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Preparation and characterization of vanillin-crosslinked chitosan therapeutic bioactive microcarriers

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ABSTRACT

Chitosan microspheres with diameter of 14.3–48.5 μm were prepared by emulsion method and using natural vanillin as cross-linking agent. The surface morphology and microstructure of the microspheres were characterized by scanning electron microscopy, X-ray diffraction and Fourier-transform infrared spectroscopy, *etc.* The hollow microspheres showed a well-defined spherical shape with median diameter of 30.3 μm and possessed a uniform surface with micro-wrinkles, which is in favor of the drug release. Interpenetrating network cross-linking mechanism might result from the Schiff base reaction and the acetalization of hydroxyl and carbonyl between chitosan and vanillin. Berberine, as a model drug, was loaded in the microspheres and released in a sustainable manner. The drug loading ratio could change from 9.16% to 29.70% corresponding to the entrapment efficiency of 91.61% to 74.25%. *In vitro* cell culture study using MG63 cells and *in vivo* implantation clearly showed that the microspheres could provide favorable cell attachment and biocompatibility. The results confirm that the drug-loaded vanillin-crosslinked chitosan microspheres could be a worthy candidate either as carriers of drugs and cells, or as therapeutic matrix for bone repair and regeneration.

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1. Introduction

Osteomyelitis with bone loss presents a special challenge for treatment and reconstruction. A staged reconstruction technique consists of initial debridement and local–systemic antibiotic therapy, followed by bone reconstruction [1]. An ideal system for local delivery of antibiotics should provide sustained delivery of higher concentration of antibiotics by diffusion to avascular area and yet minimize the risk of systemic toxicity associated with traditional method of intravenous delivery [2]. Surgical removal of infected bone often results in considerable bone loss and skeletal deficiency. Small bone defects can spontaneously regenerate to the original anatomic configuration, however, regeneration of defects that exceed a certain size is difficult to reconstruct following resolution of the infection. In the final stage of reconstruction, bone substitutes have to be selected by clinicians to treat large defect cases [3]. To solve these problems, polymer based drug delivery systems have been developed. A major benefit of bioactive carrier system is the ability to regenerate bone in the defect while allowing local antibiotic release at a sustainable or controllable rate. This combination could eliminate the need for subsequent bone reconstruction to fill large bone defects [4]. At the moment, the microsphere technology is the core technology designed to fill the defects and to release therapeutic drugs in a predictable manner [5]. The microspherical particles possess high physicochemical integrity; loading of drugs can be achieved through chemical crosslinking, blending or simple adsorption [6], and bone-bonding bioactive apatite crystals can be incorporated during preparation. In particular, the microspheres can be refined by varying polymer molecular weight and concentration, degree of crosslinking, or by chemical modification of the polymer matrix to achieve sustained and controlled drug release [7].

Chitosan (CS) has presented great potential applications in adsorption and isolation of protein, catalytic carrier, enzyme immobilization, and controlled drug release in the form of fibers, membranes, microspheres, and capsules [8]. The most attractive properties of CS are related to its biodegradability and good biocompatibility, which makes CS and its derivatives be extensively used in biomedical fields, such as for wound healing, drug delivery and tissue engineering, particularly for developing nano-/microspheres as carrier systems [9]. As a co-polymer, CS is made up of linear β-(1 → 4) glycosidic linkage which is similar in structure to cellulose [10]. In addition, CS has also been shown to facilitate cell adhesion and proliferation, and osteogenic differentiation of

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mesenchymal stem cells [11]. CS has one primary amino and two free hydroxyl groups for each C6 unit. Cross-linking is a common way to modify the CS structure and improve its controlled-release and mechanical properties [12]. The available amino and hydroxyl groups on CS are active sites capable of forming a number of linkages [13]. In cross-linked CS, the polymer chains are interconnected by cross linkers, forming a three dimensional network structure. To date, various dialdehydes, such as glyoxal and glutaraldehyde [14], are used to perform the covalent cross-linking on NH_2 sites, forming stable imine bonds between amine groups of CS and aldehyde groups *via* a Schiff reaction [15]. Researchers have evaluated cross-linking of chitosan microspheres with glutaraldehyde as well as glyoxyal for the controlled delivery of centchroman. Their studies demonstrated that drug release rates may be changed not only by the degree of cross-linking of the microspheres, but also by the type of cross-linker used [16]. However, the main drawback of dialdehydes is related to their toxicity [17]. For example, glutaraldehyde can cause irritation to mucosal membranes because of its toxicity. Therefore, considering the biocompatibility, many investigations have developed new reagents to cross-link chitosan. Researchers also reported that chitosan microspheres showed a sustained release for centchroman for 50 h with sodium hexameta polyphosphate (SHMP) as physical cross-linker [18]. However, physically crosslinked microspheres have exhibited inferior release properties due to their weak electrostatic interactions between anions and chitosan. Therefore, it is necessary and crucial to search for alternative cross-linkers for chitosan microspheres. Currently, vanillin (4-hydroxy-3- methoxybenzaldehyde) is a popular flavor extracts broadly used in food [19]. The aldehyde groups in vanillin and the amino groups in CS may undergo Schiff base reaction and form a network structure to favor the stabilization and controlled release [20]. Its biocompatibility when used for biomaterial preparation needs to be further assessed.

