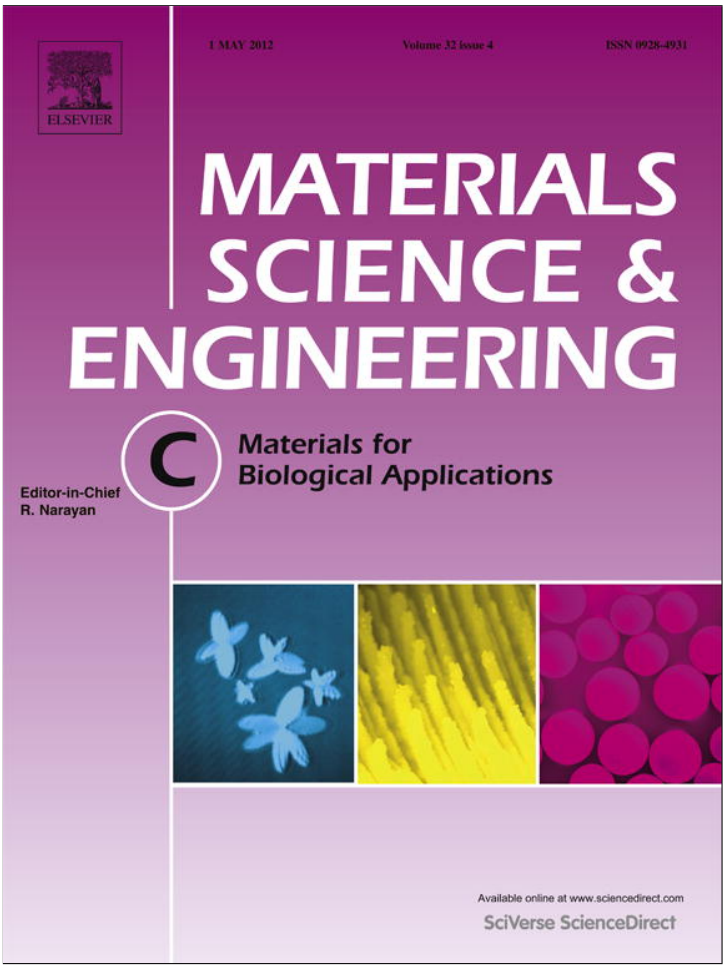

Materials Science and Engineering C 32 (2012) 994–1000

Contents lists available at SciVerse ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Radiation synthesis of gelatin/CM-chitosan/β-tricalcium phosphate composite scaffold for bone tissue engineering

Ying Zhou [a,b], Ling Xu [a,b,*], Xiangmei Zhang [b], Yinghui Zhao [b], Shicheng Wei [b,c,**], Maolin Zhai [d]

[a] College of Engineering, Peking University, Beijing 100871, China
[b] Center for Biomedical Materials and Tissue Engineering, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China
[c] Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology, Peking University, Beijing 100081, China
[d] Beijing National Laboratory for Molecular Sciences, Department of Applied Chemistry, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China

ARTICLE INFO

Article history:
Received 6 April 2011
Received in revised form 7 August 2011
Accepted 4 February 2012
Available online 10 February 2012

Keywords:
Gelatin
Carboxymethyl chitosan
β-tricalcium phosphate
Ultrasonic treatment
Radiation crosslinking
Bone tissue engineering

ABSTRACT

A series of biodegradable composite scaffolds was fabricated from an aqueous solution of gelatin, carboxymethyl chitosan (CM-chitosan) and β-tricalcium phosphate (β-TCP) by radiation-induced crosslinking at ambient temperature. Ultrasonic treatment on the polymer solutions significantly influenced the distribution of β-TCP particles. An ultrasonic time of 20 min, followed by 30 kGy irradiation induced a crosslinked scaffold with homogeneous distribution of β-TCP particles, interconnected porous structure, sound swelling capacity and mechanical strength. Fourier Transform Infrared Spectroscopy and X-ray Diffraction analysis indicated that β-TCP successfully incorporated with the network of gelatin and CM-chitosan. *In vivo* implantation of the scaffold into the mandible of beagle dog revealed that the scaffolds had excellent biocompatibility and the presence of β-TCP can accelerate bone regeneration. The comprehensive results of this study paved way for the application of gelatin/CM-chitosan/β-TCP composite scaffolds as candidate of bone tissue engineering material.

