Crosslinking of Electrospun Fibrous Gelatin Scaffolds for Apatite Mineralization

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Received 8 February 2010; accepted 9 May 2010
DOI 10.1002/app.32769
Published online 27 July 2010 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Fibrous gelatin scaffolds fabricated via electrospinning followed by crosslinking were used as substrates for apatite mineralization. Gelatin macromolecules were confined by their fibers and further restricted by the crosslinked structure while proper flexibility could be attained upon hydration. After 4 or 5 days of mineralization, partially carbonated hydroxyapatite was proved to deposit uniformly on the surface of the fibers. The property of the substrate, such as stiffness of the scaffolds and flexibility of macromolecules chain, was changed by different crosslinking ways. The influences of these properties on the formation of apatite were also investigated. Results showed that a relatively less rigid interface and more flexible chain acquired by glutaraldehyde solution crosslinking seemed to favor the nucleation of minerals and to reduce the size of the inorganic products. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 786–793, 2011

Key words: electrospinning; gelatin; fibrous scaffold; crosslinking; mineralization; apatite

INTRODUCTION

A natural bone is a composite, mainly made up of fibrous collagen framework and hydroxyapatite (HA) minerals, which grow in and on the surface of these collagen fibers.[1,2] A "matrix-mediated" mineralization is believed to obtain this composite.[3,4] Macromolecules substrates control the nucleation, polymorphism, growth, chemical composition, shape, and dimensions of the crystals deposited on them. Fabrication of substitutes, which mimicks the components and structure of the extracellular matrix (ECM) of the natural bone by means of matrix molecules modulation and *in situ* mineralization, is very important for bone tissue engineering. As is well known, the topographic structure of the polymer substrate plays an important role on nucleation of HA. What is more, some properties of the substrate, such as flexibility of macromolecules chain and stiffness of the matrix interface, are likewise learnt to impact the mineralization process as well.[5,6] Addadi and Weiner proposed that biomacromolecules locked in an relatively ordered conformation present favorable sites for heterogeneous nucleation of calcium minerals, and relatively rigid interfaces of biomaterial may induce biomineralization processes through selective electrostatic accumulation of the mineral ionic components and by structural correspondence with specific planes in the crystal.[5] Hence, structures and properties of the matrix are two significant issues involved in mineralization. Unfortunately, few investigations have been focused on the latter one.

Among various matrices for mineralization, there is no doubt that collagen, as a key ingredient of natural bone, is an ideal material for mineral deposition. However, the drawbacks of high price and immune antigenicity limit its application. Gelatin, which is a particular denatured derivate of collagen, shares much similarity to collagen molecules and is always used instead because of its lower immune antigenicity and lower cost.[7,8] So far, gelatin matrices including gelatin hydrogel, gelatin casting film, etc were used as substrates for mineralization.[9–13] Unfortunately, these substrates were short of fibrillar feature, which is extremely important in promoting formation of bonelike apatite and producing analogue of natural bone.[14] Continuous fibrous structures can be generated via electrospinning conveniently.[15–17] Several fibrous scaffolds electrospun from different synthetic materials have already been utilized as mineralization substrates.[18–20] However, there is a certain gap between the nature of these materials and that of collagen. Due to their relatively high hydrophobicity and lack of functional groups, it is not easy to immerse them in an aqueous solution containing inorganic ions, and a trouble is to be caused in homogeneous distribution of minerals within the fiber. Apart from these, relatively few

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Contract grant sponsor: Natural Science Foundation of Tianjin, China; contract grant number: 07JCYBJC02900.

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efforts were made on the influence of the properties of the substrate on mineral formation.

In this study, ultrafine fibrous scaffolds of gelatin produced via electrospinning followed by crosslinking were used as substrates for apatite mineralization. Formation was studied of minerals on this kind of fibrous structure. Some properties of the matrix were affected by different crosslinking ways, and effects were also investigated of these properties on apatite deposition. This work may provide new supplement for understanding the role of matrix in the mineralization procedure. Composites obtained here resembled the apatitic crystals and collagen fibrils in calcified biological tissues to some extent, and may have potential applications in the future.

