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Preparation of chitosan/nano hydroxyapatite organic–inorganic hybrid microspheres for bone repair

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ABSTRACT

In this work, we encapsulated icariin (ICA) into chitosan (CS)/nano hydroxyapatite (nHAP) composite microspheres to form organic–inorganic hybrid microspheres for drug delivery carrier. The composition and morphology of composite microspheres were characterized by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and differential scanning calorimetry– thermogravimetric analysis (DSC–TGA). Moreover, we further studied the performance of swelling properties, degradation properties and drug release behavior of the microspheres. ICA, the extract of traditional Chinese medicine-epimedium, was combined to study drug release properties of the microspheres. ICA loaded microspheres take on a sustained release behavior, which can be not only ascribed to electrostatic interaction between reactive negative hydroxyl (—OH) of ICA and positive amine groups ($—NH_2$) of CS, but also depended on the homogeneous dispersion of HAP nanoparticles inside CS organic matrix. In addition, the adhesion and morphology of osteoblasts were detected by inverted fluorescence microscopy. The biocompatibility of CS/nHAP/ICA microspheres was evaluated by the MTT cytotoxicity assay, Hoechst 33258 and PI fluorescence staining. These studies demonstrate that composite microspheres provide a suitable microenvironment for osteoblast attachment and proliferation. It can be speculated that the ICA loaded CS-based organic–inorganic hybrid microspheres might have potential applications in drug delivery systems.

1. Introduction

During recent decades, the organic–inorganic hybrid materials have attracted particular interest because of their excellent properties, such as surface functionality, composition, excellent mechanical properties and so on [1–3]. Simultaneously, biopolymer has frequently been used for the design of drug delivery formulations [4,5]. However, the burst release of drugs and weak mechanical properties were still big challenge in the application of drug delivery system [6]. It was mainly ascribed to the natural characteristics of the biopolymers, the weak interaction between the biopolymers and the drugs and the quick disintegration of the biopolymer carriers in the process of release [7–9]. Recently, the novel organic–inorganic hybrid composite microspheres, as a promising controlled drug delivery carrier, have been increasingly exploited to tackle the aforementioned problem owing to their unique structures and properties [10,11].

Chitosan (CS), the second-most abundant polysaccharide, has been proposed as a potential candidate for drug delivery applications because of its several outstanding characteristics such as biocompatibility, none immunogenicity, none cytotoxicity, mucus adhesion, and low cost [12–15]. CS is a natural positively charged polysaccharide which is biodegradable under the auxiliary of lysozyme [16]. Hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) is the main inorganic component of natural bone and has been studied extensively for medical applications as a function of excellent biocompatibility, bioactivity and osteoconductivity [17,18]. In our previous research, the nano HAP crystallized in situ from the CS matrix can achieve homogeneous distribution of nHAP in the CS organic matrix to form the nanohybrid scaffold [19]. The in situ scaffold possesses excellent bone conductibility and could accelerate the process of osteogenesis [20]. This shows the great advantage of the in situ method [21]. Besides, we prepared a sodium alginate–chitosan–geniposite (Alg–CS–Gip) composite

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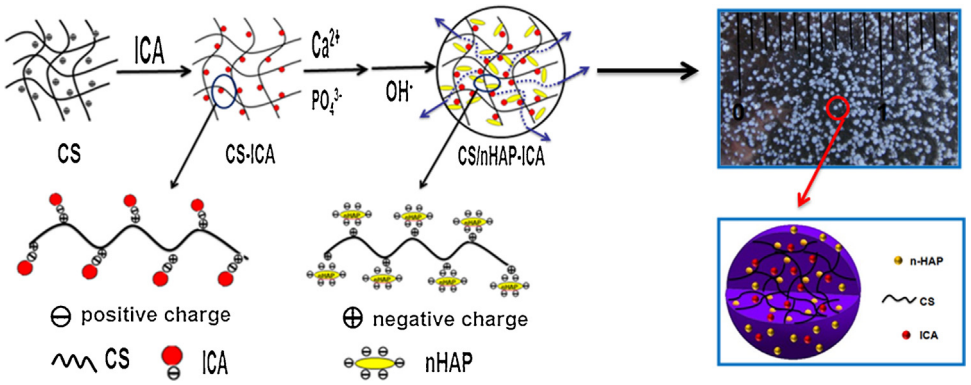

Fig. 1. The schematic representation of the in situ formation mechanism of CS/nHAP/ICA microspheres.

microsphere with the core-shell structure through the high voltage static microcapsule forming device. The simple and efficient microsphere forming process, mild forming condition and high activity of biomass are regarded as the advantages of this method [22]. ICA, the extract of traditional Chinese medicine-epimedium, has been proved as an osteoinductive agent for regeneration. Fan et al. [23] and Yang et al. [24] found that the combination of ICA and scaffold was biologically active and could promote the proliferation of osteoblasts in vitro bioactivity assay.

