Materials Science and Engineering C 78 (2017) 420–428

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Preparation of poly(lactic-*co*-glycolic acid) and chitosan composite nanocarriers via electrostatic self assembly for oral delivery of insulin

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ARTICLE INFO

Article history:
Received 26 January 2017
Received in revised form 18 April 2017
Accepted 19 April 2017
Available online 20 April 2017

Keywords:
Diabetes
Insulin
Nanocarriers
Oral delivery
Hypoglycemic effect

ABSTRACT

To improve insulin bioavailability and overcome multiple barriers for oral delivery of insulin, the composite nanocarriers (PLGA/FA-CS) prepared from poly(lactide-*co*-glycoside) (PLGA) and folic acid modified chitosan (FA-CS) were fabricated via electrostatic self-assembly method. The resultant composite nanocarriers exhibited low cytotoxicity against HT-29 cells and excellent stability against protein solution. 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 the as-prepared composite nanocarriers may be a promising drug delivery system for oral administration of insulin and other biomacromolecules.

1. Introduction

There are 415 million people with diabetes in 2015, and this is expected to rise to 552 million by 2030 according to a report from International Diabetes Federation (IDF) [1–5]. Diabetes has become one of the most lethal diseases in some counties, especially in developing countries. Insulin is commonly used to treat diabetes [6]. The subcutaneous needle injection is the preferred insulin administration method. However, it is inconvenient and painful, often leading to poor patient compliance [7]. Oral delivery has received a greatly attention and become the best desirable method of insulin administration which does not include painful and uncomfortable interventions [8]. Unfortunately, the oral availability of insulin is very poor, the result may be, for instance, enzymatic degradation, cellular mucus obstruction and susceptibility to gastrointestinal fluids. As the previous reports [9,10], the oral bioavailability of unprotected insulin is very low (e.g. lower than 1–2%).

Recently, several strategies have been made to overcome these issues to increase the bioavailability of insulin [11–19]. Among these strategies, polymeric nanocarriers may represent a closer approach to reach a successful oral insulin delivery nanosystem because they offer drug protection and facilitate drug absorption through the intestinal mucosa. For instance, the chitosan (CS)-based multifunctional nanocarriers have been shown to successfully incorporate insulin [13]. The vast exploration of CS-based nanocarriers over the last years has also demonstrated that this biopolymer plays an important role in insulin oral bioavailability. CS, a natural cationic polysaccharide is consider a promising carrier in drug delivery system due to its low toxicity, biodegradability and low immunogenicity, which possesses much desirable properties for the manufacture of drug delivery systems [20–22]. In order to further enhance the cell permeability of CS and delivery performance, it is necessary to modify a variety of targeting ligands on chitosan, such as RGD for tumor targeting [23]. Folic acid (FA) is often used as a target for cell membranes and promoting nanocarriers endocytosis via the folate receptor due to its stably, inexpensive and generally poorly immunogenic chemical with a high affinity to the folate receptor [24–25]. More importantly, the folate receptor is significantly expressed on many human epithelial cancer cell surfaces, including kidney, lung and colon cancers cell. Macromolecules conjugation with folic acid can enhance their uptake and targeting ability [26–28].

Poly(lactide-*co*-glycolide) (PLGA) is recognized by US Food and Drug Administration and is considered safe for clinical use, as pharmaceutical excipients included in the United States Pharmacopoeia (USP). PLGA nanoparticles are very popularity in drug delivery because of their ability associate and controlled release of proteins [29–32]. Moreover, PLGA nanoparticles have potential as transmucosal transporter applied to nasal and oral administration in previous report [33, 34]. However, the certain disadvantages of PLGA nanoparticles are their instability in biological fluids and ability of penetration the mucus and epithelial barriers are feebleness due to the negative surface

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http://dx.doi.org/10.1016/j.msec.2017.04.113
0928-4931/

charge of PLGA nanoparticles limits the interaction with the mucus layer or cell membrane, which have the negative surface [35–37]. Attaching a positively charged polymer to the surface of PLGA nanoparticles by electrostatic interaction may be an effective method to modify the surface charge of PLGA nanoparticles, which could improve the application and property of nanoparticles.

Herein, the composite nanocarriers (PLGA/FA-CS) prepared from poly(lactide-*co*-glycoside) (PLGA) and folic acid modified CS (FA-CS) were fabricated via electrostatic self-assembly method. PLGA and CS, which are two of the most widely investigated biomedical materials, were employed as the biological polyelectrolytes. The capsules were fabricated with multilayers by LBL self-assembly of PLGA and GC through electrostatic interaction. The stability of insulin and insulin release were investigated in detail. The cell cytotoxicity and cellular uptake of PLGA/FA-CS were also investigated. Moreover, the pharmacokinetics and hypoglycemic effect on diabetic SD rats were also performed.

2. Experimental

2.1. Materials and animals

Chitosan (CS), a white powder of about 60 mesh, was supplied by Zhejiang Yuhuan Biochemical Co. Ltd. (China). PLGA polymer (poly(lactic acid)/poly(glycolic acid) = 50:50; MW = 20,000) was purchased from Jinan Daigang Co. Ltd. (Shangdong, China). 1-Ethy-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxy succinimide (NHS), poly(vinyl alcohol) (PVA), folic acid (FA), sodium chloride (NaCl), hydrochloric acid (HCl), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), fluorescein isothiocyanate (FITC), porcine insulin (30 IU/mg) and pepsin from porcine stomach (4500 U/mg protein) were purchased from Shanghai Aladdin Chemical Reagent Co. (China). Deionized water was used in all experiments. All other reagents and chemical were of analytical grade. Folic acid conjugated chitosan (FA-CS) was prepared using a method described previously (Fig. S1) [38].

