solution with a mild preparation process, which is desired for encapsulation of biomolecules. The hydrophobic core can serve as a drug reservoir, which enables sustained release, while the hydrophilic region of the polymer stabilizes the nanostructure in the aqueous environment. However, the graft copolymer micelles possess the drawback of rapid dissociation under diluted conditions in the body fluids, which limit their effectiveness (Ohya et al., 2010; Qin et al., 2002). Moreover, unless modified with other ligands, most of the amphiphilic graft copolymers lack of the specific affinity with the mucosal tissue, and therefore hardly increase the bioavailability of encapsulated drug after oral administration.

Nanoparticles (NPs) based on *N*-trimethylatedchitosan (TMC) have been reportedly to increase the oral bioavailability of insulin due to their ability to cause reversible opening the tight junctions (TJs) (Sadeghi et al., 2008). The chitosan or TMC based NPs could be

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easily formed by via mild ion-crosslinking self-assembly strategy with satisfactory encapsulation efficiency of insulin (Yun et al., 2013). However, these NPs possessed inherent defects of poor stability and premature drug release, especially in the environment with high ionic strength (Lin et al., 2008; Zhang et al., 2014). Due to the complicated contents in the digestive intestinal fluid, this fact limited the efficiency of chitosan-based NPs for its in vivo application.

Recently, epithelium-targeting was proposed to be an efficient way to further enhance the oral absorption of the NPs (Yun et al., 2013). The decoration of the particles with specific ligand, especially peptide ligands, is expected to lead to greater binding with the epithelium and subsequent uptake. CSKSSDYQC (CSK) peptide has been identified to specifically recognize goblet cells, which is the second large cell population on epithelium (Kang et al., 2008). Our group recently reported a TMC-based epithelium-targeting NP by conjugating TMC with CSK peptide (Jin et al., 2012). The CSK modified TMC NPs produced a 1.5-fold higher relative bioavailability compared to unmodified ones in diabetic rats. Moreover, our previous studies demonstrated that the targeting recognition was partially affected by the existence of mucus, which may due to the disintegration of the NP within the mucus (Jin et al., 2012).

In the present study, we developed a novel self-assembled polyelectrolyte complex NPs with micelles cores for insulin delivery, which combined the strengths of both graft copolymer micelles and epithelium-targeting TMC based NPs. Amphiphilic dodecylamine-graft-γ-polyglutamic acid (PGA-*g*-DA) copolymers were synthesized and formed into insulin-loaded micelles, and then coated with TMC materials via electrostatic interaction. Moreover, TMC material was further modified with goblet cell-targeting CSK peptide to further enhance the delivery efficiency. With the introducing of drug-loaded micelles, improved stability and ameliorated drug release profile were expected. For in vitro evaluation of intracellular uptake and transepithelial transport, Caco-2/HT29-MTX-E12 co-cultured cell model were used, which consist of both absorptive enterocyte-like Caco-2 cells and the mucus-producing goblet cell-like HT29-MTX-E12 cells. This model also enables the evaluation the behavior of NP in the mucus layer. Internalization mechanism and the opening of tight junctions mediated by the NPs were also investigated. Finally, orally administered NPs elicited a hypoglycemic response and increase of serum insulin level in diabetic rats.

2. Materials and methods

2.1. Materials

γ-Glutamic acid (γ-PGA, Mw = 3,840,000) (Xiamen, China) was purchased from Baierte Co., Ltd. Dodecylamine (DA) was purchased from Best Chemistry Co., Ltd. (Chengdu, China). Chitosan (deacetylation degree > 90% and molecular weight of 400 kDa) was provided by AK Biotech Co., Ltd. (Shandong, China). Porcine insulin (30 IU/mg) was purchased from Wanbang Bio-Chemical Co., Ltd. (Jiangsu, China). CSKSSDYQC peptide was chemically synthesized by Kaijie Bio-pharmaceuticals Co., Ltd. (Sichuan, China). Fluorescein isothiocyanate (FITC) and 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). *N*-acetyl-L-Cysteine was obtained from Aladdin Chemistry Co., Ltd. (Shanghai, China). 1-[3-(Dimethylamino) propyl]-3-ethylaarbodiimide hydrochloride (EDC·HCl) was gained from Meapeo Co., Ltd. (Shanghai, China). *N*-Hydroxysuccinimide(NHS), *N*-methylpyrrolidone, iodomethane and acetonitrile were all obtained from Kelong chemical Co., Ltd (Chengdu, China). Other agents were all analysis grade.