The objective of the present study is to prepare the CS microspheres by emulsion method, using vanillin as the cross linker. The physicochemical properties and *in vitro* drug release behavior of the microspheres were characterized and tested. The biocompatibility of the vanillin cross-linked CS microspheres was evaluated *via in vitro* cell culture and *in vivo* animal experiment using glutaraldehyde cross-linked CS microspheres as the control.

2. Materials and methods

2.1. Materials

CS was obtained from Jinan Haidebei Marine Bioengineering Co. Ltd. (Shandong, China) with 90% deacetylation degree. Aqueous acetic acid solution was used as the solvent for CS microspheres preparation. Vanillin and glutaraldehyde (Aladdin Co. Ltd., China) were used as the cross linkers. All chemicals and reagents (liquid paraffin, Span 80, petroleum ether (60–90 °C), isopropyl alcohol, *etc.*) used in the experiments were of analytical grade.

2.2. Preparation of microspheres

CS solution (3%, w/v) was prepared by dissolving 3 g of CS in 100 ml acetic acid solution (1%, v/v) at room temperature. The solution was stirred at 1000 rpm for 30 min to ensure complete dissolution of CS. Then the solution (20 g) was poured into the liquid paraffin suspension medium (60 ml), which contained span80 (0.9 g), tween80 (0.3 g) and magnesium stearate (0.1 g), heated at 50 °C and stirred at 1800 rpm. The formed microspheres were chemically cross-linked by vanillin or glutaraldehyde, *i.e.*, the cross linker acetone solution (10%, v/v) was added dropwise into the suspension mixture and stirred for 3 h. At the end, the CS microspheres were collected with centrifugation, and fully washed with petroleum ether followed by dimethyl carbinol to remove the residual liquid paraffin and acetone (Fig. 1). The vanillin cross-linked CS microspheres (VCM) and glutaraldehyde cross-linked CS microspheres (GCM) were dried at 50 °C for 12 h in an oven in air. The drug loaded vanillin cross-linked CS microspheres (DVCM) and drug loaded glutaraldehyde cross-linked CS microspheres (DGCM) were prepared separately by mixing the berberine into CS solution, followed by a similar procedure mentioned above.

2.3. Characterization of CS microspheres

2.3.1. Particle size analysis

The particle-size distribution of cross-linked CS microspheres was measured by laser diffractometry. The microspheres were re-dispersed in 500 ml distilled water and sized by laser particle sizer (BT-9300H, China).

2.3.2. Morphology

The optical microscope (Nikon TE-2000, Japan) was used for shape determination of the as-prepared crosslinked CS microspheres. The scanning electron microscopy (SEM, JEOL, JEM-100CX, Japan) was used to observe the microspheres dried at 60 °C, and observe the dried drug-loaded CS microspheres which were embedded in methyl methacrylate, cutting into sections of 1 mm in thickness. Samples for SEM examination were sputter coated with gold before observation.

2.3.3. X-ray diffraction analysis

The phase composition and crystallinity of microsphere samples were analyzed by X-ray diffraction (XRD, Philips X'Pert Pro MPD, Netherlands). XRD patterns were recorded through a 2θ range from 5° to 58° at a rate of 2°/min.

2.3.4. Fourier transform infrared spectroscopy

The infrared spectra of pure CS and cross-linked CS microspheres were collected on an attenuated total reflectance Fourier transform infrared spectrometry (TENSOR27, BRUKER Co. Germany). All spectra were recorded by transmittance mode (100 times scanning, 400–4000 cm^{-1}).

2.4. Swelling measurement

The swelling property of the CS microspheres was determined by immersion method. 200 mg of dry microspheres without drug were put into a beaker containing 20 ml PBS (phosphate buffered solution, 0.l M, pH 7.4) and shaking occasionally at 37 °C. At predetermined time intervals, the swollen microspheres were removed from the solution, collected by centrifugation and weighed immediately after removing surface water with filter paper. The degree of swelling (S_w) was calculated using the following equation:

$$S_w(\%) = \left(\frac{W_t - W_0}{W_0}\right) \times 100 \quad (1)$$

where W_t and W_0 represent the weight of the swollen and dry samples, respectively [21]. Each swelling test was done intriplicate. To simulate the flow of a biological liquid, the buffer solution was replaced at each time point.

2.5. In vitro *drug release*

2.5.1. Drug loading ratio and entrapment efficiency of microspheres

The microspheres (10 mg) containing drug were added into 100 ml anhydrous alcohol and heated with reflux condensation at

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