1. Introduction

Bone defects resulting from tumors, diseases, infections, trauma, biochemical disorders, and abnormal skeletal development raise significant health problems. Various biomimetic tissue engineering approaches in terms of structure and composition have been developed to replace autografting and allografting [1]. Porous scaffolds, which are similar to the structure of natural bone, could induce the formation of bone from the surrounding tissue, or to act as a template for cell growing and bone tissue regeneration [2]. Meanwhile, composites with similar components of natural bone are widely used to fabricate porous scaffolds [3].

As it is well known, the extracellular matrices (ECM) of hard tissue are composed of organic and inorganic phases. The inorganic phase consists primarily of calcium phosphate such as hydroxyapatite (HA), while the main composition of organic phase is type I collagen and small amount of ground substance including glycosaminoglycans (GAGs), proteoglycans and glycoproteins [4,5]. Herein, gelatin, CM-chitosan and β-TCP were adopted to concoct the organic–inorganic composites.

Collagen is a major organic component of ECM. Gelatin, a hydrolysis derivative of collagen, is widely used in biomedical field due to its lower antigenicity and more stable physicochemical properties [6]. Chitin and chitosan have been extensively studied for tissue engineering applications because of its excellent biocompatibility, biodegradability and osteoconductivity [7,8]. However, acetic acid or organic solvents should be applied for the processing of chitin or chitosan, which would impart certain cytotoxicity to the final product [9]. CM-chitosan, a water-soluble derivative of chitosan, has the merits of chitosan and has improved biocompatibility over chitosan [10].

HA has extensively proved its osteoconductivity as the major calcium phosphate constituent of native bone [11]. However, its poor biodegradability prevents the ingrowth of natural bone and may leads to deformity after an extended period [12]. By contrast, β-tricalcium phosphate (β-TCP), a high temperature phase of tricalcium phosphate with 1.5 Ca/P mole ratio, has 10 times higher degradation rate than that of HA. It is capable of promoting osteogenesis and accelerating bone regeneration through a process of dissolution and absorption [13]. Therefore, considering its biomimetic composition and structure, a gelatin/CM-Chitosan/β-TCP composite porous scaffold was designed for bone tissue engineering application.

In the preparation of inorganic/organic composites, the pretreatment procedure would significantly influence the compatibility of the components, resulting in rather different physicochemical properties. Ultrasonic treatment has been frequently used to improve the distribution of inorganic particles in organic phase. However, the impact of

* Correspondence to: L. Xu, College of Engineering, Peking University, Beijing 100871, China. Tel.: +86 10 62769915.
** Correspondence to: S. Wei, Center for Biomedical Materials and Tissue Engineering, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China. Tel./fax: +86 10 62753404.
E-mail addresses: lingxu@pku.edu.cn (L. Xu), sc-wei@pku.edu.cn (S. Wei).

0928-4931/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.msec.2012.02.029

ultrasonic treatment was rarely discussed in the field of bio-composites for bone tissue engineering.

To prepare hydrogels and scaffolds, several crosslinking method have been developed, for example, thermal heating, ultraviolet irradiation, chemical crosslinking and radiation crosslinking. The merit and demerit of each method were discussed in our previous work [14]. Among them, radiation crosslinking technique can prepare hydrogels and scaffolds without any cytotoxic additive, which may serve as promising biomedical material [15]. In our previous work, gelatin/CM-chitosan hybrid hydrogel with open and interconnected porous structure, which had potential to be applied as wound healing material, was prepared by radiation crosslinking [14].

In this study, gelatin/CM-chitosan/β-TCP scaffolds were prepared by radiation crosslinking and lyophilizing. Effect of ultrasonic treatment on β-TCP distribution and morphology of the scaffolds were studied. Physical properties of the scaffolds such as the porous structure, compressive strength, structural stability and swelling behavior were investigated. Besides, the biocompatibility of the scaffolds was preliminary evaluated by *in vivo* implantation into the mandible of beagle dog. The scaffold is expected to be a novel biodegradable bone tissue engineering material according to the bionic principle.

