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A composite hydrogel system containing glucose-responsive nanocarriers for oral delivery of insulin

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ABSTRACT

Development of an oral delivery strategy for insulin therapeutics has drawn much attention in recent years. In this study, a glucose-responsive nanocarriers for loading of insulin has been prepared firstly. The resultant nanocarriers exhibited relative low cytotoxicity against Caco-2 cells and excellent stability against protein solution. The insulin release behaviors were evaluated triggered by pH and glucose *in vitro*. In order to enhance the oral bioavailability of insulin, the insulin-loaded glucose-responsive nanocarriers were further encapsulated into a three-dimensional (3D) hyaluronic acid (HA) hydrogel environment for overcoming multiple barriers and providing multi-protection for insulin during the transport process. The hypoglycemic effect for oral delivery of insulin was studied *in vivo*. After oral administration to the diabetic rats, the released insulin from hydrogel systems containing insulin-loaded glucose-responsive nanocarriers exhibited an effective hypoglycemic effect for longer time compared with insulin-loaded nanocarriers.

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1. Introduction

Diabetes is a common global disease that causes immense suffering for individuals and heavy burden for the health care system. To minimize complications such as organ degeneration, diabetic patients are required to inject insulin to maintain the normal blood glucose level in the body [1]. Insulin is a peptide that consisted of 51 amino acids, and it has the ability to control the blood glucose level by facilitating the uptake of glucose [2]. Despite 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. Despite their widespread use, such injections must pass through the skin or mucosal barrier, resulting in dermal trauma and pain. Furthermore, it also causes inconvenient, resulting in poor patient compliance [2,3]. Development of an oral delivery strategy for insulin therapeutics has draw much attention to both patients and the protein therapeutics industry. It not only alleviates the pain caused by injections, but can also mimic the physiological fate of insulin and may provide a better glucose homeostasis [4].

Despite significant interest, the oral route has not yet been widely adopted and applied because of the human body's natural mechanisms for breaking down ingested protein (such as insulin) into amino acid fragments [5]. And multiple physicochemical barriers (enzymatic degradation, tight junction, mucous lining, *etc.*) in gastrointestinal (GI) tract, also limit the bioavailability of oral insulin [6]. In order to overcome these obstacles mentioned above and enhance the oral bioavailability of insulin, many oral strategies using different nanocarriers have been attempted to develop in enhancing efficiency. Among them, polymeric nanocarriers have gained a lot of attention in oral insulin delivery [7–12]. They can offer drug protection and facilitate drug absorption through the intestinal mucosa due to the increasing the time and amount of interaction with the mucus layer of the intestine [8].

Nanocarriers of natural and synthetic biodegradable polymers [6, 13–19] and lipids [20] have been used to date for oral delivery of insulin. For example, Lowman et al. [21] were prepared insulin-loaded poly((methacrylic acid)-grafted-poly(ethylene glycol)) hydrogel microspheres that greatly increased insulin transport. Chitosan, a kind of biodegradable natural polymer which is related to the protonation of amine groups in acidic pH, also has been studied in for oral delivery of insulin due to its cationic nature facilitating effective encapsulation of biomolecules like proteins and drugs and prolongs the drug resident time in the GI tract [6]. Alginate microparticles produced by emulsification method was also used as the carrier systems for the drug delivery of insulin [22]. Although the relatively successful applications of nanocarriers to deliver insulin, it still suffer from low bioavailability compared to injection route, resulting in wasted drug and therefore higher cost [23,24]. Recently, methacrylic acid (MAA)-based

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nanocarriers, such as poly(methacrylic acid-grafted-ethylene glycol (P(MAAg-PEG)) and poly(methacrylic acid-co-*N*-vinylpyrrolidone) (P(MAA-co-NVP)) have been extensively studied as delivery systems for protein therapeutics [25]. At a neutral pH, the carboxyl group in MAA is deprotonated which creates an ionic repulsion between the polymer chains (*i.e.*, decomplexed state). Thus, the mesh size of the nanocarriers increases, allowing the release of an entrapped drug. These systems can therefore respond to pH shift between stomach and small intestine (from ~pH 2 to 7) as an environmental trigger to release and deliver protein to the targeted site of delivery [25]. Furthermore, the tunable physicochemical characteristics of these polymer networks allow for modulation of biological activity through attachment of targeting ligands and mucoadhesion enhancers [26].

Although intense research activities using nanocarriers for oral delivery of insulin have been reported, the loaded insulin is still faced the degradation threats from the acid or enzyme in GI tract. Thanks to their profitable performance, including excellent biocompatibility, high water content, and capacity to degrade into safe products, hydrogels have attracted great attention and have been extensively used in several biomedical applications [27]. In order to enhance the oral delivery efficiency of insulin, we wonder if nanocarriers can be further encapsulated into a three-dimensional (3D) hydrogel environment, which provided a simple strategy to ensure (i) providing the multiple protection for insulin, and (ii) delaying the decomposition of nanocarriers, *i.e.* long enough to allow the diffusion of the nanocarriers in GI tract.

In the present work, we focus on enabling oral delivery of insulin using a composite hydrogel system containing insulin-loaded glucose-responsive nanocarriers. The nanocarriers have multi-responsive properties, in which the favorable equilibrium swelling behavior between acidic and neutral pH environments due to the protonation or deprotonation of carboxyl groups in acrylic mononers [5,28,29], the affinity recognition between phenylboronic acid (PBA) groups and glucose molecules [30,31], and epithelial cell-targeting of folic acid (FA) [32–34]. Then, the novel hydrogel system containing glucose-responsive nanocarriers for oral insulin delivery are 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 hypoglycemic effect on diabetic Spraguee Dawley (SD) rats using insulin-loaded composite hydrogel system are investigated *in vivo*.

