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Preparation of chitosan-based multifunctional nanocarriers overcoming multiple barriers for oral delivery of insulin

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ABSTRACT

To overcome multiple barriers for oral delivery of insulin, the chitosan-based multifunctional nanocarriers modified by L-valine (LV, used as a target ligand to facilitate the absorption of the small intestine) and phenylboronic acid (PBA, used as a glucose-responsive unit) have been designed and evaluated in this study. The resultant nanocarriers exhibited low cytotoxicity against HT-29 cells and excellent stability against protein solution. The insulin release behaviors were evaluated triggered by pH and glucose *in vitro*. The chemical stability of loaded insulin against digestive enzyme were established in presence of simulated gastric fluid (SGF) containing pepsin and simulated intestinal fluid (SIF) containing pancreatin, respectively. The uptake behavior of HT-29 cells was evaluated by confocal laser scanning microscope. After oral administration to the diabetic rats, an effective hypoglycemic effect was obtained compared with subcutaneous injection of insulin. This work suggests that L-valine modified chitosan-based multifunctional nanocarriers may be a promising drug delivery carrier for oral administration of insulin.

1. Introduction

There are 415 million adults were living with diabetes in 2015 and this number is expected to increase to around 642 million or one in ten adults by 2040, according to a report from International Diabetes Federation (IDF) [1]. It has become one of the most lethal diseases in some counties, especially in the developing countries. Insulin is commonly used to treat diabetes [2]. Although the significant developments in insulin therapy over the past few decades, subcutaneous injection of insulin remains the preferred approach for the treatment of insulin-requiring diabetic patients due to ease of administration [3]. However, such injections must pass through the skin or mucosal barrier, resulting in dermal trauma and pain [4]. Thus, focusing on the development new route of administration (oral or pulmonary) or reducing the injection doses are beneficial to reduce the inconvenience and drawbacks associated with this conventional method [5–7]. Oral diabetes drugs, pills you take by mouth, are commonly prescribed to help treat diabetes. Currently, there are 6 groups of oral medicine, including biguanides, sufonylureas, meglitinides, alpha-glucosidase inhibitors, dipeptidyl peptidase 4 inhibitor and thizalidinediones. For example, Glimepiride, common brand name as Amaryl, is a kind of sulfonylureas drug. Nausea and upset stomach may occur after oral treatment. Acarbose (alpha glucosidase inhibitor), common brand name as Percose, is often used to control high blood sugar in people with diabetes. Diarrhea, gas, upset stomach, constipation, or stomach pain may occur in the first few weeks of treatment. At present, suitable oral formulations of proteins are still under development, facing countless challenges despite all the efforts, time and money spent on the research. Major obstructions in developing oral or pulmonary insulin formulations are either enzymatic barriers or physical barriers (*i.e.* intestinal epithelium), which oral insulin has to overcome [8]. Because insulin is a peptide that consisted of 51 amino acids, it can get deteriorated by gastric pH and intestinal enzymes, and even intestinal epithelial cell membranes serve as absorption barrier for intact peptide structure resulting in <1% bioavailabity of total insulin taken orally [9–12].

To overcome these problems mentioned above, various approaches are tried which include micro and nanoparticles [13–15], liposomes [16,17], gastrointestinal patches [18] and permeation enhancers [19,20]. Among them, the use of biocompatible and biodegradable materials has been described as a promising strategy toward oral administration of proteins and peptides [21,22]. Due to favorable nature of chitosan as a positively charged biocompatible, nontoxic, and mucoadhesive

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polymer, many researchers selected chitosan as an oral drug carrier [23–29]. Chitosan is a mucoadhesive polycationic polymer composed of *N*-acetyl-d-glucosamine and d-glucosamine. It, with an intrinsic pK_a of 5.5, will thus lose its charge and spontaneous assembly into nanoparticles in an aqueous solution [30,31]. For example, together with tripolyphosphate (TPP) or even just polyelectrolyte complexation with insulin is the most common methods to produce chitosan nanoparticles. The interaction of chitosan and polyanions leads to a spontaneous formation of nanoparticles in an aqueous media and mild conditions, with no need for using organic solvents or heat, avoiding cytotoxicity concerns and threats to insulin stability, thus being the main advantages of these carriers [32]. Another study conducted by Mukhopadhyay et al. showed an average assimilation efficiency of insulin with self-assembled chitosan nanoparticles of approximately 85% [33]. The nanoparticles retained insulin efficiently in simulated gastric conditions with a significant amount of insulin released in simulated intestinal conditions. These insulin-loaded nanoparticles were effective for dropping the blood glucose level when administrated *in vivo*.

