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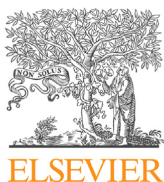

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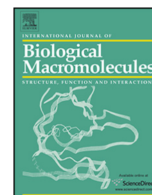

Surface functional modification of self-assembled insulin nanospheres for improving intestinal absorption

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ABSTRACT

In this work we fabricated therapeutic protein drugs such as insulin as free-carrier delivery system to improve their oral absorption efficiency. The formulation involved self-assembly of insulin into nanospheres (INS) by a novel thermal induced phase separation method. In consideration of harsh environment in gastrointestinal tract, surface functional modification of INS with ε-poly-L-lysine (EPL) was employed to form a core–shell structure (INS@EPL) and protect them from too fast dissociation before their arriving at target uptake sites. Both INS and INS@EPL were characterized as uniformly spherical particles with mean diameter size of 150–300 nm. The process of transient thermal treatment did not change their biological potency retention significantly. In vitro dissolution studies showed that shell cross-linked of INS with EPL improved the release profiles of insulin from the self-assembled nanospheres at intestinal pH. Confocal microscopy visualization and transport experiments proved the enhanced paracellular permeability of INS@EPL in Caco-2 cells. Compared to that of INS, enteral administration of INS@EPL at 20 IU/kg resulted in more significant hypoglycemic effects in diabetic rats up to 12 h. Accordingly, the results indicated that surface functional modification of self-assembled insulin nanospheres with shell cross-linked polycationic peptide could be a promising candidate for oral therapeutic protein delivery.
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1. Introduction

Insulin, a major protein hormone consisting of 51 amino acids, is secreted by the β-cells of the pancreas and plays a crucial role in controlling diabetes. The most common form of insulin therapy is daily subcutaneous injection to diabetic patients. This frequent injection always remains a painful treatment leading to the patient noncompliance and unsatisfactory metabolic regulation [1,2]. Oral delivery is the preferred route of long-term administration because it offers several advantages over other routes such as convenience, less invasive and expensive. Moreover, the oral route will imitate the physiologic first hepatic uptake [3]. Unfortunately efficient absorption of oral insulin is difficult to achieve because proteins are generally water-soluble compounds with abundant hydrophilic polar groups on their surface, which lead to a low permeability through the lipidic intestinal mucosa and a poor absorption percentage via the GI tract [4,5].

Therefore there are clinical situations in which novel formulations with capability of allowing proteins transport across the intestinal epithelium into the blood and insignificant potential toxicological issues are needed. Among the possible considerations to achieve sufficient oral bioavailability of these therapeutic agents, such as micro-emulsions [6], liposome [7], polymeric and lipid nanoparticles [8,9], self-assembled nanospheres from the therapeutic proteins provide us a promising alternative. These "pure" protein particles are defined three dimensional architectures in nanoscale, which come from packing of the biologic macromolecules through noncovalent interactions, such as the hydrophobic interactions, hydrogen bonding, van der Waals forces, as well as electromagnetic interactions [10]. Thus these self-assembled protein nanospheres may offer the comparable delivery efficacy and should not present any safety issues raised by the chemical vehicles, especially repeated dosing over the long term is required. The simplicity and mildness of the self-assembly process indicate the potential labor and cost advantages over the conventional delivery systems. Moreover, the effects of particle size on

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permeability of mucous membrane have been verified by previous reports that smaller particles less than 500 nm were more readily absorbed than larger particles by both Caco-2 cells and by intestinal tissues [11].

Despite of the encouraging potential pharmaceutical applications of self-assembled protein nanospheres, exposure of these naked cargos into the harsh gastrointestinal tract still remains a challenge. Due to the weak intermolecular forces, the self-assembled therapeutic proteins are susceptible to the surrounding factors (e.g. physiological pH and ionic strength) and apt to dissociation before their arriving at target uptake sites [12]. Various strategies have been employed to overcome these barriers, such as chemical cross-linking using aldehydes [13] and physical encapsulation with biodegradable polymers [14,2]. However, these approaches have incurred either loss of protein bioactivity or the additional expense of protein stabilizing carriers.

We have therefore fabricated hydrophilic protein drugs as free-carrier delivery system to improve their oral bioavailability. The formulation involved self-assembly of therapeutic protein into nano-scale first. To endow the protein nanospheres with improved both release behaviors and penetration performance, the followed surface modification with ε-poly-L-lysine, a homopolymerized cationic peptide with pK_a of ∼10.8, through a unique ionically cross-linking process was employed. In all studies, insulin was used as a biomacromolecular motif, which is of short serum half-life (4–6 min) and thus needed to be frequent subcutaneous administration in the management of diabetes mellitus. The characteristics of the self-assembled nanospheres, including morphology, particle size, second structure conformation and release behaviors in vitro were studied. To evaluate the absorption efficiency of the developed formulations, both the in vitro transport across caco-2 monolayer and in vivo serum pharmacokinetics of peroral administration of self-assembled nanospheres with various modification degrees were also investigated.