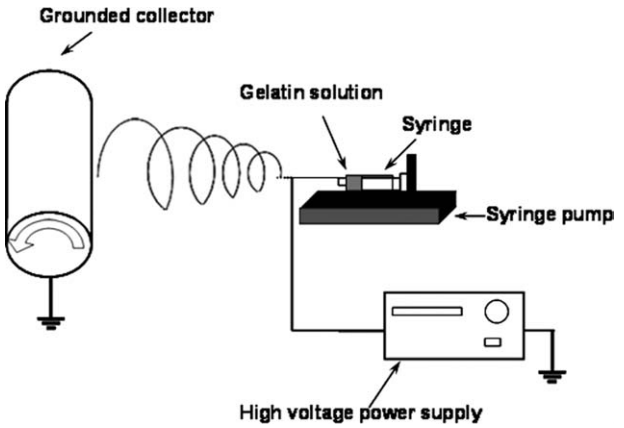

Figure 1 Schemetic diagram of electrospun scaffold experimental setup.

EXPERIMENTAL METHODS

Materials

Gelatin type B (~ 225 Bloom) from bovine skin in powder form was purchased from Sigma (St. Louis, MO). 2,2,2-trifluoroethanol (TFE, purity 99.9%) was obtained from Weihai Newera Chemical (Shandong, China). Glutaraldehyde (GTA, 50% (v/v), aqueous solution) was a product of Fuchen Chemical Factory (Tianjin, China). Tris (hydroxymethyl) aminomethane (Tris) was from Aoboxing Biotech (Beijing, China). $CaCl_2$, $Na_2HPO_4{\cdot}2H_2O$, and NaOH were all purchased from Amresco Inc. (Solon, OH). Ultrapure water (resistivity 18.24 MΩ·cm) was used throughout the experiment.

Preparation of electrospun scaffold

Gelatin fibers were electrospun according to the literature concerned.[21,22] A schemetic diagram of electrospun scaffold experimental setup is shown as Figure 1. Briefly, a transparent solution (10% w/v) for electrospinning was prepared by dissolving gelatin in TFE and by stirring it at room temperature up to 24 h. The solution was added into a syringe attached with a clinic-shaped metal capillary, and a feeding rate of 0.8 mL/h was set by means of a syringe pump (KI-75439-0005, Cole-Parmer Instrument, Vernon Hills, IL). The applied voltage was controlled at 10 kV by a high-voltage power supply (DW-P503-4AC, Tianjin Dongwen High-voltage Electrical Source Factory, Tianjin, China). The distance between the spinneret and the grounded collector was 15 cm. The obtained samples were dried in a vacuum oven at room temperature for 24 h to remove residual solvent, and the thickness of the resulting electrospun scaffold was around 80 μm.

Crosslinking of electrospun scaffold

The electrospun scaffolds were placed above a Petri dish containing GTA aqueous solution in a sealed container. They were crosslinked in the GTA vapor at ambient temperature (29°C) for 48 h. After crosslinked, the samples were washed with excessive water and then exposed in a fume hood for 24 h to remove residual GTA and dried in the air. To obtain a kind of substrate with some different properties, another method to crosslink the scaffolds was employed by using GTA absolute ethanol solution (GTA solution) as described elsewhere.[23] Briefly, electrospun scaffolds were immersed in this GTA absolute ethanol solution at 4°C for 48 h. The volume ratio of absolute ethanol to 50% GTA aqueous solution was 30 (v/v). The ratio of 50% GTA aqueous solution volume to the mass of gelatin was 0.03 (v/w). Thereafter, the crosslinked scaffolds were washed with excessive water, placed in the fume hood for 24 h, and dried in the air.

To estimate the degree of crosslinking, crosslinked scaffolds were weighed and then submerged in water for various periods of time at 29°C. The mass loss of these samples was determined according to the following equations:

$$\text{Mass loss } (\%) = \frac{m_i - m_d}{m_i} \times 100, \qquad (1)$$

where m_d is the mass of the sample in its dry state after submersion in water and m_i is the initial mass of the sample in its dry state.

A tensile test of the scaffold was carried out by using a 100 N load cell at a speed of 5 mm/min at 29°C. The effective length of the samples was 40 mm. At least six samples were tested for each type of crosslinked scaffolds.

Mineralization

The alternate mineralization process[24,25] was used to nucleate and grow apatite on gelatin fibrous substrates. Crosslinked scaffolds (1.5 cm × 1.5 cm) were

soaked in 0.4 mol/L $CaCl_2$ solution buffered with Tris (Ca solution) for 12 h. After washed thoroughly with water, the samples were in succession soaked in 0.24 mol/L Na_2HPO_4 solution buffered with Tris (P solution) for another 12 h. The pH of the above solutions was about 11–13. Each mineralization cycle proceeded for 24 h and was repeated several times at 29°C depending on the specifications of the experiment. At the end of each day, the mineralized specimens were washed thoroughly with water and dried in the air for further investigations.

