Fabrication and properties of mineralized collagen-chitosan/hydroxyapatite scaffolds

Haiguang Zhao, Lie Ma*, Changyou Gao* and Jiacong Shen
Department of Polymer Science and Engineering, Zhejiang University, Key Laboratory of Macromolecular Synthesis and Functionalization, Ministry of Education, Hangzhou 310027, China

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A hydroxyapatite (HAp)/biopolymer composite scaffold was fabricated by mineralizing a cross-linked collagen/chitosan, which was pre-mineralized with Ca\(^{2+}\) and phosphate salts, in simulated body fluid (SBF) for only 24 hr. A self-organized structure similar to bone is expected. Microstructures of the crosslinked collagen/chitosan scaffold, the pre-mineralized collagen–chitosan scaffold (CCS), and the mineralized collagen-chitosan/HAp scaffolds (MCCHS) were characterized by scanning electron microscopy (SEM), revealing non-alteration of the porous structure and formation of the HAp particles. X-ray diffractometer (XRD) confirmed the crystalline structure of the HAp. Thermal gravimetric analysis found that more HAp particles were formed when the CCSs were pre-mineralized in a higher concentration of Ca\(^{2+}\). Water-uptake ratio of the crosslinked CCS was ~160, decreased to ~120 after incubating in Ca\(^{2+}\) solution, and further decreased to ~20 after mineralization. Mechanical strength of the CCS was improved significantly after the in situ mineralization too. The method introduced here may be potentially applied to obtain other biopolymer/HAp composite in a short period. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: collagen; chitosan; biomaterials; hydroxyapatite; biomineralization

INTRODUCTION

Bone loss and defects are frequently encountered in clinics. Several methods such as bone substitutes of xenografts, allografts, and autografts have been applied in clinics to repair the bone defects. However, these bone substitutes often suffer from the problems of antigenicity and limitation of donor sites. In the past few decades, bone tissue engineering and regenerative medicine has appeared as a new approach in regenerating bone tissues completely. Many artificial bone equivalents are being used as bone graft substitutes. Among these applications, tissue engineering biomaterials are indispensable, which function as scaffolds with adequate mechanical strength, osteoconductivity, and controllable biodegradability. Natural and synthetic polymers such as polysaccharides, poly(α-hydroxy ester), hydrogels, and thermoplastic elastomers are among the categories of often applied bone scaffolds. Other important ones are bioactive ceramics such as calcium phosphates and bioactive glasses. The calcium phosphate compounds including hydroxyapatite (HAp), α-tricalcium phosphate (α-TCP), and β-tricalcium phosphate (β-TCP) are more extensively applied as the bone substitutes. HAp is a major constituent of bone which has osteoconductivity. However, to overcome its brittleness and poor processing property, more recently, attention has been paid to the composites of polymers and ceramics, with the aims of increasing the mechanical stability and improving tissue interaction too.

It is known that collagen fibril and HAp are the main components of bone, thus their composite is more promising to elicit good bone tissue reaction. A typical way to obtain their composites is to drop a calcium-containing alkaline solution to a phosphoric acid solution containing dissolved type I collagen. However, such collagen/HAp composites are demonstrated little bone tissue reaction because of lack of bioactive surface. A bioactive surface would accelerate the bone growth, thereby shortening the healing time. Nowadays, simulated body fluid (SBF), which is a tris-buffer solution with inorganic ion concentrations almost equal to those of human plasma, is used to form a bioactive surface on the bone scaffold. However, formation of the HAp requires the existence of surface functional groups which induce nucleation of HAp. Therefore, the time is rather long in order to obtain large amounts of bone-like HAp in the collagen scaffold.

Based on these considerations, in this work a crosslinked collagen–chitosan is used to synthesize a collagen–chitosan/HAp scaffold (CCHS) in situ first, and then to obtain the mineralized collagen–chitosan/HAp scaffold (MCCHS) by incubation in the SBF (Fig. 1). The in situ formed HAp can function initially as the nucleus, thus the growth of HAp in the SBF can be accelerated. Moreover, the resulted MCCHS...
has a similar structure and composition as the nature bone. Properties of the CCHS and MCCHS such as microstructures, water-uptake, and mechanical strength shall be evaluated as well.

