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Research paper

Preparation and evaluation of alginate–chitosan microspheres for oral delivery of insulin

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ABSTRACT

The alginate–chitosan microspheres with narrow size distribution were prepared by membrane emulsification technique in combination with ion (Ca^{2+}) and polymer (chitosan) solidification. The preparation procedure was observed, and the physical properties (particle size distribution, surface morphology, chitosan distribution, zeta potential) of the microspheres were characterized. Subsequently, the microspheres were employed to load model peptide of insulin. The effect of loading ways on the loading efficiency and immunological activity of insulin were investigated. It was shown that the higher loading efficiency (56.7%) and remarkable activity maintenance (99.4%) were obtained when the insulin was loaded during the chitosan solidification process (Method B). Afterward, the release profile *in vitro* for the optimal insulin-loaded microspheres was investigated. Under the pH conditions of gastrointestinal environment, only 32% of insulin released during the simulated transit time of drug (2 h in the stomach and 4 h in the intestinal). While under the pH condition of blood environment, insulin release was stable and sustained for a long time (14 days). Furthermore, the chemical stability of insulin released from the microspheres was well preserved after they were treated with the simulated gastric fluid containing pepsin for 2 h. Finally, the blood glucose level of diabetic rats could be effectively reduced and stably kept for a long time (~60 h) after oral administration of the insulin-loaded alginate–chitosan microspheres. Therefore, the alginate–chitosan microspheres were found to be promising vectors showing a good efficiency in oral administration of protein or peptide drugs.

1. Introduction

Pharmaceutical proteins or peptides are becoming an important class of therapeutic drugs with lower side effects and specific treatment effects [1]. However, due to their large molecular weight and size, they show poor permeability characteristics through various mucosal surfaces and biological membranes. Moreover, rapid clearance in liver and other body tissues by proteolytic enzymes as well as inherently chemical and physical instability are also factors which result in the low bioavailability associated with the oral delivery [2]. Among several attempts proposed to improve oral bioavailability, microencapsulation represents a promising strategy [3].

Recently, the natural polysaccharide, alginate, is used as a carrier material for protein and peptide drugs and has attracted increasing attentions due to its excellent biocompatibility, mucoadhesive biodegradability and mild gelation conditions [4]. It possesses a unique property of mild gel-formation in the presence of multivalent cations such as calcium ions in aqueous media, which takes place at junctions in the G–G sequence rich chain region known as the "egg-box junctions" [5,6]. However, the loose network of bead results in a major limitation of drug-leakage through the pores during alginate-Ca bead preparation [7]. Fortunately, the mechanical properties and permeability of alginate-Ca bead can be effectively improved by a polycation, such as chitosan. Chitosan is a naturally occurring polysaccharide comprising D-glucosamine and N-acetyl-glucosamine with unique polycation characteristics [8,9]. Upon mixing with the alginate, the strong electrostatic interaction of amino groups of chitosan with the carboxyl groups of alginate leads to the formation of chitosan–alginate complex. The complexation reduces the porosity of alginate beads and decreases the leakage of the encapsulated drugs [10,11]. On the other hand, this complex exhibits pH-sensitivity that release of macromolecules from alginate beads in low pH solutions is significantly reduced, thereby being used as an oral delivery vehicle [12].

To date, several methods have been performed for the preparation of alginate–chitosan microspheres, such as spray drying [13], coacervation technique [14] and emulsification/solidification

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technique [15]. However, limitations are found in the practical application. For example, the size of particles prepared by the spray drying and coacervation technique is too large to be effectively absorbed in the intestinal tract [16]. Although smaller particles can be obtained by emulsification/solidification technique, the emulsion is usually prepared by high-energy instruments or methods with high shear force, which may result in deactivation of polypeptide drugs. In addition, the microspheres prepared by spray drying and emulsification/solidification technique have broad size distribution, leading to possible side effects and poor repeatability [17] during the practical applications.

To overcome the disadvantages mentioned above, the membrane emulsification technique was applied to prepare alginate–chitosan microspheres with narrow size distribution. It is a unique method to mildly provide emulsion with uniform droplet size. Then, the droplets can be solidified to form microspheres or microcapsules by various solidification techniques. Nevertheless, in the case of alginate microsphere, the solidification process of alginate droplets in W/O emulsion is a big challenge because it is totally different from the direct solidification by $CaCl_2$ solution. In this study, we developed a two-step solidification process with $CaCl_2$ mini-emulsion in W/O system followed by chitosan in aqueous phase. The microspheres properties of particle size, surface morphology, chitosan distribution and zeta potential were characterized in detail. As a model peptide, insulin was loaded in the microsphere by three methods. Consequently, an effective insulin loading method with higher activity retention was developed. Further evaluations of the insulin-loaded alginate–chitosan microsphere were performed *in vitro* and *in vivo* (diabetes rats).