Characterization

The morphologies of the electrospun scaffold and mineralized samples were examined with a scanning electron microscope (SEM, Philips XL30; Philips, The Netherlands). Average fiber diameter was determined by Adobe Photoshop 7.0 software from 50 different fibers on the scanning electron microscope (SEM) micrographs. The element analysis was carried out by an energy dispersive spectrometer (EDS) which was directly connected to SEM. Further observation of the mineral layer was performed on a field emission SEM (FESEM, LEO 1530VP; Carl Zeiss, Germany) at a higher magnification. The mineralized samples were also analyzed by fourier transform infrared (FTIR) spectroscopy. Data was collected with a FTS3000 spectrometer (BIO-RAD). The measurements were taken in the wave number range of 4000–400 cm^{-1} at a resolution of 4 $cm^{-1.}$ An X-ray diffraction (XRD, D/MAX-2500, Rigaku, Japan) analysis was performed with Cu Kα radiation, the λ of which was 0.15 nm, at a current of 100 mA and at an accelerating voltage of 40 kV. The spectra were recorded in the 2θ range of 20°–60° with a scanning speed of 4°/min.

RESULTS AND DISCUSSION

In nature, the mineral crystalline during tissue calcification is initialized by heterogeneous nucleation.[25] Comparatively rigid interface was said to present favorable sites for heterogeneous nucleation and to offer structural correspondence with specific planes in the crystal.[5] Nevertheless, it was also found that the tails of the collagen fibers, compared to the backbone regions of the triple-helices were expected to be relatively flexible and were suggested as suitable for combining with ions and accommodating apatite aggregates.[26,27] Hence, the matrix should be neither too stiff nor too soft and somehow can give a certain degree of flexibility for molecules to induce the mineralization. Here, in this work, ultrafine fibrous topographic structures were introduced to gelatin matrix, and a stiff network was formed after chemical crosslinking. In this structure, gelatin macromolecules were confined into many single fibers and further restricted by the crosslinking network, thus becoming more regular to some extent in this manner. Therefore, this matrix provided a comparatively stiff fibrous interface for minerals deposition. On the other hand, upon hydration, molecules chain in the fibers was endowed with some flexibility, which may enable functional groups on the gelatin fibers to accumulate ionic components selectively.