Male Sprague Dawley (SD) rats weighing 230–250 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%. HT-29 cells were gained from Shanghai R&S Biotechnology Co. Ltd. (China). They were cultured in Dulbecco's Modified Eagke Medium (DMEM) (Beyotime Biotechnology, China) containing 10% fetal bovine serum (FBS) and 1% non-essential amino (Beyotime Biotechnology, China).

2.2. Preparation of PLGA/FA-CS composite nanocarriers

Insulin loaded PLGA/FA-CS nanocarriers were prepared using a double emulsion method, which with a slight modification from a previous method [39–40]. Briefly, 1 mL of 4 mg/mL insulin solution (was dissolved in solution of HCl (0.01 M, pH = 2.0) and pH of insulin solution was adjusted to 7.0 using 1 M NaOH solution) was added to 1 mL ethyl acetate oil phase containing 20 mg of PLGA. The phase one of W/O emulsion was formed after ultrasound sonication (200 W) for 60 s in ice bath. Then, 2 mL of 1% (W/V) solution of poly(vinyl alcohol) (PVA) containing 1 mg preceding folic acid conjugated CS (contain 1% glacial acetic acid) was droped and mixed by a slight shaking fully followed by ultrasound sonication (200 W) for 60 s in ice bath to produce a W/O/W double emulsion. Finally, the double emulsion was dropwised into 6 mL 1% solution of PVA, and the emulsion solution was mixed by magnetic stirring for 1 h at room temperature. After 1 h, the remaining ethyl acetate was removed from vacuum rotary evaporation at 40. Aliquots of nanocarriers suspension were washed twice with deionized water by centrifugation (12,000 rpm, 4 °C, 30 min) and resuspension or freeze-drying obtained nanoparticles. The blank PLGA/FA-CS nanocarriers were prepared using the same method exception of insulin solution.

2.3. Characterization

The size and ζ-potential of nanoparticles were measured by Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). All measurements were performed in triplicate. Nanocarriers surface morphology were performed using scanning electron microscope (SEM) (Zeiss, ULTRA-55, Germany) and transmission electron microscopy (TEM) (JEOL, JSM-2100, Japan). To ensure encapsulation efficiency (EE) and drug loading capacity (DLC) of insulin-loading nanocarriers (insulin-PLGA/FA-CS or insulin-PLGA), the nanocarriers were isolation the deionized water suspension medium by centrifugation (12,000 rpm, 4 °C, 30 min). The quantity of free insulin in supernatant liquid was measured using UV spectrophotometry method [41]. Aliquot 3 mL of the supernatant were measured absorbance by using UV spectrophotometry (λ = 276, AuCy, UV1901PC, Shanghai). The absorbance of insulin was recorded and the concentration of free insulin in the supernatant was calculated from a standard curve of insulin. The EE and DLC were calculated using the following formula:

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

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

2.4. Enzyme inhibition in simulated gastrointestinal fluids

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to United States Pharmacopeia 35. The SGF and SIF were 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) pancreation, pH 6.8, separately. In order to test the protective properties of the PLGA/FA-CS nanocarriers against enzymatic degradation of insulin, the insulin-PLGA/FA-CS nanocarriers were dispersal in SGF and SIF. Aliquots of insulin-PLGA/FA-CS nanocarriers at a concentration of 0.5 mg/mL were dispersed in SGF and SIF, respectively. The mixed liquor were incubation at 37 °C follow agitation at 100 rpm on an orbital shaker for 30 min and 60 min. The enzyme reaction was stopped immediately by the added o.1 M NaOH. Sample were collected at 30 min and 60 min, which it by ultracentrifugation and remove the supernatant fluid using UV to test the absorbance of insulin in supernatant fluid, then follow by determination of remained insulin concentration using the standard curve.

2.5. Insulin release in vitro

The insulin release from nanocarriers (Ins-PLGA/FA-CS) were studied with concentration of 1.0 mg/mL in SGF (without pepsin) and SIF (without pancreatin), respectively at 37 °C under agitation at 100 rpm. At predetermined time, samples were collected and remove from the supernatant fluid. The release insulin was calculation through the UV absorbance and using the insulin standard curve. The experiments were done in triplicates. The release percent of insulin was then estimated by dividing the amount of supernatant by the total amount of the Ins-PLGA/FA-CS nanocarriers added.

2.6. Cytotoxicity

In order to test the cytotoxicity of PLGA/FA-CS nanocarriers, which were evaluated the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [42]. To measure the cells viability, the HT-29 cells were seeded in 96-well plates, which the cell density at a 1×10^4 per well-plates with 200 μL of growth medium including 10% fetal bovine serum (FBS) and continue to grow overnight at 37 °C in 5% CO_2 incubator. Then cells attached to wells after 24 h, subsequently, the media of 96-well plates were removed and 200 μL insulin-loading PLGA/FA-CS nanocarriers and blank nanocarriers solution were added to that well for 48 h, respectively, with the concentrations of 0, 20, 50, 100, 250, 500 and 1000 μg/mL (group 1). Then, the concentration of