2. Materials and methods

2.1. Materials

Sodium alginate was purchased from Acros Organics (New Jersey, USA). According to the manufacturer, the alginate contained 65–75% guluronic acid (G) subunits and 25–35% mannuronic acid (M) subunits and had a molecular weight of 450–550 kDa, specifically with a viscosity of 485 cP for a 1 wt% aqueous solution. Chitosan was ordered from Yuhuan Ocean Biochemical Co., Ltd. (Zhejiang, China), and the degree of deacetylation is 89% and MW (viscosity-average molecular weight) is 150 kDa. A fast acting insulin analog (IA) (25.8 IU/mg), which is identical to human insulin except for inversion of ProB28–LysB29, was kindly provided by Gan & Li Biotechnological Co., Ltd. (China). Alex Fluoresce-488 was from Invitrogen reagent corporation (USA), and Streptozotocin (STZ) from Alexis Biochemicals (USA). PO-500 (Hexaglycerin penta ester) was supplied by Sakamoto Yakuhin Kogyo Co., Ltd. (Japan). KP-18C (C_{18} silane coupling agent) was provided by Shin-Etsu Chemical Co., (Japan). Other reagents were of analytical grade.

2.2. Preparation of blank alginate–chitosan microspheres

The experimental set-up in details for membrane emulsification process is referred to our previous work [17]. The schematic preparation process of alginate–chitosan microspheres is shown in Fig. 1. Briefly, SPG (Shirasu Porous Glass) membrane with a specific pore size (7.0 μm) was premodified to be hydrophobic with KP-18C (C_{18} silane coupling agent). Five milliliters of 1.0 wt% alginate in acetic acid buffer solution (pH = 4.2) was used as the water phase. Under the pressure of nitrogen, the water phase was pressed through the membrane into 50 ml of oil phase (liquid paraffin and petroleum ether in a volume ratio of 7:5; emulsifier: 4 wt% PO-500) to form uniform-sized droplets. Then, the mini-emulsion of $CaCl_2$ prepared by dispersing 1.5 ml $CaCl_2$ solution

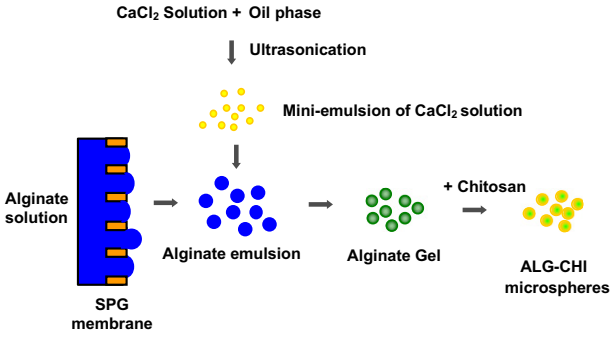

Fig. 1. A schematic presentation of the preparation process of blank alginate–chitosan microsphere. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(0.5 mol/l) into 10 ml oil phase (the same recipe as mentioned above) by ultrasonication (S450D, Branson Ultrasonics Corporation, USA) was added into the sodium alginate emulsion as the first-step solidification. The solidification process was continued for 5 h under stirring of 300 rpm. The solidified alginate gels were collected and washed two times with petroleum ether and four times with distilled water by centrifugation (1000*g*, 5 min, 25 °C) and then dispersed in 5 ml of 1.6 wt% chitosan acetic acid buffer solution (pH = 4.2) for 1 h under stirring of 300 rpm as the second-step solidification. Finally, the alginate–chitosan microspheres were washed twice with 1 wt% aqueous acetic acid to remove the residual chitosan on the surface and then dried by lyophilization (Labconco Freezone Plus 6, USA) at 0 °C for 48 h.

2.3. Sample characterization

An optical microscope (XSZ-H_3, ChongQing Opitical & Electrical Instrument Co., Ltd., PR China) installed with picture capturer (wv-CP230, Panasonic Co., Ltd., Japan) was used to monitor the droplets before and after solidification. The surface morphology of the dried samples was observed by a scanning electron microscopy (SEM, JEM-6700F, Japan). The size distribution was analyzed by a Mastersizer 2000 laser particle analyzer. Polydispersity was determined by the SPAN factor expressed as

$$\mathrm{SPAN} = [\mathrm{D}(v,90) - \mathrm{D}(v,10)]/\mathrm{D}(v,50) \quad (1)$$

where D(v, 90), D(v, 10) and D(v, 50) are volume size diameters at 90%, 10% and 50% of the cumulative volume, respectively.

The ξ-potential was measured by ZetaPlus 21421 (Brookhaven Instruments Corporation, USA). The average pore size of microspheres was measured by BET method (ASAP 2020, Micromeritics, USA). It was found that the compound of chitosan–glutaraldehyde exhibited fluorescence [18]. TCS SP2 Laser Scanning Cofocal Microscopy (LSCM, Leica) was used to observe the chitosan distribution in the blank alginate–chitosan microsphere. In detail, 1 ml of 50 wt% glutaraldehyde solution was slowly dropped into as-prepared alginate–chitosan microspheres dispersed in 5 ml distilled water at a stirring speed of 300 rpm for 1 h, and then the glutaraldehyde-crosslinked microspheres were washed twice with distilled water. Different content of chitosan led to varied fluorescence intensity of microspheres, so the chitosan in samples prepared by different conditions in orthodox experiments were also quantified by LSR flow cytometer (Becton Dickinson). The results are shown in Supplementary material 2.

2.4. Insulin loading

Insulin (pI = 5.35–5.45) was positive on the condition of microsphere preparation, while alginate was negative. If mixing the