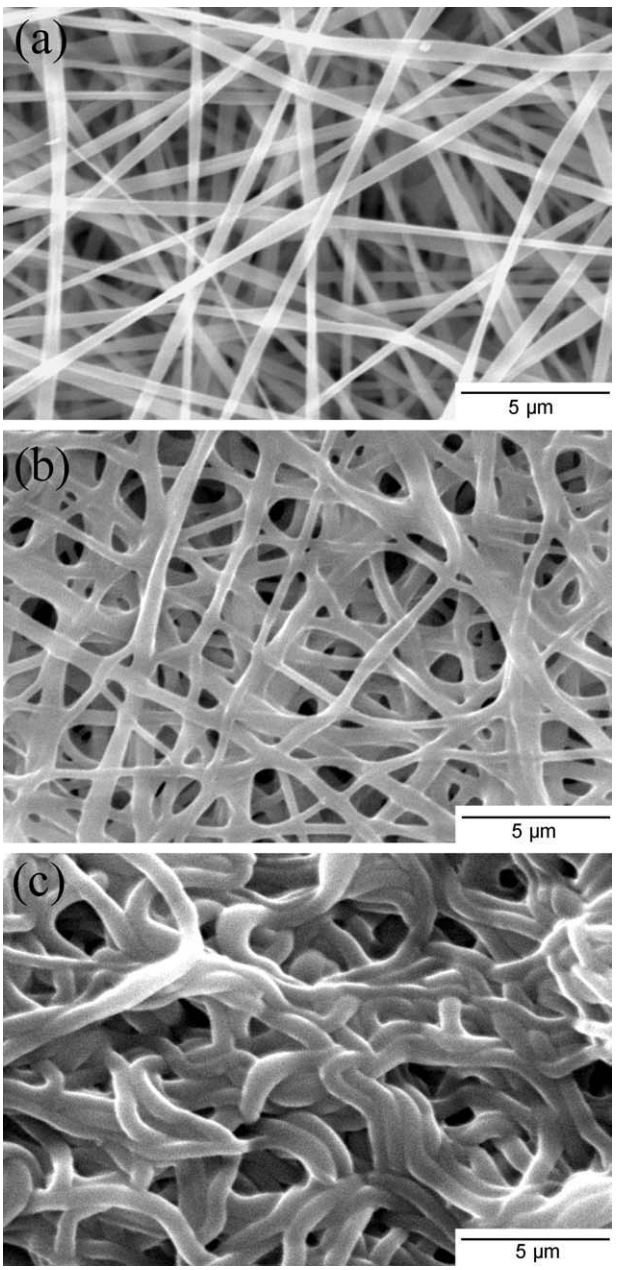

Figure 2 SEM micrographs of the electrospun gelatin fibrous scaffolds before and after crosslinking: (a) eletrospun samples, (b) GTA vapor crosslinked samples, and (c) GTA solution crosslinked samples.

From the SEM micrograph, it can be seen that uniform and fine gelatin fibers of which the average diameter was 327 ± 59 nm with random orientation were obtained through electrospinning [Fig. 2(a)].