2. Materials and methods

2.1. Materials

Gelatin (product G1890, 300 g Bloom, porcine skin, Type A) was purchased from Sigma Chemical Co. Ltd. CM-chitosan powder (degree of deacetylation 96.5%; Mw 70,000) was purchased from Qingdao Honghai Co. Ltd., China. β-TCP (average diameter of 5 μm) was obtained from Shanghai Rebone Biomaterials Co., Ltd, China. Other reagents used here were all of analytical grade.

2.2. Preparation of gelatin/CM-chitosan/β-TCP composite scaffolds

The gelatin/CM-chitosan/β-TCP composite scaffolds were typically prepared as follows. Firstly, gelatin and CM-chitosan powders with a weight ratio of 2:3 were mixed homogeneously by deionized water at 50 °C with a concentration of 10%. Next, β-TCP was dispersed by deionized water to form a suspension with a concentration of 10%, and then stirred at room temperature for 0.5 h and treated by ultrasonication (40 kHz, 250 W) for certain time intervals. After that, the β-TCP suspension was poured into the gelatin/CM-chitosan solution under agitation. In the end, the mixture was mixed by an ARE-310 hybrid mixer (Japan Thinky Co., Ltd.) for 10 min to form a homogenous polymer solution. According to our previous work [14], to get promising physical properties, the total polymer concentration was fixed to 10 wt.%. Thus prepared solutions were filled into test tubes (inner diameter of 10 mm) and subjected for γ-irradiation with 30 kGy using a ^{60}Co radiation facility, which was performed at room temperature at a dose rate of 20 Gy min^{-1}. The hydrogel was denominated with the β-TCP fractions in the solid part, and Table 1 showed the composition of the hydrogels.

Table 1
The composition of the gelatin/CM-chitosan/β-TCP hydrogels.

β-TCP fraction in the solid part	Composition of the hydrogel			
	Gelatin (wt.%)	CM-chitosan (wt.%)	β-TCP (wt.%)	Total solid content (wt.%)
0%	4	6	0	10
5%	3.8	5.7	0.5	10
10%	3.6	5.4	1	10
20%	3.2	4.8	2	10
30%	2.8	4.2	3	10
40%	2.4	3.6	4	10

2.3. Characterization of the scaffolds

2.3.1. Swelling behavior in deionized water and phosphate buffer solution

The hydrogels were cut into cylinders with diameter of 10 mm and thickness of 5 mm, then the samples were immersed in beakers containing 150 mL deionized water or phosphate buffer solution (PBS) (0.15 mol L^{-1}, pH = 7.2) at 37 °C. After soaking for desired time interval (described in Figs. 3 and 4), the samples were withdrawn from the solution, gently removed surface solution by filter paper. The degree of swelling was calculated using Eq. (1). Six parallel samples were measured to achieve an average value.

$$\text{Degree of Swelling} = \frac{m_2}{m_1 \times 10\%} \times 100\% \quad (1)$$

where m_1 and m_2 represent weight of the sample before and after swelling, respectively.

2.3.2. Porosity

Cylinders of the hydrogels with the size of Φ 10 × 5 mm were immersed in deionized water for 24 h and then lyophilized. Liquid displacement method was used to determine the porosity [16]. Briefly, the specimen was put into a conical flask, and then the conical flask was evacuated to let the dehydrated ethanol sucked in the porous scaffolds. The system was kept in sealed condition for 48 h until the scaffold was saturated with ethanol. The porosity of the sample was calculated using Eq. (2):

$$p = \frac{m_4 - m_3}{\rho V_1} \times 100\% \quad (2)$$

where m_3 and m_4 represent the weight of the sample before and after immersing in ethanol, and V_1 is the volume of the sample, ρ is the density of dehydrated ethanol. The scaffolds were measured triplicate to get an average.

2.3.3. Mechanical properties

The compressive strength of the samples was tested by universal material testing instrument (Instron 5843). Φ 10 × 10 mm cylinders of the hydrogels and lyophilized scaffolds were prepared. The compressive test was conducted with a constant strain rate of 1 mm min^{-1} until 90% reduction in specimen height. The compressive strength was calculated from the stress–strain curve. Compressive modulus was calculated as the slope of the initial linear portion of the curve. The samples were measured triplicate to get an average.

