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Bioinspired double polysaccharides-based nanohybrid scaffold for bone tissue engineering

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ABSTRACT

The fabrication of bone scaffolds with interconnected porous structure, adequate mechanical properties and excellent biocompatibility presents a great challenge. Herein, a hybrid nanostructured chitosan/chondroitin sulfate/hydroxyapatite (ChS/CSA/HAP) in situ composite scaffold was prepared by in situ fabrication and freeze-drying technique. The composition and morphology of scaffold were characterized by Fourier transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD) and scanning electron microscopy (SEM). It proved that the low crystallinity of HAP crystals were uniformly distributed in ChS/CSA organic matrix and the nanostructured hybrid scaffold exhibited good mechanical property. The biocompatibility and in vitro bioactivity were detected by MTT-assay, maturation (alkaline phosphatase (ALP) activity), Hoechst 33258 and PI fluorescence staining. In vitro tests indicated that the hybrid scaffold not only promoted the adhesion and proliferation of osteoblasts, but also improved the growth of the osteoblasts. Therefore, it is promising for bone repair application in bone tissue engineering.

1. Introduction

In recent decades, the repair of large bone defects resulting from trauma, traffic accidents and surgical resection is still an urgent and complicated problem in orthopedic surgery [1,2]. Fortunately, owing to its success in the repair and regeneration of bone defects [3–5], bone tissue engineering (BTE) has been drawn much attention. As we all know, natural bone is a natural mineralized biological material consisting of around 65 wt% apatite mineral, 25 wt% organic compound and 10 wt% water [6,7]. Hence, the ideal bone scaffolds for BET ought to have interconnected porous structure, adequate mechanical properties and excellent biocompatibility [8,9], and also similar chemical composition, crystallinity and crystallographic texture to natural bones [5], which are especially important in the reconstruction of critical-sized bone defects in the wounded positions. So bone substitutes and scaffolds become essentially important in clinical orthopedic applications, especially in large bone defect healing.

Hydroxyapatite (HAP) and Chitosan (ChS) are of great interest in the bioactive biomaterials in BTE in that they have excellent biocompatibility with human body [10]. Chitosan (ChS) is the second-most abundant natural polysaccharide with alkalescence and positive charge which is composed of β-1,4-linked D-glucosamine (deacetylated section) and *N*-acetyl-D-glucosamine (acetylated section) [11]. Owing to its outstanding characteristics, such as inherent wound healing properties, biocompatibility, biodegradability, suitability for cell in growth, intrinsic antibacterial nature [12,13] non-toxicity and low cost [11], ChS has been widely used as a scaffold in orthopedic and other biomedical applications. Due to excellent biocompatibility and in vitro bioactivity [14,15], HAP ($Ca_{10}(PO_4)_6(OH)_2$), which is the main ingredient of the inorganic component in natural bone, has been extensively applied in medical fields and demonstrated great biological efficacy [16]. However, its disadvantage, such as brittleness, makes it hard to shape and limits its usage [17]. In order to overcome this challenge, previous studies have indicated that nano-crystalline HAP combined with synthesized or natural polymer could increase the adhesion and proliferation of osteoblasts [18]. Chondroitin sulfate (CSA), which is biodegradable, is another major sulfated gly-

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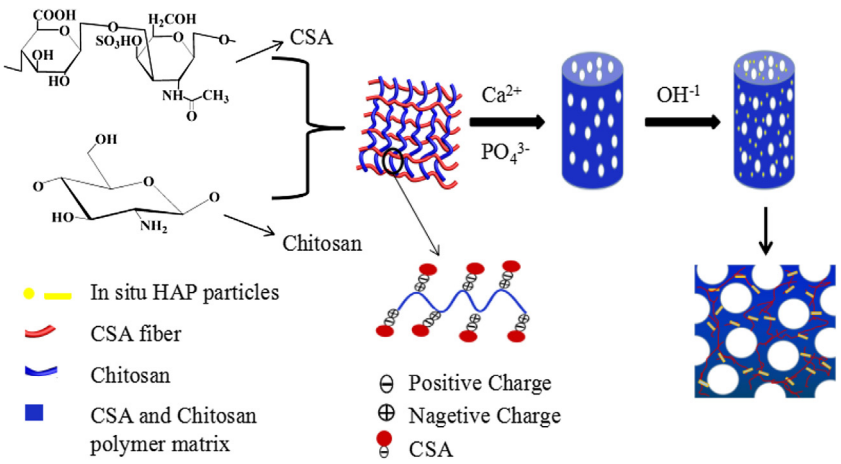

Fig. 1. The schematic representation of the in situ formation mechanism of ChS/CSA/nHAP hybrid scaffold.

cosaminoglycan. It includes of b-1,3-linked *N*-acetyl galactosamine (GalNAc) and b-1,4-linked D-glucuronic acid (GlcA) with certain position(s) sulfated [19,20]. In addition of chondroitin sulfate in scaffold may improve the ability of the secretion of proteoglycan and collagen [21]. CSA is an ECM component with high negative charge density that could be combined with ChS to produce devices with biomedical applicability [22].

