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A cell-penetrating peptide mediated chitosan nanocarriers for improving intestinal insulin delivery

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ABSTRACT

To overcome barriers for oral delivery of insulin, the chitosan(CS)-based nanocarriers with a novel cell penetrating peptide (SAR6EW) have been prepared and evaluated in this study. Characterization measurements showed that SAR6EW/CS/insulin-NPs displayed global particles with smooth surfaces and an average diameter about 150 nm. The entrapment efficiency and loading rates of insulin were 75.36% and 7.58%, respectively. Insulin could be released constantly from SAR6EW/CS/insulin-NPs *in vitro*. Furthermore, SAR6EW/CS/insulin-NPs could facilitate the uptake of insulin and induce a significantly higher internalization of insulin via adding clathrin and caveolae mediated endocytosis. In addition, *in vivo* hypoglycemic studies showed that orally administrated SAR6EW/CS/insulin-NPs produced a better hypoglycemic effect as compared with CS/insulin-NPs in diabetic rats. Meanwhile, no significant cytotoxicity of the nanoparticles was observed. In conclusion, SAR6EW-mediated chitosan nanocarriers showed sufficient effectiveness for oral delivery of insulin. This delivery system is also promising for the delivery of other protein drugs by oral administration.

1. Introduction

An oral formulation of insulin would be a beneficial alternative to injections for diabetic patients because it would alleviate pain, mimic the physiological fate of insulin, and may provide a better glucose homeostasis (Khan, 2003; Babiker & Datta, 2011). Ramineni, Cunningham, Dziubla, & Puleo (2013) reported that oral administration could simulate the physiological hypoglycemia mechanism by directly delivering insulin into the liver after absorption. Thus far, oral delivery is acknowledged as the most convenient insulin delivery method due being noninvasive and patient-friendly properties. Nevertheless, there are two main limitations using the oral route for insulin delivery. One is degradation by gastrointestinal proteolytic enzymes, and the other is poor mucosal permeability due to its high molecular weight and lack of lipophilicity (Wong, Martinez, & Dass, 2016). Therefore, it is very important to overcome these barriers by developing an efficient oral insulin delivery system.

Cell-penetrating peptides (CPPs) have attracted increasing attentions as a promising vehicle for the systemic delivery of biomolecules across intestinal mucosa (Tashima, 2016). CPPs can facilitate the transduction of macromolecules such as siRNA (Tai & Gao, 2016), proteins (Bolhassani, Jafarzade, & Mardini, 2016) and liposomes (Torchilin, Rammohan, Weissig, & Levchenko, 2001) into cells. Some researchers have reported that CPPs can enhance intestinal insulin absorption simply by co-administration as a physical mixture without intermolecular cross-linking (Kamei, Morishita, & Takayama, 2009; Khafagy, Morishita, Isowa, Imai, & Takayama, 2009). The noncovalent electrostatic interaction exhibits the same permeation efficiency as a covalent chemical conjugation. Among some types of CPPs, R8, arginine-rich peptides, can significantly enhance intestinal insulin absorption without negative effects on the intestinal mucosa (Morishita, Kamei, Ehara, Isowa, & Takayama, 2007; Liu et al., 2013). To improve CPPs cargo delivery efficiency, structure-activity studies must be performed. In our previous study, we chemically modified R8 and substituted different amino acids of R8 to obtain the amphiphilic lipopeptide (SAR6EW). The chemical structure of SAR6EW was showed in Scheme 2. These modifications increased the permeation of insulin both *in vitro* and *in vivo* (Zhang, Li, Han, Hu, & Zhang, 2015).

Nanoparticles are a promising approach for oral delivery of proteins due to small size range, sustained release and ability to enhance drug stability and absorption. Chitosan (CS) is a cationic polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). CS is a positively charged biocompatible, non-

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toxic, and mucoadhesive polymer. CS is being actively investigated for the preparation of particulate drug delivery systems (Makhlofa, Tozuka, & Takeuchi, 2011; Wang et al., 2016; Li et al., 2017). For example, it contributed to oral absorption of drug by providing electrostatic interaction with negatively charged mucin. CS nanoparticles can be synthesized by a variety of methods. In contrast to chemical cross-linking, CS NPs can be produced spontaneously by ionic interaction with negatively charged polyanions followed by inter- and intra-molecular cross-linking (Lin et al., 2007; Amidi, Mastrobattista, Jiskoot, & Hennink, 2010). This method avoids possible cytotoxicity and hazards of protein instability induced by chemical cross-linking agents.

The aim of this study is to prepare chitosan nanoparticles, carrying the cell penetrating peptide, SAR6EW, to facilitate the permeation of insulin. We made the nanoparticles using the ionic gelation technique. Firstly, we verified that nanoparticles were successfully obtained through SEM and FTIR. Next, the ability of nanoparticles to enhance the intestinal bioavailability of insulin was evaluated *in vitro* in a Caco-2 cell monolayer and in *vivo* in diabetic rats. Finally, the toxicity of the nanoparticles was investigated.

