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Formation of nano-hydroxyapatite crystal *in situ* in chitosan–pectin polyelectrolyte complex network

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ABSTRACT

Hydroxyapatite (HA)/polysaccharide composites have been widely used in bone tissue engineering due to their chemical similarity to natural bone. Polymer matrix-mediated synthesis of nano-hydroxyapatite is one of the simplest models for biomimetic. In this article, the nano-hydroxyapatite/chitosan–pectin (nHCP) composites were prepared through *in situ* mineralization of hydroxyapatite in chitosan–pectin polyelectrolyte complex (PEC) network. The formation processes of nHCP were investigated by X-ray diffraction (XRD) analysis. The interactions between nHA crystal and chitosan–pectin PEC networks were studied using Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC). The morphology and structure of nHA crystal were characterized by XRD and Transmission Electron Microscope (TEM). Results suggested that the interfacial interactions between nano-hydroxyapatite crystal and chitosan–pectin PEC network assist the site specific nucleation and growth of nHA nanoparticles. The nHA crystals grow along the *c*-axis. In this process, pH value is the main factor to control the nucleation and growth of nHA crystal in chitosan–pectin PEC networks, because both the interactions' strength between nHA crystal and chitosan–pectin and diffusion rate of inorganic ions depend on the pH value of the reaction system. Apart from the pH value, the chitosan/pectin ratio and $[Ca^{2+}]$ also take important effects on the formation of nHA crystal. An effective way to control the size of nHA crystal is to adjust the content of pectin and $[Ca^{2+}]$. It is interesting that the Zeta potential of nHCP composites is about −30 mV when the chitosan/pectin ratio≦1:1, and the dispersion solution of nHCP composites has higher stability, which provides the possibility to prepare 3D porous scaffolds with nHCP for bone tissue engineering.

1. Introduction

Hybrid organic/inorganic materials can offer great advantages because of their wide variety of properties, and these materials are often found in living body especially in hard tissue. For example, bone and teeth are actually organic/inorganic composites mainly made up of nano-hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, nHA) and collagen fibers [1]. In living organisms, tens to hundreds of nano-blocks, under the control of an organic matrix, combine into self-assembled biomaterials. This process exhibits a high level of spatial control as the mineralization usually takes place in a confined reaction environment. Fabrication of materials that resemble bone, even at the lowest level of hierarchical organization, is more difficult because it involves two dissimilar organic and inorganic nanophases that have a specific spatial relation with respect to one another. A "matrix-mediated" mineralization is believed to imitate this structure [2]. This process is mimetic to prepare the ideal bone tissue substitute *in vitro*. And "matrix-mediated" mineralization is very important for understanding the fundamental science of biomineralization and development of new materials with tailored structure and properties. This confined reaction environment is constructed by biomolecules — mainly by polymers [3]. These polymers can provide HA-forming ability and control mineralization events; their main task is to control the nucleation and growth of hydroxyapatite crystal. It seems possible to control the structure and arrangement of polymer to modulate the morphology of HA *in vitro* just as what may occur *in vivo*. Interfacial molecular interactions at polymer–HA interface can assist the site specific nucleation and growth of nanoparticles. In association with lattice matching, these interactions also determine the crystallographic structure and orientation of nucleating of HA [4]. Recently, many researchers found that size [5,6], surface properties [7] and crystallinity [8,9] of HA in polymer–HA composites greatly affect cell-material interactions. In addition, mechanical properties of these composites depend on the molecular weights of polymers [10] as well as the interactions between HA crystal and polymer chain [11]. They

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are expected to act as novel artificial bone substitutes exhibiting not only bioactivity but also mechanical performance analogous to natural bone.

Polysaccharides, existing in living organism, are often used as template to study mineralization behaviors of hydroxyapatite crystal. Many functional groups of these polysaccharides, such as hydroxyl, carboxyl, sulfonic group and amino group, are known to induce apatite nucleation and influence growth of HA crystal. And concentration of polysaccharides, type and concentration of inorganic ions, pH value and temperature are also the main factors to control mineralization process. For example, Liu et al. [12] found that staple-fiber-like HA crystals can be obtained at a low concentration of chondroitin sulfate template, while flake-like HA crystals are synthesized at a higher concentration (0.5 wt.%) of chondroitin sulfate.