In this paper, we reported a convenient method to prepare ChS/CSA/nHAP in situ composite scaffold based on double polysaccharides by in situ fabrication and freeze-drying technique. Fig. 1 showed the schematic diagram about the in situ formation mechanism of ChS/CSA/nHAP composite scaffold. Results of the composition and morphology indicated in situ formed nHAP particles were crystallized on a natural origin ChS and CSA polymer matrix, and uniformly distributed in the scaffold. These can be explained that between positive amine groups (-NH_2) of ChS and reactive negative hydroxyl radical (• -COOH) of CSA occurred electrostatic interaction, which leaded the homogeneous dispersion of HAP nanoparticles in the ChS/CSA organic matrix. Results of MTT assay, maturation (alkaline phosphatase (ALP) activity), Hoechst 33258 and PI fluorescence staining demonstrated that the scaffold had good biocompatibility and could enhance the expression of osteoblast cells functions. It indicated that the ChS/CSA/nHAP hybrid scaffold might have potential applications in bone repairing systems for bone tissue engineering.

2. Materials

Chitosan (ChS) was obtained from Shanghai Bio Life Science &Technology Co., Ltd. Chondroitin sulfate (CSA) was provided by BIO BASIC INC. The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-Hydroxysuccinimide (NHS) were supplied by GL Biochem. (Shanghai) Ltd. Beta-Glycerol Phosphate Disodium Salt Pentahydrate was purchased from Guangzhou QiYun Biological Technology Co., Ltd. $Ca(NO_3)_2{\cdot}4H_2O$ (A.R.) and $K_2HPO_4{\cdot}3H_2O$ (A.R.) were dissolved in de-ionized water to form 2 mol l^{-1} and 1.2 mol l^{-1} solution, respectively. A 2% (V/V) acetic acid solution was prepared before used.

3. Methods

The ChS/CSA/nHAP in situ scaffolds were similarly prepared by in situ crystallization technology using our previously published method [23]. Briefly, 1 g ChS and 0.11 g CSA were added into 1 ml of 1% acetic acid to form the homogeneous solution, followed by addition of 3 ml $Ca(NO_3)_2$ and 3 ml K_2HPO_4 aqueous solution under stirring, and agitation till the mixed solution was obtained. Then the crosslinking agent EDC/NHS according to the ratio of EDC: NHS: COOH = 2:1:1 were added into the above solution to crosslink for 5 h under room temperature so as to obtain the precursor mixture. The precursor mixture was transferred into a 24-well plate and kept at 4 °C for another 10 h. And the mixture was frozen at −20 °C and later lyophilized to form the beginning porous scaffold. The porous scaffold was immersed into 2.5 wt% NaOH ethanol/de-ionized water for 10 h at room temperature in order to make in situ crystallization. The porous scaffold was rinsed with de-ionized water till the pH became neutral. Lastly, the scaffold was lyophilized again to obtain the final ChS/CSA/nHAP in situ scaffolds. The preparative procedure of the organic ChS/CSA scaffold which was the control group of cytological research was followed by the preparation of ChS/CSA/nHAP in situ composite scaffold.

3.1. Physicochemical characterization of the scaffolds

The morphology and microstructure of the scaffolds were characterized by scanning electron microscopy (Nova Nano SEM 230, FEI, USA).

The phase composition of the scaffold was detected by X'Pert MPD Pro, PANalytical, Netherlands with 40 KV and the scanning range from 10° to 70° using Co radiation (λ = 0.178 nm).

The interaction between the groups among the components present in the scaffolds was analyzed by the Fourier transform infrared spectroscopy (FT-IR) (Nicolet Nexus spectrometer) with the scanning range from 4000 to 400 cm^{-1}.

3.2. Mechanical properties

The compressive strengths of the in situ scaffolds were tested by a universal material testing machine (AG-1, Shimadzu) at a cross-head speed of 0.5 mm/min. And when the strain was 20%, the compressive stress was recorded. Each measurement was repeated 3 times.

3.3. Porosity

The porosity of in situ scaffolds was tested by a liquid displacement method [24]. A sample which was weighted in air (m_1) was immersed in a weighing bottle containing enough ethanol in order to submerge the sample for several minutes. And under vacuum condition, the bottle was transferred to a glass desiccator, which was better to impel the ethanol into the scaffold pores. The ethanol-impregnated scaffold was weighed in air (m_2) and in ethanol (m_3)