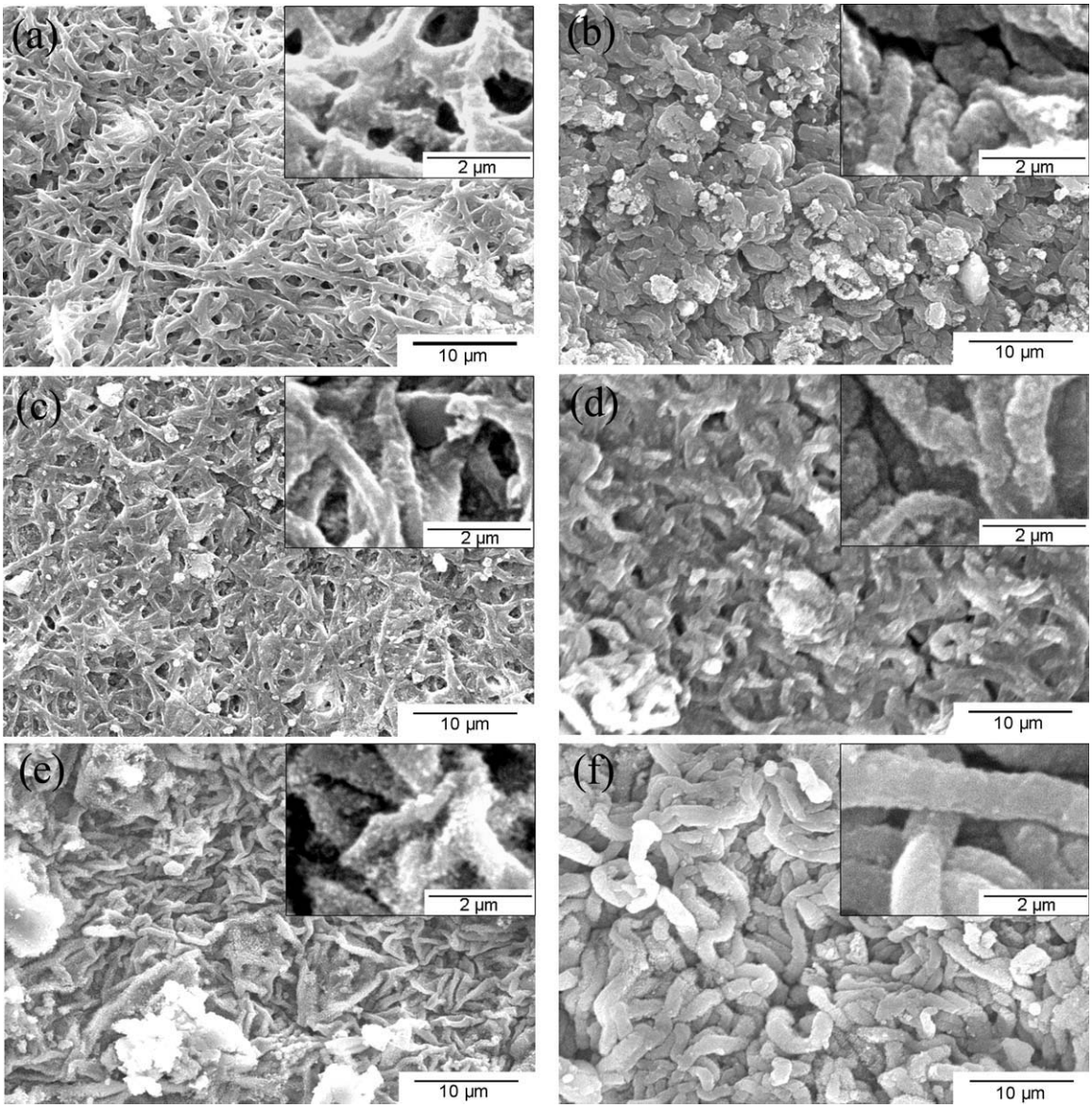

Figure 3 SEM micrographs of crosslinked scaffolds after mineralization for 1(a,b), 3(c,d), and 5(e,f) days, respectively. (a,c,e) GTA vapor crosslinked samples; (b,d,f) GTA solution crosslinked samples.

Gelatin is water soluble and even a drop of water will destroy the fibrous structure on the scaffold, and therefore crosslinking is needed.[22] To obtain different properties of the matrix, the electrospun scaffolds were crosslinked by GTA vapor and GTA solution respectively [Fig. 2(b,c)]. An average diameter of fibers increased to 528 ± 76 nm (vapor crosslinked) and 690 ± 89 nm (solution crosslinked) after the crosslinking.

SEM micrographs in Figure 3 show the visible observation of the mineralization process. It can be seen that the mineral phase is generated along the fiber axe. On the first day, inorganic particles separated on the fiber surface and some of them aggregated together. As the mineralization time was prolonged, there were more and more minerals till a uniform layer of HA was obtained on the surface of the fibers, wrapping the entire fiber in it. Needle-like crystals with a diameter of less than 100 nm was seen.

The FTIR spectra of the samples mineralized for 5 days (Fig. 4) provided further information about the formation of HA and the interaction of HA and gelatin. Take for example samples mineralized on GTA solution crosslinked scaffolds, there appeared typical absorption bands of phosphate vibration at 1034 $cm^{-1.}$ Also the PO_4^{3-} related bands at 608 and 555 cm^{-1} demonstrated the existence of the apatite phase.[8,28] Weak peaks, denoted the vibrational mode of the CO_3^{2-}, were observed at 1406 and 878 cm^{-1}, meaning that the PO_4^{3-} sites of the HA structure were partially substituted by CO_3^{2-} groups.[8] On the

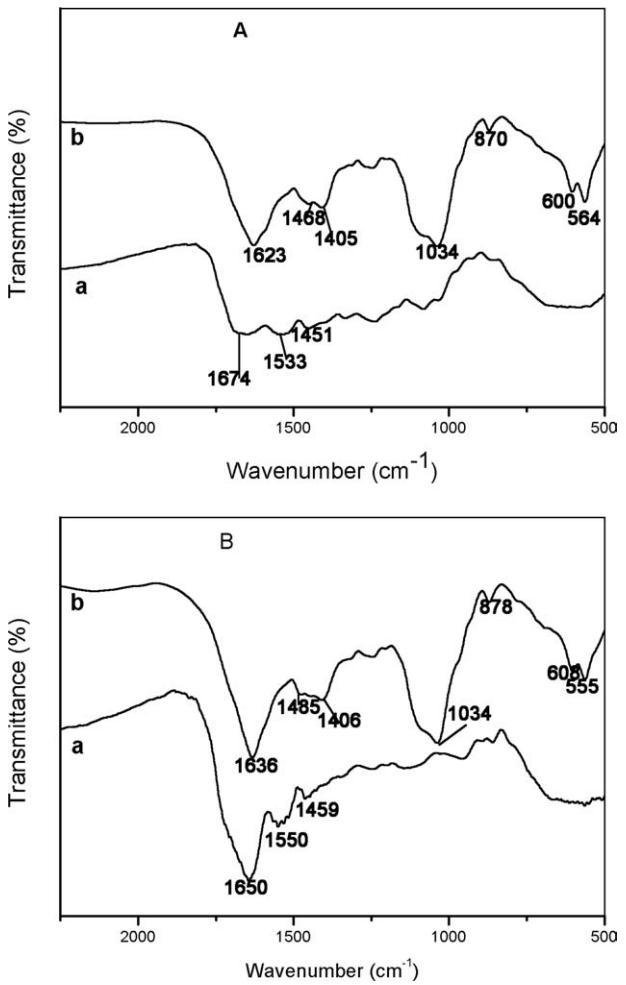

Figure 4 FTIR spectra of crosslinked gelatin fibrous scaffolds before (a) and after mineralization for 5 days (b). A: GTA vapor crosslinked samples; B: GTA solution crosslinked samples.