Due to the gradual degradation kinetic profiles of the composite nanoparticles *in vivo*, coupled with hydrophobic interactions between the hydrophobic drug and hydrophobic segment of the polymer matrix, the unfavorably slow drug release may be happen. The successful design and synthesis of stimulus responsive chitosan-based intracellular delivery systems is in high demand to enhance the oral absorption while simultaneously preventing unwanted toxicities. In order to overcome such issue, an alternative glucose sensor moiety can be introduced from a synthetic component, phenylboronic acid as a potential candidate for such an objective, which is due to the fact that the boronic acid binds reversibly to diols to form a cyclic boronic ester in aqueous media [34–38]. Moreover, the boronic acid is a biocompatible functional group with low cytotoxicity and low immunogenicity. In addition, the oral bioavailability of insulin is always very poor because of the following barriers: insulin is susceptible to digestion by proteases in the gastrointestinal (GI) tract; the GI tract is coated with a mucus layer that may prevent macromolecules or drug delivery systems from reaching beneath the epithelial cells [39–41]. Because of the poor cellular uptake of a hydrophilic macromolecule in epithelial cells, insulin can hardly permeate the epithelial cell layer in the intestine. Fortunately, it has been reported that conjugating L-valine increases its transportation in intestinal cells of rats [42]. For instance, L-valine modified chitosan in the nanoparticles greatly contributed to high encapsulation efficiency for protein drug, enhancement of drug absorption, prolonged drug residence in the gut, and favorable enzymatic inhibition [43,44]. The L-valine-conjugated drug enhances oral bioavailability of drug, implicating the great potential of L-valine-conjugated carrier in absorption of drug [45].

The aim of this investigation is to prepare multifunctional nanocarriers, in which chitosan used as nanocarriers backbone, phenylboronic acid (PBA) used as hydrophobic and glucose-sensitive unit, and L-valine (LV) used to facilitate the absorption of the small intestine. They can be easily obtained *via* graft reaction [46] and, thus, spontaneously form nanocarriers in aqueous solution. Then, the novel oral drug delivery system is further developed for overcoming multiple barriers during the insulin delivery in GI tract. The insulin release behaviors are evaluated triggered by pH and glucose *in vitro*. And the hypoglycemic effect against diabetic Sprague Dawley (SD) rat models is evaluated *in vivo*. Herein, using L-valine modified chitosan as nanonarriers for oral delivery of insulin exhibit some advantages. Firstly, chitosan is natural polymeric material. It exhibit higher biocompatible, nontoxic and biodegradable properties compared with synthesized ones. Secondly, although chitosan is a mucoadhesive polycationic polymer, the modification of L-valine to the chitosan is beneficial to prolong their gastrointestinal retention time and promote the uptake of insulin before elimination from the intestinal canal.

2. Experimental

2.1. Preparation of chitosan-based multifunctional nanocarriers

Firstly, carboxymethyl chitosan (CMCS) was prepared according to a similar method described elsewhere [47]. Then, the terminal carboxyl groups of CMCS were conjugated to the amino groups of 3-APBA and L-Valine to synthesize CMCS-PBA-LV. Briefly, 300 mg CMCS was dissolved in 40 mL of deionized water containing EDC and NHS. The reaction proceeded under stirring for 2 h at 0 °C to form active esters of CMCS. 3-APBA (500 mg in DMF) and L-Valine (100 mg in deionized water) were added to the above solution and stirred for 24 h to get the reaction complete. The mixture was subjected to a 5-fold dilution with deionized water and filtered. The filtrate was dialyzed against deionized water using a dialysis bag (MWCO = 3500 Da) for 48 h and freeze-dried to obtain CMCS-PBA-LV. The final yield of CMCS-PBA-LV was around 87%.

2.2. Preparation of insulin loaded chitosan-based multifunctional nanocarriers

The insulin-loaded CMCS-PBA-LV nanoparticles were prepared by the similar route except the adding certain amount of insulin. Briefly, insulin was dissolved in HCl solution (0.01 M, pH = 2.0) at a concentration of 1 mg mL^{-1}, and the pH was adjusted to 8.0 using 1 M NaOH solution. Then, the insulin solution was mixed with 1% CMCS-PBA-LV solution. Then the sodium tripolyphosphate (0.5%) was added which was continued stirring (1000 rpm) at room temperature for 30 min, yielding an opalescent suspension. The resultant solution was centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was removed for the determination of entrapment efficiency and the formed insulin-loaded multi-responsive nanocarriers were freeze-dried and stored at 4 °C in the dark until further use. The FITC-insulin loaded CMCS-PBA-LV nanocarriers were prepared by the same method.

2.3. Insulin release in vitro

The insulin release studies were carried out in pH = 1.2, 6.8 and 7.4 phosphate buffer solution (PBS) with temperature controlled at 37 °C. The insulin loaded nanocarriers were suspended in the above buffer solution (2.5 mg mL^{-1}) containing different concentration of glucose. Aliquots of 200 μL were taken at one-hour intervals and the released insulin was estimated by means of Lowry method [48]. 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.

2.4. Enzyme inhibition studies

To investigate the protective properties of the CMCS-PBA-LV against enzymatic degradation of insulin, the insulin-loaded nanocarriers (1%) were dispersed in (Simulated Gastric Fluid) SGF (USP) and (Simulated Intestinal Fluid) SIF (USP). The SGF and SIF were consisted of 35 mM NaCl, 80 mM HCl, 0.3% (w/v) pepsin, pH = 1.2 and 50 mM KH_2PO_4, 15 mM NaOH, 1.0% (w/v) pancreatin, pH = 6.8, respectively. Aliquots of insulin solution (3 mL each, 98.6 μg L^{-1}), insulin-loaded nanocarriers (INCs) containing the same content insulin in 1 mL of 0.08 M HCl were dispersed in SGF and SIF, respectively. The mixtures were incubated under agitation at 100 rpm on an orbital shaker for 30 min and 60 min in the 37 °C water bath. The enzymatic reaction was stopped immediately by the addition of 0.1 M NaOH. Samples (100 μL) were collected at 30 min and 60 min, then followed by determination of remained insulin concentration using the insulin ELISA kit [49].