The polysaccharide chitin is found in nature as a major component of organic fraction of several biocomposites in which an organic matrix is associated with an inorganic fraction. And in the mineralized tissue either α or β form is present. Chitin and chitosan, the latter being obtained by partial deacetylation of chitin, possess the inherent physical and biological characteristics that may render them useful as components in tissue replacement material. And they have a crucial role in the hierarchical control of biomineralization processes [13]. The chitin and chitosan molecules contain C=O, OH, and NH_2 groups and oxygen atoms, which show high affinity to the charged groups of octacalcium phosphate (OCP), the compartmentalized space on the layered chitin governs the orientation of the OCP crystals [14], the OCP crystal structure consists of alternating hydrated and apatite layers, which resemble HA. When pH<5, the calcium phosphate is calcium hydrogen phosphate dehydrate (DCPD) in chitosan-phosphorylated/chitosan system, on the other hand, alkaline conditions facilitate the formation of HA [15]. We found that the size of nHA crystal formed on the chitosan–gelatin network film surface can be modulated via adjusting the charge types and density of chitosan–gelatin template, moreover, the reaction temperature is very important for the formation of HA on chitosan–gelatin films [16]. The pectins are polyanions which consist of linear regions of (1-4)-R-D-galacturonosyl units and their methyl esters, interrupted by (1-2)-R-L-rhamnopyranosyl units, which has been recently attracting much attention as novel biomaterials to improve the proliferation of osteoblast [17,18]. Moreover, the carboxyl groups of pectin play important roles in the process of mineralization, which have a catalytic effect for heterogeneous apatite nucleation. But the methoxyl degree of pectin separated from different kinds of plants is often diverse. Ichibouji et al. [19] found that the apatite-forming ability on various pectins is in the order of (pectic acid)<(apple-derived pectin)<(citrus-derived pectin) due to an increase of the amount of carboxyl group. And low methoxyl pectin can be used to fabricate hydrogels by forming so-called egg-box structure with Ca^{2+} ions.

Although both chitosan and pectin could assist the formation of HA and control growth of HA crystal, the modulation capacity is limited owing to the single nucleation site when they are used separately. A polyelectrolyte complex (PEC) hydrogel is formed from anionic pectin and cationic chitosan under specific pH conditions [20]. The strength of interactions between chitosan and pectin could be controlled via adjusting pH. The various functional groups and adjustable interactions in chitosan–pectin PEC system could provide multiple nucleation sites and growth space for HA crystal. Therefore, in this study, the hydroxyapatite/chitosan–pectin nanocomposites (nHCP) were prepared. This strategy has distinct advantages: the synthesis method is simple and low-cost; the morphology and structure of nHA crystal can be modulated by chitosan–pectin PECs network. And the formation process of nHA crystal could be controlled by pH and ageing time. Moreover, excellent biocompatibility of chitosan and pectin endows the nHCP composites potential application in bone tissue engineering.

2. Materials and methods

2.1. Materials

Chitosan (CS, mean molecular weight 2.0×10^5 Da, deacetylation, >85%) was supplied by Qingdao Medical Institute (Qingdao, China). Pectin (citrus-derived) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytic grade.

2.2. Hydroxyapatite/chitosan–pectin nanocomposites preparation

Chitosan was dissolved in acetic acid solution (1% v/v) and pectin was dissolved in deionized water. Then $Ca(NO_3)_2$ was added into pectin solution and NaH_2PO_4 was added into chitosan solution, at a molar ratio of Ca/P=1.67. After 5 h, the NaH_2PO_4/chitosan solution was added drop wise into $Ca(NO_3)_2$/pectin solution with magnetic stirring. The pH was adjusted to 5, 7, 9 and 13. At special ageing time (Table 1), the precipitate was separated by centrifugation and washed with deionized water to pH=7.0. Further, the precipitate was lyophilized. All these steps except lyophilization were conducted at room temperature. The detail preparation parameters were listed in Table 1.

2.3. Characterization

The formation process of HA crystal was investigated by X-ray diffraction (XRD) analysis (Rigaku D/max 2500v/pc) under the operating conditions of 40 kV and 200 mA. The chemical interactions between nHA crystal and functional groups of chitosan–pectin PEC were estimated by FTIR (MAGNA-560, Nicolet, USA). The crystallite morphology of the nHCP composites was analyzed by Transmission Electron Microscope (TEM, JEM-100CX II, Japan) and selected area electron diffraction (SEAD) operated at 100 kV. Thermal characterization was carried out using Differential Scanning Calorimeter (DSC, Netzsch, Germany). Zeta potential of nHCP aqueous solution was performed on Zetasizer Nano S, Malvern Instruments. The stability of nHCP in aqueous solution was observed after dispersing the nHCP composites in deionized water under ultrasonication (sonication power 400 W and work time 450 s).

3. Results and discussion

3.1. Interaction between hydroxyapatite and chitosan–pectin PEC

The FTIR spectroscopy was performed to understand the interactions between nHA crystals and chitosan–pectin PECs. Fig. 1 shows the FTIR curves of nHCP synthesized in different chitosan–pectin PECs. All these nHCP composites give typical peaks of phosphate vibration at

Table 1
nHCP composites and their preparation conditions.

Samples	Chitosan/pectin(w/w)	$[Ca^{2+}]$ (mol/L)	pH	Reaction time (h)
nHCP-1	1:0	0.2	13	48
nHCP-2	7:3	0.2	13	48
nHCP-3	5:5	0.2	13	48
nHCP-4	3:7	0.2	13	48
nHCP-5	0:1	0.2	13	48
nHCP-3(0.1)	5:5	0.1	13	48
nHCP-3(0.3)	5:5	0.3	13	48
nHCP-3(4 h)	5:5	0.2	13	4
nHCP-3(8 h)	5:5	0.2	13	8
nHCP-3 (12 h)	5:5	0.2	13	12
nHCG-3(48 h)	5:5	0.2	13	48
nHCG-3(pH5)	5:5	0.2	5	48
nHCG-3(pH7)	5:5	0.2	7	48
nHCG-3(pH9)	5:5	0.2	9	48
HA	–	0.2	13	48