2.3.4. Scanning electron microscope

The morphology of the scaffolds was observed with Scanning Electron Microscope (SEM) (HITACHI S-4800, Japan) at an accelerating voltage of 1 kV. Hydrogels were immersed in deionized water for 24 h and then lyophilized before observation.

2.3.5. Fourier transform infrared spectroscopy

The hydrogels were lyophilized and ground to a fine powder. Fourier Transform Infrared Spectroscopy (FTIR) analysis was performed using a Nicolet Magna-IR 750, with a Nicolet NicPlan IR microscope attachment (resolution 2 cm^{-1}, scan 64 times) and a MCT/A detector with a range of 700–4000 cm^{-1}.

2.3.6. X-ray Diffraction

Powder X-ray Diffraction (XRD) measurements were performed using a Philips X'Pert Pro diffractomter with a 3 kW ceramic tube as

the X-ray source (Cu K_α) and an X'celerator detector. Gelatin/CM-chitosan, gelatin/CM-chitosan/β-TCP composite hydrogels were lyophilized and compacted into pellets for XRD analysis.

2.4. In vivo implantation

2.4.1. Application procedure

In vivo implantation was carried out to evaluate the biocompatibility of the scaffold. Before implantation, Φ 4×4 mm cylinders of the gelatin/CM-chitosan, gelatin/CM-chitosan/β-TCP composite hydrogels (30% β-TCP fraction) were lyophilized, and then the porous scaffolds were cut into 2 mm length disks and sterilized by γ-radiation.

Animal testing was performed in accordance with the procedures approved by the Institutional Animal Care and Use Committee of Peking University. Mature beagle dogs (11.4±1.8 kg) were anesthetized by pentobarbital at the dose of 1 ml·kg^{-1}. The medial aspect of each mandible was exposed by dissection and periosteal flap was lifted. Holes of 4 mm in diameter, drilled in both buccal sides of the mandible between premolar and molar, were filled with gelatin/CM-chitosan and gelatin/CM-chitosan/β-TCP composite scaffolds respectively. The cross sections of the scaffolds should cling to the wound surface, and the specimens (approximately Φ 4×2 mm) should comfortably press fit to the hole. After that, sutured the periosteal flap, applied topical prophylactic antibiotics and closed the wound. The beagle dogs were sacrificed at 4 weeks, and the specimens were removed along with surrounding bone and stored in 4% buffered formalin.

2.4.2. Micro-computed tomography

The quantitative analysis of new bone fraction was performed using a Micro-computed tomography (micro-CT) system (Skyscan 1076). Specimens were scanned over a 180° rotation with an exposure time of 15 min and an X-ray source of 40 kV/120 μA.

The original 3D images were reconstructed and analyzed with NRecon, CTvol and CTan software packages. In the 3D analysis, total volume (TV) and bone volume (BV) were measured directly. Volumetric density (BV/TV, %) was calculated from BV and TV, which represented the quantity of regenerated new bone.

2.5. Statistical analysis

All data were expressed as mean ± standard deviations (SD). Statistical significance of differences between means was determined by one-way analysis of variance (ANOVA). P-values less than 0.05 denote statistical significance.

3. Result and discussion

3.1. Preparation of gelatin/CM-chitosan/β-TCP scaffolds

Ultrasonic treatment is an effective way to improve the diffusion of inorganic phase into organic phase, and further influence the morphology of the composite. In this study, gelatin/CM-chitosan/β-TCP solutions with 30% β-TCP fraction were treated by ultrasonic wave for different time length. The SEM images of the scaffolds in Fig. 1 show that the pore size of scaffolds distributed in the range of 150–550 μm and had little correlation with ultrasonic time. It is known that bone substitutes possessed porous structures with pore diameters in the range of 100–800 μm will benefit bone regeneration [17]. The macropores in gelatin/CM-chitosan/β-TCP scaffolds can promote the formation of internal mineralized bone, while the micropores and the interconnected pores serve for nutrient delivery.

