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Effect of *in situ* formed hydroxyapatite on microstructure of freeze-gelled chitosan-based biocomposite scaffolds

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ABSTRACT

New *in situ* highly-porous chitosan/hydroxyapatite (CS/HA) biocomposite scaffolds have been prepared via freeze-gelation technique. Different content of *in situ* synthesized hydroxyapatite within chitosan solution was obtained by changing the amount of calcium and phosphate precursors. The composition of precipitated inorganic phase was characterized by X-ray diffraction analysis (XRD) and Fourier transformed infrared spectroscopy (FTIR), while morphology of scaffolds was imaged by scanning electron microscopy (SEM). SEM observations of cross section and surface area of prepared scaffolds have shown different microstructure and topography regarding to the HA content, which plays an important role in cell adhesion and proliferation, and nutrient transport. The MTT assay of scaffolds with different content of hydroxyapatite has shown no toxicity which is one of the main requirements for potential biomedical application. Likewise, the presented synthesis allows preparing the scaffolds with large and very well interconnected pores without obtaining toxic intermediate products.

1. Introduction

Regeneration and repair of damaged bone and cartilage draw large attention in tissue engineering. So far, the most promising therapy for defect reparation is based on seeding and *in vitro* culture of primary osteoblast cells (preosteoblasts) on synthetic or natural three-dimensional scaffolds. High porosity (larger than 90%) with large pore size (100–200 μm) and interconnected porous structure for cells and nutrients transport is one of the important factors for scaffold engineering [1,2].

Numerous investigations involving different scaffold preparation methods for chitosan/composite systems have been performed such as freeze-drying [3,4], electrospinning [5,6], particle leaching [7,8], foaming process [9] and combination of two different methods (freeze-drying and 3D printing [10,11], freeze-extraction and thermally induced phase separation [12], freeze-drying and particle leaching [13], electrospraying/freeze-drying [14]).

Freeze-gelation is an easy method for producing scaffolds from polymer or composite hydrogels for tissue engineering applications. It is based on thermally induced phase separation of ice crystals and polymer matrix within frozen polymer

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solution. The resultant porous structure is obtained by simultaneous gelling the hydrogel and removing the ice crystals (frozen solvent) under freeze conditions, in a non-solvent bath. Freeze-gelation method allows scaffold preparation with various porosity, and pore size and shape, depending on working parameters: (1) freezing temperature, (2) cooling rate (deep freezer or liquid nitrogen), (3) bathing temperature, (4) bathing time, and (5) polymer nature. Hsieh et al. [15] have prepared different scaffolds based on chitosan, alginate and carboxymethyl cellulose using different cooling modes (fast/slow), and have concluded that cooling rate is predominant factor that affects pore size and indirectly mechanical properties of the scaffolds. Although freeze-gelation is not so used method for scaffold preparation, it takes less time and energy regarding to the freeze-drying technique.

Several studies have used freeze-gelation as a method for producing large pore size and good pore interconnectivity [16–19], but neither of reviewed studies obtained visual pores larger than 100 μm.

Porosity of hydrogels has a major influence on mechanical properties, which are the main drawback regarding the load-bearing application in tissue engineering. The addition of another polymer or crosslinking agent [20–22] could improve compression strength, but alteration of microstructure (pore size and shape, interconnectivity) and decrement of biocompatibility (cytotoxicity) of material is inevitable [23].

In our previous research paper [24], we have presented an easy method of synthesizing *in situ* HA within chitosan solution using non-toxic and cost effective precursors. In this way, we have obtained homogeneous colloidal composite solution by avoiding the HA-particle agglomeration, which is the main problem in usual polymer–hydroxyapatite particles blending. Besides remarkable antibacterial and antifungal activity, and enzymatic biodegradability [23], the pH-dependable solubility of chitosan in aqueous solution has allowed homogeneous precipitation of *in situ* carbonated hydroxyapatite (CHA). The aim of this work has focused on the chitosan ability to form a hydrogel in basic aqueous solution, which simultaneously represents physical crosslink of chitosan matrix. In following paper, producing chitosan/hydroxyapatite (CS/HA) composite scaffolds with suitable porosity has been obtained from composite suspensions via freeze-gelation method. Amount of HA precursors within chitosan solution for *in situ* HA precipitation has been varied. The effect of content of *in situ* synthesized HA on scaffold's microstructure has been studied. In addition as composites with potential application in the regeneration of bone, non-cytotoxicity was assessed by culturing fibroblasts L929 with extracts of different scaffolds following standard conditions. To authors' knowledge, this is the first time of presenting the influence of *in situ* synthesized HA on microstructure and topography of freeze-gelled chitosan-based composite scaffolds.

2. Experimental procedure

2.1. Materials

Chitosan (CS, $\overline{M}_W = 100,000–300,000$ g mol^{-1}, DD = 0.95–0.98, Acros Organics), calcium carbonate ($CaCO_3$, calcite; TTT), urea phosphate ($(NH_2)_2CO–H_3PO_4$; Aldrich Chemistry), acetic acid (HAc; POCH), sodium hydroxide (NaOH, Gram–Mol) and ethanol (EtOH, 96%, Kefo) were all of analytical grade.

2.2. Synthesis of chitosan/hydroxyapatite (CS/HA) biocomposites

Based on our previous study [24], syntheses of CS/HA composites with different content of HA precursors were done as follows: specific amount of calcite was suspended in 1.2 wt% chitosan solution (prepared in 0.36% acetic acid) at ambient temperature. Then, appropriate amount of urea phosphate, respect to the Ca/P ratio 1.67, was added into the suspension and temperature was set at 50 °C. Stirring was continued for 4 days. The precursor's amount was adjusted to obtain 0, 10, 20, 30, 40, 50 and 60 wt.% of final *in situ* precipitated HA. To characterize the composition of *in situ* synthesized inorganic phase, one portion of each prepared composite suspension was solvent-casted at 37 °C, i.e. suspensions were dried at 37 °C to obtain composite films (SC composites). The other portion of suspension was used for porous composite scaffold preparation (FG composites). Pure chitosan and composite films with thickness of 0.2–0.5 mm obtained by solvent-casting are denoted as 0-SC, 1-SC, 2-SC, 3-SC, 4-SC, 5-SC and 6-SC (Table 1).

Table 1
Sample's denotation regarding the materials composition.

System	Sample abbreviation	Precursor's amount (%)		Ca/P ratio	Final HA amount (%)
		Calcite	UPH		
Chitosan	0-SC; 0-FG	0	0	0	0
Chitosan/10HA	1-SC; 1-FG	9.95	9.49	1.67	10
Chitosan/20HA	2-SC; 2-FG	19.94	19.09	1.67	20
Chitosan/30HA	3-SC; 3-FG	29.92	28.80	1.67	30
Chitosan/40HA	4-SC; 4-FG	39.90	38.61	1.67	40
Chitosan/50HA	5-SC; 5-FG	49.89	48.55	1.67	50
Chitosan/60HA	6-SC; 6-FG	59.89	58.60	1.67	60

SC: solvent-casting method; FG: freeze-gelation method.