other hand, the characteristic absorption bands around 1650 cm^{-1}, assigned to amide I (C=O) in crosslinked gelatin samples, shifted to 1636 cm^{-1} after mineralization for 5 days. Also the amide II peak at 1550 cm^{-1} can hardly be detected in the spectrum of mineralized products. These suggested that C=O groups of gelatin may have interacted with inorganic minerals. In addition, the symmetrical stretching mode of carboxylate groups (COO^-), which was located at 1459 cm^{-1} in the crosslinked gelatin samples, shifted to 1485 cm^{-1} after mineralization, indicating the chemical interactions between COO^- and HA.[29] All this showed that the gelatin macromolecule chains in the fibrous network were flexible enough for their carboxyl groups to be accessible to ion attachment from the aqueous solution.

X-ray diffraction patterns of the mineralized samples are shown as Figure 5. At the beginning, almost no obvious characteristic peaks assigned to inorganic crystals were detected. As mineralization proceeded, apart from some peaks resulted from the crosslinked scaffolds, characteristic diffraction peaks were observed of HA centered at 2θ of 25.8° (002), 31.8° (211), 32.9° (112), and 40° (310). However, these peaks appeared relatively broadened with respect to those recorded from the HA crystal standard. With the increase of mineralization time, the intensity of these characteristic peaks increased. And the peaks also became sharper and more obvious. What is more, characteristic reflection of (222) and (213) for HA centered at 46.5° and 49.8° can also be detected at the patterns mineralized for 4 and 5 days. These indicated HA minerals with low crystallinity were formed while the crystallinity of the apatite increased with mineralization time. Ca/P molar ratios of the obtained mineral were also detected. Ca/P after 2 days of mineralization was 1.50 and 1.45 for the minerals obtained on GTA vapor crosslinked scaffold and on solution crosslinked scaffold, respectively. With mineralization time increasing to 5 days, Ca/P ratios became 1.57 (vapor crosslinked samples) and 1.66 (solution crosslinked samples), showing a tendency to approach 1.67 (stochiometric Ca/P value of HA). Theoretically, during apatite formation in aqueous media, amorphous calcium phosphate precursor gradually converts into stable HA.[30] The substitution of HPO_4^{2-} for PO_4^{3-} or Na^+ for Ca^{2+} may cause the deviation of Ca/P ratio. With the mineralization going on, the deficiency caused by the substitution of Na^+ and HPO_4^{2-} decreased, and the mineral phase converted into the most stable form.

In a previous research, no crystal but amorphous calcium phosphate was found after several days of mineralization on gelatin casting film, while only stretched films with molecules orientation and ordered pattern gave HA crystalline XRD effect.[13] In this research, the relatively stiff network with gelatin macromolecules assembling into many fibers seemed to effectively induce the formation of HA crystals. Moreover, properties of the substrate, here changed by different crosslinking ways, also influenced the mineralization as well. Returning again to consider the SEM micrographs in Figure 3, it can be seen that crystals deposited on GTA solution treated scaffolds exhibited a denser body while a more porous body was obtained on GTA vapor crosslinked samples. The morphology discrepancy is significant when mineralization was carried out after 4 days. The morphology of the mineral layer after 4 days of mineralization was examined by FESEM at higher resolution (Fig. 6). It is clearly shown that the average diameter of the needle-like crystals was slightly larger on GTA vapor crosslinked network than on GTA solution crosslinked sample. Meanwhile, the fiber morphology of the mineralized samples treated by GTA solution was comparatively clearer and fiber seemed more round and robust. XRD patterns