MATERIALS AND METHODS

Materials
Chitosan ($M_w = 1.0 \times 10^5$–$1.7 \times 10^5$, 75–85% deacetylation degree) was purchased from Sigma. Collagen type I was isolated from fresh bovine tendon by a trypsin-digestion and acetic acid dissolution method. All other chemicals were used as received. Triple-distilled water was used throughout the experiment.

Preparation of collagen–chitosan scaffold (CCS)
Collagen and chitosan were dissolved in 0.5 M acetic acid to form a 0.5% (w/v) solution in a mass ratio of 9:1. After deaeration under reduced pressure to evolve entrapped air bubbles, the collagen–chitosan composite was injected into a home-made mold, frozen in a refrigerator at $-20^\circ C$ for 2 hr, and then lyophilized for 48 hr to obtain a porous CCS. After incubated in 0.5 M acetic acid solution until the scaffold regained its swelling state, the CCS was crosslinked with 0.25% (w/v) GA at $4^\circ C$ for 12 hr. The crosslinked scaffold was extensively rinsed with water to remove the excess GA, and then was freeze-dried to obtain the dried crosslink-CCS.

Preparation of CCHS
The crosslinked CCS was incubated in 0.5 M acetic acid until the swelling state was regained, and then was incubated in 0.05, 0.1, 0.25, or 0.5 M CaCl$_2$ solution for 1 hr. A diammonium hydrogen phosphate (NH$_4$)$_2$HPO$_4$ solution with different concentration (0.03, 0.06, 0.1, or 0.3 M) was quickly dropped into the CaCl$_2$ solution having CCS at a final Ca/P molar ratio of 1.67. After the pH value of the mixture was adjusted to 11, the reaction was allowed to take place at 25°C for 30 min. The obtained scaffold was extensively washed with water, frozen at $-20^\circ C$ for 2 hr, and lyophilized for 48 hr to obtain the dried CCHS.

Preparation of MCCHS
The CCHS was incubated in 0.5 M acetic acid until the swelling state was regained, and then extensively washed with triple-distilled water. The scaffold was then soaked in a SBF (2.5 mM Ca$^{2+}$, 142.0 mM Na$^+$, 1.5 mM Mg$^{2+}$, 5.0 mM K$^+$, 192.8 mM Cl$^-$, 4.2 mM HCO$_3^-$, 1.0 mM HPO$_4^{2-}$, 0.5 mM SO$_4^{2-}$, 50 mM (CH$_2$OH)$_3$CNH$_2$, and 45.0 mM H$^+$) at pH 7.4 and 37°C in a shaking flask. After incubated for 12–24 hr, the scaffold was extensively washed with water, and frozen at $-20^\circ C$ for 2 hr and lyophilized for 48 hr to obtain the dried MCCHS.

Characterization
Fourier transform infrared (FTIR) spectra were recorded on a BRUKER VECTOR22 spectrophotometer. KBr tablets containing the samples were prepared and used for the measurement. The crystalline phase of the CCHS and MCCHS was determined in an X-ray diffractometer (XRD, RIGAKU/MAX-C). Spectra were recorded from $2\theta = 10^\circ$ to $80^\circ$ at a scanning rate of 2 $^\circ$/min and a step size of 0.02 $^\circ$. Microstructure of the samples were observed under scanning electron microscopy (SEM, JEOL JEM) after the samples were coated with an ultrathin gold layer. Thermal stability of the samples were evaluated by thermogravimetry (NET.ZSCH STA 409PG). The samples weighting 10–15 mg were heated from 50$^\circ$ C to 800$^\circ$ C with a heating rate of 10$^\circ$/min in nitrogen atmosphere. The weight retention degree is defined as the ratio of remained weight at a given temperature to the initial weight. The hydroxyapatite (HAp) weight ratio is defined as the weight of HAp/the weight of CCS. The HAp weight ratio was also obtained by a microbalance. The water-uptake ratio was obtained by incubation of the samples ($W_0$) in water at room temperature for 2 hr. After the surface water was adsorbed by a filter paper, the weight ($W_t$) was determined by a microbalance. The water-uptake ratio is defined as ($W_t - W_0$)/$W_0$. Each value was averaged from five parallel measurements. Dynamic rheological properties of the samples were measured by an advanced rheometric expansion system (ARES, Rheometric Scientific Inc.) at 1% strain with 20 mm parallel plates. Before each rheological test, linear dependence of viscoelasticity on frequency at the test temperature was checked. For dynamic
measurements, an increasing oscillatory frequency ranging from 0.1 to 100 Hz at a fixed recorded oscillatory strain of 1% at 37°C was applied, and the storage (G') and loss (G'') modulii were recorded.