Caco-2 cells were gained from institute of Biochemistry and Cell Biology (Shanghai, China). HT29-MTX-E12 cell line was a kind gift from Dr. David Brayden (University of Dublin, Ireland). Male Sprague–Dawley rats weighing 220 ± 20 g were supplied by Experimental Animal Center of Sichuan University (protocol number for animal study: CSDGZ-10). The rats were housed at a room temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%.

2.2. Polymer synthesis

The amphiphilic graft copolymer (PGA-*g*-DA) was synthesized via the reaction of carboxyl groups of γ-PGA with amine groups of DA in the presence of EDC·HCl (Shima et al., 2014). Briefly, γ-PGA (4 mmol) was dissolved in a mixture solution of water (4 mL) and DMSO (8 mL), then EDC·HCl (6mmol) was added under stirring to active the acid moieties of γ-PGA for 1 h. DA (6mmol) was subsequently added, and the reaction was allowed to continue for 24 h at 40 °C. The obtained product was dialyzed (MWCO: 8000–14,000) against 50% ethanol solution for 7 d, and then transferred to deionized water for another 2 d. Finally, the dialyzed solution was subsequently lyophilized and analyzed by ^{1}H NMR (UNITY INOVA-400, Varian Inc., USA). The critical micelle concentration (CMC) of synthesized PGA-*g*-DA in aqueous medium was tested by fluorescence spectroscopy using pyrene as a probe (Duhem et al., 2012). A fluorometer (RF-5301 PC, Shimadzu Co., Japan) was used to record the fluorescence emission spectrum of pyrene. The excitation wavelength was set at 339 nm, while the slit was 5.0 nm. The intensity of emission was monitored at a wavelength range of 350–500 nm. The ratio of the intensities (I_1/I_3) of the first peak (I_1, 373 nm) to the third peak (I_3, 384 nm) was calculated.

TMC were synthesized as previously described (Jin et al., 2012). Briefly, TMC was obtained by methylation of amine groups of CS with methyl iodide (CHI_3) in a strong base solution (sodium hydroxide, NaOH) using *N*-methylpyrrolidone (NMP) as solvents. The reaction proceeded for 2 h at 60 °C. The product was purified by dialysis and then lyophilized (Free Zone 2.5 L, LABCONCO Inc., USA). The degree of quaternization was calculated from the integration of ^{1}H NMR. The obtained TMC was conjugated with CSK peptide via amide bond formed among the residual primary amino groups on TMC and carboxyl groups on CSK peptide. TMC (0.27 mmol), EDC·HCl (0.45 mmol) and NHS (0.45 mmol) were dissolved in 10 mL of water filled with nitrogen. Then CSK peptide (0.045 mmol) was added in the solution. The resultant mixture was left to react for 3 d at room temperature in dark followed by dialysis and lyophilization. The obtained TMC-CSK was identified by ^{1}H NMR.

2.3. Preparation and characterization of insulin-loaded NPs

2.3.1. Preparation of insulin-loaded NPs

The insulin loaded NP with was prepared in a two-step way. Firstly, insulin-loaded PGA-*g*-DA micelles were prepared using dialysis strategy. Briefly, PGA-*g*-DA (14 mg) was dissolved in a mixture solution of DMSO (1.5 mL) and phosphate buffer (1 mL, pH 7.4, 0.02 M). Insulin was dissolved in hydrochloric acid solution (HCl, pH 2.0), and then added drop-wisely in the pre-mixed solution. Thereafter, the solution was kept stirring for 30 min at room temperature, and then dialyzed (MWCO: 8000–14,000) against water for 24 h at 4 °C to obtain micelles. Subsequently, the prepared micelle core was coated with TMC or TMC-CSK using ionic crosslinking method. Briefly, the micelles solution was added drop-wisely to the TMC or TMC-CSK aqueous solution. Then, the mixture was added with tripolyphosphate (TPP) and magnesium sulphate ($MgSO_4$), and was keep stirred for 30 min. The NPs prepared with TMC or CSK-TMC was termed as T-NPs or CSK-NPs, respectively.