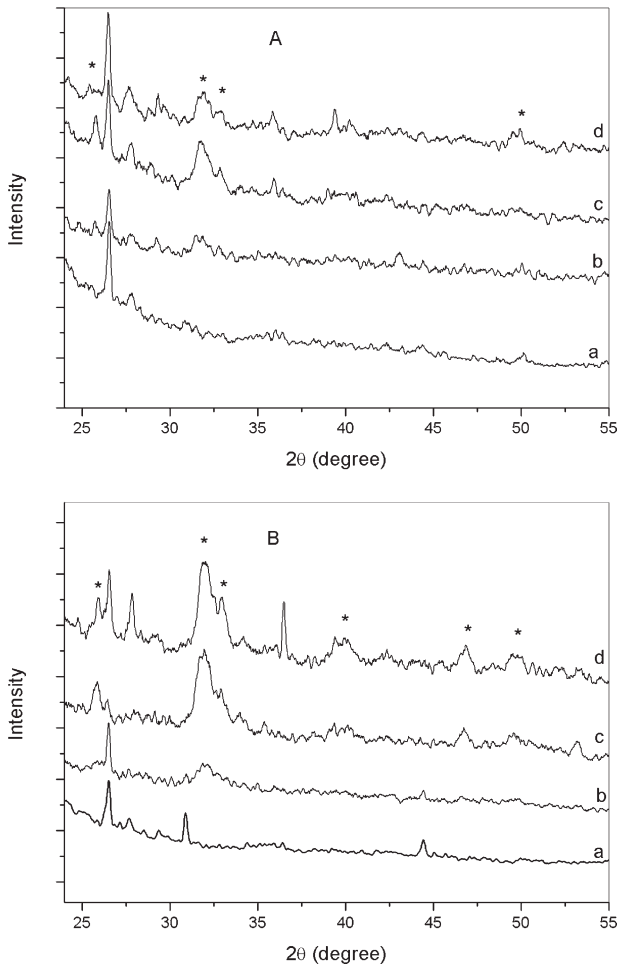

Figure 5 XRD patterns of crosslinked scaffolds before (a) and after mineralization for 2(b), 4(c), and 5(d) days, respectively. A: GTA vapor crosslinked samples; B: GTA solution crosslinked samples.

showed that characteristic diffraction peaks of HA formed on GTA solution treated scaffold were more obvious and the major peaks were sharper and more intensive, indicating comparatively higher crystallinity of HA deposited on GTA solution treated scaffolds. Crystallite size in a direction perpendicular to the crystallographic plane was also approximately estimated by Scherrer's formula.

$$D = \frac{K\lambda}{\beta \cos \theta}, \tag{2}$$

where D denotes the average crystallite size, λ represents the X-ray wavelength (0.15 nm), θ is the diffraction angle, K is shape constant (0.89), and β is the full width of the characteristic diffraction peak at half of the maximum intensity (rad). HA obtained in polymer matrices mainly exhibits in elongated needle-like shapes and inclines to grow in the c-axis, which corresponds to the (002) reflection peak.[25] Therefore, the size of HA crystalline was usually calculated by (002) reflection peak.[20,25] In this study, the calculated crystallite size perpendicular to (002) plane was about 17.0 nm for the crystals formed on GTA solution crosslinked samples after 4 days of mineralization, smaller than crystals obtained on vapor treated ones (28.3 nm). These quite agreed with the result of morphology observation of mineral layer (Fig. 6). Moreover, the Ca/P ratio of the crystal formed on GTA solution treated fibers was 1.66 while it was 1.57 on vapor crosslinked fibers. Therefore, the crystals generated on the GTA solution crosslinked scaffolds showed more similarity to HA in the aspects of crystal structure and composition.

To reveal the different properties of the fibrous network, the stiffness of the scaffolds was studied and the crosslinking degree of gelatin matrix was also evaluated. According to our test, the tensile modulus and the tensile strength of the GTA solution crosslinked samples were 560.8 ± 56.6 MPa and 7.8 ± 2.3 MPa, both of which were lower than the values of GTA vapor crosslinked network (866.0 ± 71.1 MPa and 18.3 ± 1.1 MPa). This suggested that GTA vapor treated scaffold was stiffer than GTA solution crosslinked samples. On the other hand, the flexibility of molecules chain was determined by the