2. Experimental

2.1. Materials and animals

Porcine insulin (30 IU/mg) was purchased from Wanbang Bio-Chemical Co., Ltd. (Jiangsu, China). Pepsin from porcine stomach (4500 U/mg protein) was obtained from Sigma–Aldrich (USA). Insulin ELISA kit was purchased from Shanghai Duma Biotechnology Co., Ltd. (China). Fluorescein isothiocyanate (FITC) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were all purchased from Sigma Aldrich (St. Louis, MO, USA). 4-Vinylbenzeneboronic acid (VPBA), ethylene glycol dimethacrylate (EGDMA), acrylic acid (AA), poly(ethylene glycol) acrylate (APEG), sodium chloride (NaCl), hydrochloric acid (HCl), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), folic acid (FA), sodium dodecyl sulfate (SDS), divinyl sulfone (DVS), N, N′-Dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), hyaluronic acid (HA), *N*, *N*-dimethylformamide (DMF), acetonitrile, sodium sulfate (Na_2SO_4), potassium sulphite (K_2SO_3) and potassium persulfate ($K_2S_2O_8$) were obtained from Shanghai Aladdin Chemical Reagent Co. (China). Folic acid conjugated APEG (APEG-FA) was synthesized using DCC/DMAP as activation agents according to the reported method [35]. FITC was covalently linked to insulin in a way as described previously [36]. MilliQ water was used in all experiments.

Caco-2 cells were gained from Shanghai cell bank of Chinese Academy of Sciences. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Beyotime Biotechnology, China) containing 10% fetal bovine serum (FBS) and 1% non-essential amino (Beyotime Biotechnology, China). Male Sprague Dawley (SD) rats weighing 220 g were supplied by Experimental Animal Center of Zhejiang Academy of Medical Sciences. The rats were housed at a room temperature of 22 °C and a relative humidity of 50%.

Artificial gastric fluid (AGF, pH = 1.2) and artificial intestinal fluid (AIF, pH = 6.8) were prepared as follows. 7 mL of HCl (37 wt%) and 2.0 g of NaCl were dissolved in 1000 mL of deionized water to prepare AGF. To prepare AIF, KH_2PO_4 (6.8 g) was dissolved in 500 mL of deionized water, and the pH was then adjusted to 6.8. The solution was then diluted to 1000 mL by the addition of deionized water.

2.2. Preparation of glucose-responsive nanocarriers

The blank multi-responsive nanocarriers (respected by NCs) is described as follows. Typically, APEG-FA (0.262 g, 0.1 mmol), VPBA (0.06 g in 400 μL DMF, 0.4 mmol), AA (0.045 g, 0.6 mmol), EGDMA (0.006 g, 0.03 mmol), SDS (0.01 g, 0.0347 mmol), $K_2S_2O_8$ (0.01 g) and K_2SO_3 (0.009 g) were dissolved in H_2O (10.0 mL). The mixture was firstly stirred for 10 min at room temperature and then sonicated for 5 min to obtain milky emulsion. The emulsion was immersed in liquid nitrogen followed by three cycles of freeze-pump thaw procedures, then sealed and submerged in an oil bath at 30 °C for 24 h. The obtained product was dialyzed against deionized water for 3 days using a dialysis bag (MWCO = 3500 Da). The final product was freeze-dried for 24 h with a yield of over 84%. The structure and composition of the products were confirmed by ^{1}H NMR and FT-IR analysis (Fig. S1-S3).

The insulin-loaded multi-responsive nanocarriers (respected by INCs) were prepared by the similar route except the adding certain amount of insulin before polymerization. Briefly, insulin was dissolved in HCl solution (0.01 M, pH = 2.0) at a concentration of 1 mg/mL, and the pH was adjusted to 8.0 using 1 M NaOH solution [37,38]. Then, the insulin solution was mixed with monomer and initiator solution. The mixture was stirred and sonicated at room temperature for 20 min, yielding an emulsion. The resultant emulsion was sealed in a Schlenk tube after three cycles of freeze-pump thaw procedures and submerged in an oil bath at 30 °C for 24 h. The resultant solution was centrifuged at 10,000 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 multi-responsive nanocarriers were prepared by the same method.

2.3. Preparation of hydrogel system containing of glucose-responsive nanocarriers

The 0.05 g of insulin-loaded multi-responsive nanocarriers (respected by INCs) was dispersed in 5 mL HCl solution with pH = 2.0. And 0.10 g of HA was dissolved in 10 mL NaOH solution with pH = 5.0. Two solutions were mixed under high speed stirred at 8000 rpm for 5 min at room temperature. Then, the crosslinking agent, divinyl sulfone (DVS) (10, 20 and 40 μL in 1 mL H_2O, respected by INCs-IHA-10, INCs-HA-20 and INCs-HA-40, respectively), was dropped into the mixed solution under middle speed stirred at 2000 rpm for 30 min. The prepared hydrogel system was dialyzed against HCl solution (pH = 1.0) for 3 days using a dialysis bag (MWCO = 3500 Da) to remove the unreacted DVS. The final product was freeze-dried for 24 h. For the comparison, the free HA hydrogels with same degree of cross-linking (respected by HA-10, HA-20 and HA-40, respectively) were prepared using the above method except for adding of INCs.

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