Here, we presented one-step method for preparing ICA-loaded CS/nHAP composite microspheres by the high voltage static microcapsule forming device. Fig. 1 displays the schematic representation of the in situ formation mechanism of CS/nHAP/ICA composite microspheres. The composition and morphology of composite microspheres were characterized by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and differential scanning calorimetry–thermogravimetric analysis (DSC–TGA). These determined that ICA loaded microspheres take on a sustained release behavior, which can be ascribed to electrostatic interaction between reactive negative hydroxyl radical (—OH) of ICA and positive amine groups ($—NH_2$) of CS and the homogeneous dispersion of HAP nanoparticles inside the organic matrix–CS. Subsequently, MTT, Hoechst 33258 and PI fluorescence staining demonstrated that composite microspheres provided a suitable microenvironment for the attachment and proliferation of osteoblast. It can be speculated that the ICA loaded CS-based organic–inorganic hybrid microspheres might have potential applications in a bone repairing drug delivery systems.

2. Experimental

2.1. Materials

Chitosan (CS) was obtained from the Shanghai Bio Life Science & Technology Co., Ltd. The degree of deacetylation was 92.8% and the viscosity molecular weight was 389,000 Da. Icariin (molecular formula: $C_{33}H_{40}O_{15}$, molecular weight = 676.67) was purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). $Ca(NO_3)_2{\cdot}4H_2O$ (A.R.) and $K_2HPO_4{\cdot}3H_2O$ (A.R.) were dissolved in deionized water to form 2 mol L^{-1} and 1.2 mol L^{-1} solution, respectively. Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) and Fetal bovine serum (FBS) were purchased from the HyClone Co. (USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromile (MTT) were purchased from the Biosharp Co. (USA). The reagents were all analytical grade and used without further purification. Deionized water was used in all experiments.

2.2. Preparation of ICA-loaded CS/nHAP composite microspheres

In this experiment, the CS/nHAP/ICA microspheres were synthesized by the following process. Briefly, moderate CS powder was dissolved in 46 ml of 2% acetic acid solution stirred for 2 h to form a homogeneous CS solution. The alcohol solution of ICA was added and stirred for 1.5 h magnetically at room temperature, followed by adding $Ca(NO_3)_2{\cdot}4H_2O$ and $K_2HPO_4{\cdot}3H_2O$ solution according to the ratio of Ca/P = 1.67, stirred for 3 h, to in situ prepare the CS/nHAP/ICA precursor solution. By the high voltage electrostatic micro capsule forming device (2.8 ± 0.5 KV), precursor solution was added dropwise in a 200 ml beaker containing 4 wt% NaOH aqueous solution (as coagulation bath). The mixtures were thoroughly mixed by occasional shaking and incubated for 12 h at 37 ± 0.5 °C to form microspheres. After several cycles of centrifugation at 7000 rpm for 5 min and washing with deionized water, the media gradually became a neutral and the microspheres were lyophilized for further use.

2.3. Phase and microstructure characterization

In order to determine the crystal structure of the samples, XRD measurements of CS and CS/nHAP/ICA microspheres were conducted by a diffractometer (X'Pert MPD Pro, PANalytical, Netherlands) with 40 KV and scanning range from 10 °C to 70 °C using Co radiation (λ = 0.178 nm). Fourier transform infrared spectra (FT-IR) were used to examine the constituents of the samples, which were collected in a FT-IR spectrometer (Thermo Nicolet 360, Nicolet, USA) in the region of 4000–500 cm^{-1}. The dry samples were powdered and mixed with KBr and then pressed into pellets under reduced pressure. The surface morphologies of the CS and CS/nHAP/ICA microspheres were observed by scanning electron microscopy (SEM) (Nova Nano SEM 230, FEI, USA). Samples for SEM observation were prepared by coating with a thin layer of gold after drying a drop of particle solution on an aluminum stub.

2.4. In vitro degradation and release experiments

2.4.1. Study of thermograms

Thermograms of the composite microspheres were conducted using DSC and thermal gravimetric analysis (TGA) (TA 449C, TA Instruments-Waters LLC, USA) under constant argon purging. The samples were heated from 0 to 800 °C at a heating rate of 10 °C min^{-1} in air and a gas flow rate of 30 ml min^{-1}.

2.4.2. Study of hydrolytic degradation

In order to detect the degradability investigation of microspheres, phosphate buffer solution (PBS, pH 7.4, 37 °C) containing 500 μg/ml was used as a medium. The samples were placed in 5 ml