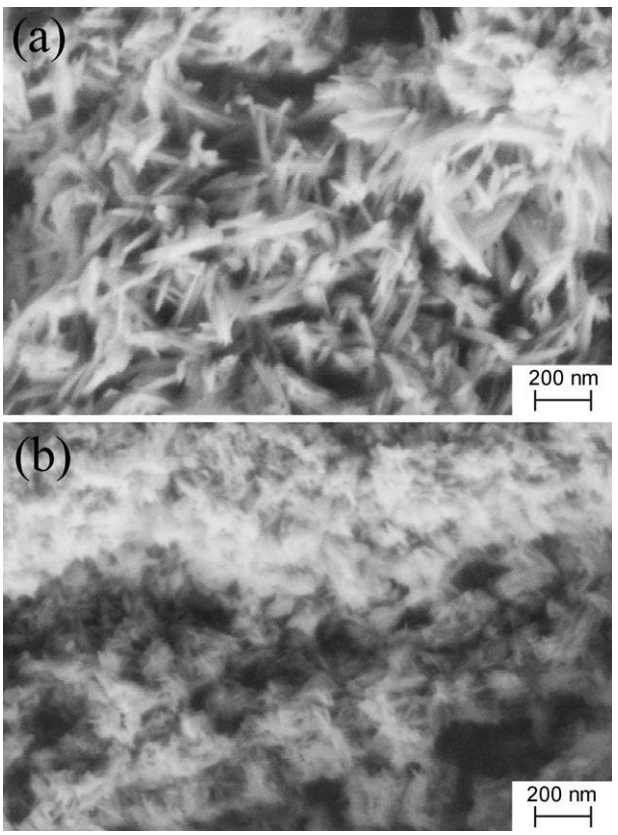

Figure 6 FESEM micrographs of mineral layer after 4 days of mineralization on GTA vapor crosslinked (a) and GTA solution crosslinked samples (b), respectively.

crosslinking degree. The lower the crosslinking degree is, the more flexible the macromolecule is. Moreover, because it was said that crosslinking degree of gelatin matrix can be approximately evaluated by determining changes in gelatin solubility,[31] mass loss of the crosslinked samples after incubation in water was studied. The results indicated that the mass loss increased with the prolongation of incubating time. For the vapor crosslinked samples, the mass loss was about 2.9 ± 0.9% on the first day of immersion in water and was gradually increased to 7.7 ± 0.3 % on the fifth day. For the solution crosslinked samples, the mass loss changed from 19.0 ± 1.4% on the first day to 26.5 ± 0.9 % on the fifth day. The mass loss of the solution crosslinked scaffolds was much larger than that of the vapor crosslinked ones. Also the dissolution rate of solution treated sample was much higher. GTA vapor can slowly penetrate into the inner part of gelatin fibers, and crosslinking reaction can proceed thoroughly. Therefore, the value of mass loss was lower and thus a higher crosslinking degree of the scaffold was reflected. Though solution was usually said to crosslink samples more rapidly,[32] the core region of single fiber may not be well crosslinked. Therefore, the mass loss of GTA solution crosslinked scaffold was relatively larger.

From above, it can be concluded that gelatin macromolecules were confined less tightly in the network through GTA solution crosslinking, i.e., relatively less rigid interface was formed through GTA solution crosslinking and the macromolecules in single fiber were more flexible upon immersing in aqueous environment. Therefore, more carboxyl groups were postulated to be available to expose to the interface to coordinate with ions, leading to the formation of a large number of nuclei for the growth of HA crystals so that these crystals can not grow very large. Moreover, as much more mineral nuclei formed on solution crosslinked fibers, denser minerals were obtained whereas a comparatively more porous structure engendered on the GTA vapor crosslinked fibers. In addition, compared with the rigid interface provided by vapor crosslinked scaffolds, a less stiff interface as a result of solution crosslinking seemed to favor the mineral structure rearrangement, which might speed up the conversion from the amorphous state to stable HA. Hence, crystals with less lattice deficiency and higher crystallinity were achieved on GTA solution crosslinked samples. In natural bones, crystals are also observed in the middle areas of the collagen fibrils apart from those on their surfaces.[2] In our case, it was reasonable to deduce that minerals could likewise nucleate inside the gelatin fibers, for ions could penetrate inside the single fiber as the scaffold was swollen. Crystals inside the fibers may provide some mechanical support to make the fiber more round and robust.

CONCLUSIONS

A gelatin fibrous scaffold was fabricated via electrospinning followed by GTA crosslinking. Gelatin macromolecules were regulated by being confined into many single fibers, mimicking the collagen substrate from component and structure to some extent. After several days of mineralization, fibers were uniformly covered by a layer of minerals. XRD and FTIR results indicated that the newly synthesized mineral was partially carbonated hydroxyapatite and had a low degree of crystallinity, which resembled the mineral in natural bones. The properties of the fibrous scaffold, such as stiffness of the matrix and flexibility of the molecules chain, affected by different crosslinking ways, influenced mineralization as well. It was found that through glutaraldehyde solution crosslinking, a relatively less rigid interface and more flexible chain can be obtained. On scaffolds with these properties, minerals were smaller and the crystallinity of them was higher.

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