2. Materials and methods

2.1. Materials

Insulin (28 IU/mg) was purchased from Xuzhou Wanbang Biochemical Pharmaceutical Co., Ltd. (Xuzhou, China). SAR6EW was purchased from Shanghai Gil Chemical Co., Ltd. (Shanghai, China). Chitosan (CS, 200 kDa, deacetylation degree 75–85%, viscosity 5–20 mPa s), and chlorpromazine (CPZ) were obtained from TCI (Tokyo, Japan). 5-(N-ethyl-N-isopropyl) amiloride (EIPA) was acquired from Sigma-Aldrich. Sodium azide was obtained from Beyotime (Nantong, China). M-β-CD was purchased from Shangdong Binzhou Zhiyuan Bio-Technology Co., Ltd (Shangdong, China). All other solvents were of analytical or chromatographic grades and were commercially available.

2.2. Preparation of nanoparticles

Chitosan (200 mg) was dissolved in 20 mL acetic acid solution (1%) under magnetic stirring for 15 h. Insulin (120 mg) was dissolved in 20 mL NaOH (0.01 mol/L) solution. An equivalent volume of insulin was added dropwise by syringe to chitosan under gentle magnetic stirring (500 rpm). The mixture was maintained under gentle stirring at 25 °C for 3 h. For the cross-linking, a TPP (tripolyphosphate) solution (27 mM) was prepared by dissolving the powder in redistilled water. TPP solution (50 mL) was then introduced to the complexing medium and stirred for 0.5 h (300 rpm) to obtain the cross-linked CS/insulin-NPs. After that, SAR6EW (5 mg/100 mL) was added to the CS/insulin-NP solution and stirred for 2 h (600 rpm) to obtain the cross-linked SAR6EW/CS/insulin-NPs.

Freeze-drying was performed for 24 h. After freeze-drying, NPs were dispersed in redistilled water at the target concentration for further analysis. The FITC-insulin loaded nanoparticles were prepared by the same method.

2.3. Characterization of nanoparticles

Characterization of nanoparticles was performed according to Wang et al. (2016) with minor modifications.

Surface Morphology. The surface morphological characteristics of CS/insulin-NPs and SAR6EW/CS/insulin-NPs were examined under a scanning electron microscope (SEM, JEOL JSM-5900, Japan) and the size distribution was analyzed by ImageJ (V1.44, http://rsb.info.nih.gov/ij/). The results were presented using a three-dimensional model.

Zeta Potential. Zeta potential of these nanoparticles were determined with dynamic light scattering (DLS; Malvern Zeta Potential Analyzer, UK). Before measurement, the particles were resuspended into deionized water. All tested nanoparticles were measured in triplicate.

Fourier transform infrared (FTIR) spectra. FTIR spectra of all samples (CS, SAR6EW, insulin, CS/insulin-NPs and SAR6EW/CS/insulin-NPs) were collected on an infrared spectrophotometer (Nexus 670, Nicolet, USA). Each sample was mixed with KBr and pressed into a KBr disk. The spectrum was recorded from 400 to 4000 cm^{-1} at a resolution of 2 cm^{-1}.

2.4. Encapsulation efficiencies (EE) and loading capacity (LC) of insulin

The samples were prepared according to the above methods. The suspension of the insulin-loaded NPs was centrifuged at 13,000 rpm for 20 min at 4 °C (L8–60 M; Beckman, Fullerton, CA). The free insulin in supernatants was filtered through a 0.4-micronsyringe filter and the protein content was estimated by Lowry's method at 595 nm using an ultraviolet spectrophotometer (UV2100, Shimadzu). The EE and LC were calculated using the following formulas:

$$LC(\%) = \frac{\text{total amount of insulin added} - \text{free insulin}}{\text{weight of nanoparticles}} \times 100$$

$$EE(\%) = \frac{\text{total amount of insulin added} - \text{free insulin}}{\text{total amount of insulin added}} \times 100$$

2.5. *Insulin release* in vitro

The insulin release studies were carried out according to Li et al. (2017) with minor modifications. In this study, pH 2.0 and 7.4 phosphate buffered solutions were selected to imitate the environment of gastric juice and intestinal juice, respectively. Phosphate buffered solution (PBS) at the pH 2.0 and 7.4 were prepared. The insulin nanoparticles were suspended in the above buffers (2.5 mg mL^{-1}) containing different concentrations of glucose. Aliquots of 200 μL were taken at 30 min intervals and the released insulin was estimated by means of the Lowry method. An equivalent volume of the fresh buffer was replaced each time after the sampling. The experiments were done in triplicates. The amount of insulin in the test solution was calculated from the insulin standard maintained during the assay.

2.6. Protection against gastric degradation

Insulin stability against trypsin degradation was evaluated in vitro to assess the protective effect of the nanoparticle formulations against gastric degradation. The CS/insulin-NPs and SAR6EW/CS/insulin-NPs were incubated in pH 6.8 buffers with trypsin at 37 °C. At pre-determined time intervals, samples were withdrawn and the enzymatic reaction stopped by adding 50 μL of ice-cold 0.5% TFA. The remaining amount of insulin was assayed with HPLC. All the experiments were performed three times.

2.7. In vitro *cellular studies*

2.7.1. Caco-2 cell culture

Caco-2 cells, derived from a human colorectal carcinoma, share similar morphology and function to intestinal epithelium. Caco-2 cells were grown in an incubator at 37 °C under 5% CO_2. Cells were cultured in T-75 flasks using Modified Eagle's Medium (MEM)