On the other hand, the surface morphology of pore walls in scaffolds shows significant difference. The aggregation of β-TCP particles was seldom appeared in scaffold with ultrasonic time of 20 min (Fig. 1(B2)), while serious aggregation was observed in other scaffolds. It is clear that under a certain time, ultrasonic treatment can promote the distribution of β-TCP particles. However, extensive ultrasonic treatment will lead to the aggregation of β-TCP. In addition, as shown in Fig. 1(B2), small pits with diameters of 1–5 μm, which might be the defects caused by β-TCP, existed in the pore wall of the scaffold. As it is well known, proper roughness of material surface can promote cell attachment [3,18]. Therefore, the morphology of scaffolds can be monitored by adjusting ultrasonic time to prepare a proper scaffold for osteoblastic cells.

The inorganic phase occupies 65% of the dry weight of natural bone and the fraction of inorganic component is significant to bone tissue regeneration. It was reported that scaffolds with higher fraction of β-TCP in a proper range could better induce bone formation and osteoconductive [19]. Fig. 2 displays the morphology of the scaffolds with different fractions of β-TCP, when the ultrasonic time was set to 20 min. As observed, the composite scaffolds had similar pore size (about 350 μm), but some agglomeration appeared in the walls of scaffolds containing higher fractions of β-TCP, which was agree with the literature results [20].

3.2. Characterization of gelatin/CM-chitosan/β-TCP composite scaffold

3.2.1. Swelling behavior

Swelling behavior of the hydrogels in solutions was investigated to evaluate their capacity to absorb tissue fluid, and to simulate their degradation in the presence of body fluid. Swelling isotherm

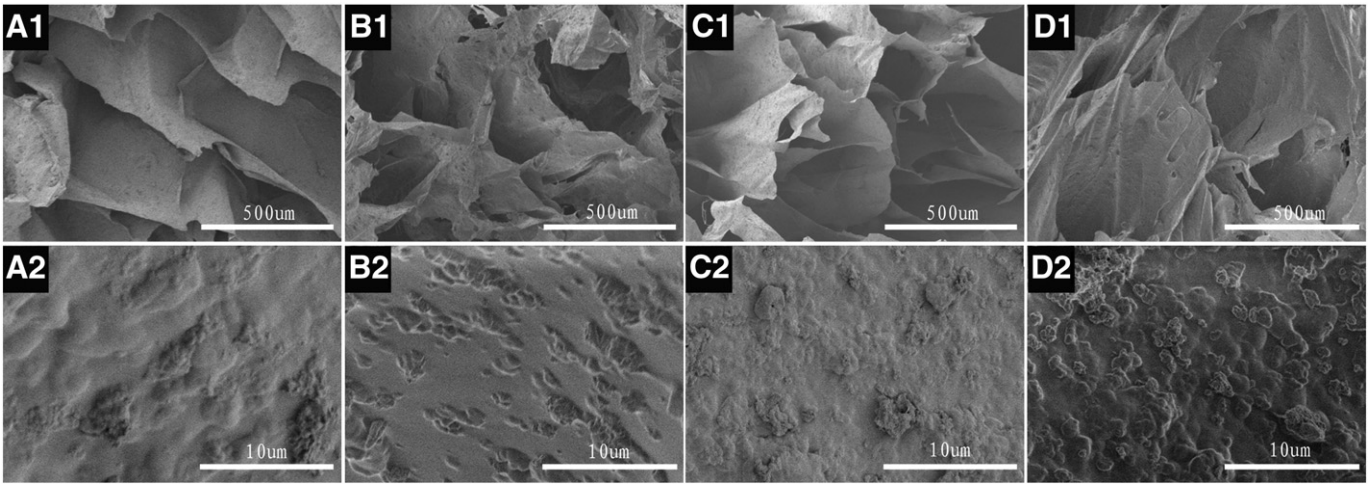

Fig. 1. SEM images of gelatin/CM-chitosan/β-TCP composite scaffolds fabricated with ultrasonic time of (A) 5 min, (B) 20 min, (C) 50 min, and (D) 2 h. The β-TCP fraction was 30%.