Statistical analysis
Experimental data were analyzed using two-population Student’s t-test. The significant level was set as p < 0.05. Results are reported as mean ± standard deviation.

RESULTS AND DISCUSSION
Interaction between collagen/chitosan and HAp
HAp could be readily synthesized by adding (NH₄)₂HPO₄ solution to the crosslinked CCS containing CaCl₂ solution, as shown in the bottom of Fig. 1. By changing the concentration of CaCl₂ solution and (NH₄)₂HPO₄ solution, the molar ratio of Ca–P can be adjusted to 1.67 which is same as that of HAp. The crosslinked CCS was incubated in 0.5 M acetic acid until swelling state was regained, and then was incubated in 0.05, 0.1, 0.25, or 0.5 M CaCl₂ solution for 1 hr. A (NH₄)₂HPO₄ solution with different concentration (0.03, 0.06, 0.1, or 0.3 M) was quickly dropped into the CaCl₂ solution having CCS at a final Ca/P molar ratio of 1.67.

Characterization of CCHS by FTIR was firstly carried out to determine the crystal structure and to confirm the presence of HAp (Fig. 2). The spectrum of HAp (Fig. 2(a)) shows typical peaks of phosphate vibration at 1030–1034 cm⁻¹, phosphate ν₄ bending vibration at 565 and 604 cm⁻¹. The spectrum of the crosslinked CSS (Fig. 2(b)) shows C=O band at 1658 cm⁻¹, amide I band at 1584 cm⁻¹, and amide II band at 1416 cm⁻¹. Peak shifting was observed after the crosslinked CCS was soaked with Ca²⁺ ions (Fig. 2(c)) and further reacted with (NH₄)₂HPO₄: the C=O band shifted to lower wavenumber at 1630 cm⁻¹, and further to 1616–1618 cm⁻¹ after formation of the CCHS (Fig. 2(d–g)). Spectra of all the CCHS (Fig. 2(d–g)) show also the typical peaks of phosphate vibration at 1030–1034 cm⁻¹, phosphate ν₄ bending vibration at 565 and 604 cm⁻¹. These results confirm the existence of active interaction between the collagen–chitosan matrix and HAp. It is likely that the Ca²⁺ firstly interacts with the C=O group, and then reacts with HPO₄²⁻ to form the HAp.

Microstructure
The initial porous and loose microstructure of the CCS has been largely preserved after GA treatment. The pore size was within the range of 80–150 μm, a size which is suitable for infiltration and proliferation of osteoblasts mesenchymal stem cells. After pre-mineralization to form HAp (Its crystalline structure shall be verified in Fig. 6) in situ (Fig. 3a–3d), the CCHS has a similar microstructure as that of the crosslinked CCS, implying that the porous structure has not been destroyed by this reaction process. Magnified images (Fig. 3e–3h) reveal the characteristic HAp nanoparticles, which distributed rather evenly in the crosslinked CCS. Pre-adsorption of Ca²⁺ with a concentration lower than 0.05 M produced HAp particles with needle-like morphology (Fig. 3e). Above that concentration, the formed HAp particles have a spherical shape, and their size was...
increased from 100 nm (0.1 M Ca\textsuperscript{2+}) (Fig. 3f) to 200 nm (0.5 M Ca\textsuperscript{2+}) (Fig. 3h). After the CCHS was mineralized in SBF for 24 hr, needle like HAp particles were formed (Fig. 4b–4e). In contrast, without the pre-mineralization, the same incubation did not produce HAp particles in the crosslinked CCS (Fig. 4a). After the pre-mineralization, growth of the HAp particles becomes more easily from the HAp nuclei, forming finally the needle-like particles.