2. Materials and methods

2.1. Materials

Porcine insulin with a biological potency of 28 IU/mg was provided by Xuzhou Wanbang Biochemical Pharmaceutical Co., Ltd. (Jiangsu, China). Insulin enzyme-linked immunoassay (ELISA) kits were supplied by Lichen Biological Science and Technology Co., Ltd. (Shanghai, China). The Caco-2 cell line, originating from a human colorectal carcinoma, was obtained from the American Type Culture Collection (ATCC37-HTB, Rockville, MD, USA) and used between passages 31 and 42. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (NY, USA). Poly (vinyl alcohol) (PVA, Av.MW 30–70 kDa, 88% hydrolysis) was supplied by Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Polylysine (ε-poly-L-lysine, EPL) with molecular weight of 3500–4700 was obtained from Bainafo Bioengineering Co., Ltd. (Zhengzhou, China). Fluorescein isothiocyanate labeled insulin (FITC-insulin), trypsin (≥10,000 U/mg), rhodamine-phalloidin and Hoechst 33258 were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents and chemicals were of analytical grade.

2.2. Self-assembly insulin nanospheres (INS)

The insulin nanospheres were spontaneously formed by a modified temperature induced phase separation [15]. Briefly, a solution buffered at pH 5.3–5.5 containing 12% PEG 2000 and 0.5% PVA was prepared and pre-heated to 75 °C. Bulk porcine insulin powder was directly added while stirring to allow complete dissolution within 3–5 min. Then the temperature of the solution was cooled down to room temperature at a controlled rate. After further 2 h of let stand, the resulting opaque suspension was subjected to centrifugation at 2000 × g for 15 min. The resulted precipitates were washed and re-suspended in 0.5% PVA (pH 6.0–6.5) by ultrasonication.

For protein content determination, the resulted particles were dissolved in 0.01 M hydrochloric acid and subjected to vortex for dissociation completely. The insulin content in the aqueous phase was measured by RP-HPLC analysis as previously described [16]. The drug recovery efficiency (R.E.) was expressed as percentage of the insulin in particles relative to the total amount used for the particle preparation.

2.3. Surface modification of insulin nanospheres

The subsequent surface modification of insulin nanospheres was performed using a modified ionic shell cross-linking procedure. Briefly, EPL stock solution was introduced into the suspension at various weight ratio of EPL/INS. After co-incubation for 1 h with constant stirring at ambient temperature, a 5% (w/v) solution of sodium tripolyphosphate (TPP) was dropped into the resultant solution and allowed to stirring further 30 min. Thereafter, the coated nanospheres were recovered from the aqueous phase by ultracentrifugation (40,000 × g for 15 min; CS120GXL, Hitch Co., Ltd., Japan) and washed two times with distilled water. Then freeze drying were employed for subsequent physical characterizations.

2.4. Morphology characterization

The surface appearance of the protein nanospheres was examined with scanning electron microscopy (SEM) analysis. All samples were mounted on aluminum stubs, followed by gold metallization using an ion sputter coater (Hitachi E101, Tokyo, Japan) and observed on a Hitachi S-2400N scanning electron microscope (Tokyo, Japan). For examination of structural morphology, a transmission electron microscope (TEM), (JEM-1200EX, Tokyo, Japan) was employed. After diluting 50-fold with the original dispersion medium of the preparation, the samples were negatively stained with sodium phosphotungstate solution (0.2%, w/v) for observation.

2.5. Particle size and surface electric potential analysis

The freeze dried nanosphere samples were re-suspended in distilled water by sonication before measurement. The obtained suspensions were subjected to examination. The particle size distribution, expressed as mean diameter and polydispersity index, was determined by photon correlation spectroscopy (PCS) using Zetasizer Nano-ZS90 (Malvern Instruments, UK). Each measurement was performed in triplicate.

2.6. Fourier transform infrared spectroscopy (FTIR)

FTIR was used to characterize the particles and the secondary structure of insulin in the nano self-assembly. The FTIR spectra were recorded on a Bruker IFS 55 spectrometer (Bruker, Switzerland) in the range of 4000–100 cm^{-1}. Samples were prepared by mixing sample powder with KBr and scanned at 2 cm^{-1} resolution. Second-derivative spectra were obtained with the derivative function of Omnic software (Nicolet, Waltham, MA). Background-corrected spectra were analyzed by second derivatization in the amide I band region for components peak frequencies. Gaussian curve-fitting was performed on the Fourier self-deconvoluted amide I band region. The secondary structure content was calculated from the areas of the individually assigned peaks and their fractions of the total area in the region.

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