Transition of the particle shape could be visualized from the image of 12 hr mineralization in the SBF (Fig. 5), confirming the particle growth nature.

**Crystalline structure**

Two groups of XRD reflection typical peaks can be utilized to detect the HAp formation: one at \(2\theta = 26.0^\circ\) and the other group is ranging from \(2\theta = 31.8^\circ\) (211) to \(2\theta = 32.9^\circ\) (112) and...
XRD spectra of the MCCHS (6(a2)–6(a5)) show that there are strong reflection peaks at 2\(\theta\) = 26.2, 31.8, and 39.8°, and present general patterns of typical low crystalline HAp. By contrast, Fig. 6(a) shows that there is no reflection peak at the HAp position, indicating that no HAp was formed in the crosslinked CCS. These results are in good agreement with the SEM observation, and confirm further that the pre-nucleation plays a crucial role in the HAp growth. Figure 6(b) shows that the reflection peak at 32.7° (Fig. 6(b1)) shifted to 31.8° (Fig. 6(b2)) after incubation in the SBF solution for more than 12 hr, confirming the gradual formation of the HAp from the CaP compounds.

Thermal stability and HAp weight increase
To reveal stability of the crosslinked CCS, CCHS, and MCCHS, TGA characterization was performed (Fig. 7a). All the samples lost their weights progressively below 260°C, and then rapidly above 260°C. The weight loss below 260°C should be attributed to the water evaporation and dehydration between the biopolymer molecules.32 Apparently, the MCCHS family has a larger weight retention ratio above 500°C treatment than that of the CCHS counterpart at the same concentration of Ca\(^{2+}\), although the absolute weight of all the samples was increased along with the increase of Ca\(^{2+}\) concentration (Fig. 7b). Quantification by microbalance obtained the same alteration principle. Therefore, these results reveal that amount of the formed HAp particles is increased along with the Ca\(^{2+}\) concentration within the investigated range. The water-uptake ratio of the CCHS was decreased rapidly from 80 (0.05 M Ca\(^{2+}\)) to 22 (0.5 M Ca\(^{2+}\)) (\(p < 0.05\)), whereas the water-uptake ratio of the MCCHS was further decreased from 52 (0.05 M Ca\(^{2+}\)) to 17 (0.5 M Ca\(^{2+}\)) (\(p < 0.05\)). Generally, the water-uptake ratio is controlled by hydrophilicity and water maintenance of the scaffold.33 Although formation of HAp particles may decrease more or less the amount of hydrophilic groups of the CCS, the main reason here should be largely the increase in the scaffold weight (\(W_0\)) as shown in Fig. 7b, which influences severely the water-uptake ratio according to its definition.

Rheological characterization
Mechanical strength of the scaffolding materials, especially in a dynamic force state, is extremely important for bone repair. Figure 9 shows that all the samples had a similar viscoelastic behavior. But the \(G'\) of CCHS and MCCHS (\(\sim\)10 kPa) was significantly larger than that of the crosslinked CCS (\(\sim\)2 kPa) when the Ca\(^{2+}\) concentration was higher than

Water-uptake property
Free exchange of nutrients is extremely important for a scaffold in bone regeneration. Water-uptake ratios of the crosslinked CCS, CCHS, and MCCHS are compared in Fig. 8. The crosslinked CCS had an initial water-uptake ratio of \(\sim\)160, decreased to \(\sim\)120 after incubated in Ca\(^{2+}\) regardless of the Ca\(^{2+}\) concentration within the investigated range.
Mineralization of collagen-chitosan scaffolds

CONCLUSIONS

Fast mineralization in a crosslinked porous CCS is accomplished by pre-mineralizing the CCS with Ca$_{2+}$ and phosphate solution, which produces numerous HAp nuclei with a size below 200 nm. The subsequent mineralization in SBF produces more HAp particles, whose amount is increased along with the concentration of Ca$_{2+}$ used in the pre-mineralization. XRD characterization confirms the structure of the formed HAp. The microstructure of the scaffold is mostly preserved, whereas the storage modulus $G'$ and loss modulus $G''$ are significantly improved after the in situ HAp mineralization. Therefore, the method introduced here is of significance to obtain a composite HAp/collagen–chitosan porous scaffold in a very short time, whose biological performance is under investigation now.

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