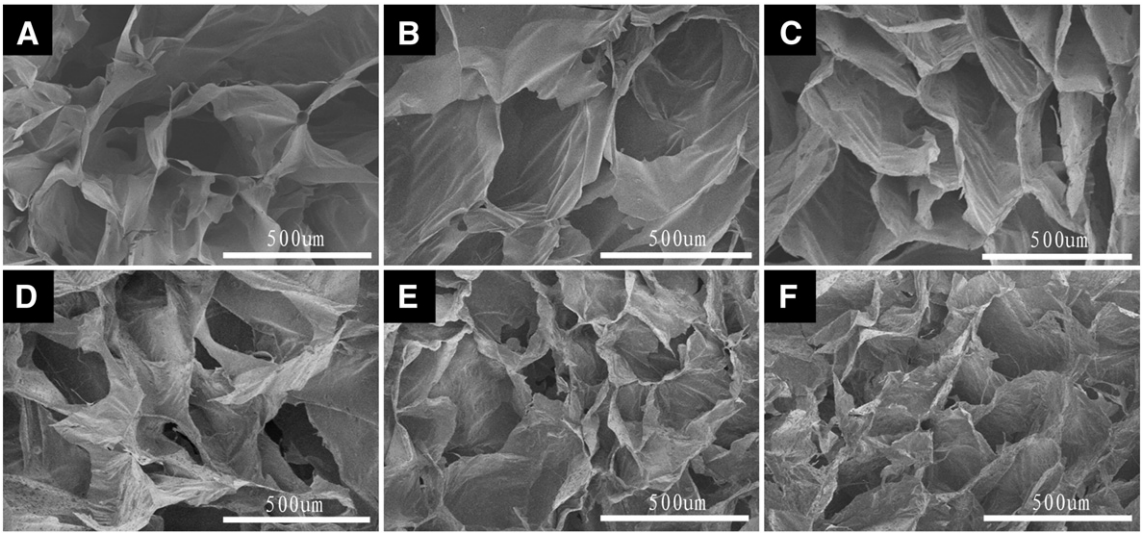

Fig. 2. SEM images of gelatin/CM-chitosan/β-TCP composite scaffolds with β-TCP fraction of (A) 0%, (B) 5%, (C) 10%, (D) 20%, (E) 30%, and (F) 40%. The ultrasonic time was 20 min.

(Fig. 3) of the hydrogels incubated at 37 °C in PBS manifests that the swelling degree of all compositions ascended rapidly initially and then leveled off after around 24 h. With continued exposure to PBS, the composites will finally disintegrate via hydrolysis, and hydrogel with higher β-TCP fraction underwent a faster degradation. Swelling of the hydrogels in deionized water (data not shown) had similar profile with that in PBS. Therefore, swelling degree was generally determined at 24 h expect in swelling kinetic study.

Upon immersing in solution, the hydrogel swelled and hydrolyzed at the same time [21]. The amine group and carboxyl group interacted with each other via hydrogen bond in the hydrogel, which favors the swelling. Meanwhile, polysaccharides are readily to be degraded by oxidation and beta-elimination surrounding by acid, alkaline or enzyme. The ions in PBS will accelerate the hydrolysis of CM-chitosan. Initially, large amount of solution diffused into the network of hydrogel, and the swelling was dominant; however, after the hydrogels reached the maximum degree of swelling, the thermodynamics driving force for solvent diffusion decreased and the hydrolysis in the interior of the hydrogel domain, leading to the collapse of hydrogel. Meanwhile, the defects caused by the releasing of β-TCP during the hydrolysis would accelerate the collapse. Therefore, gelatin/CM-chitosan/β-TCP hydrogels with 40% β-TCP fractions collapsed finally at 55 h, which was 15 h faster than hydrogels without β-TCP.

As discussed in previous sections, a temporary dynamic equilibrium between swelling and collapsing occurred at around 24 h after swelling experiment both in PBS and deionized water. In the mean time, the degree of swelling reached a maximum value and could maintain a temporary equilibrium for about 10 h. Therefore, to investigate maximum absorbable abilities, hydrogels were immersed in deionized water for 24 h, and the results were shown in Fig. 4. All the maximum degrees of swelling of samples immersed in deionized water were higher than that in PBS. It is ascribed that increasing the concentration of the swelling media will reduce osmotic swelling pressure and thereby reduce the swelling rate of the hydrogel [22].

However, the maximum degree of swelling of hydrogels immersed in deionized water and PBS both decreased with increases in the weight ratio of β-TCP, which suggested that the increasing amount of β-TCP would restrict the water adsorption. For example, as shown in Fig. 4, while the β-TCP fraction increased from 0 to 10%, the maximum degree of swelling of sample decreased from 106 to 80. However, the decrement of maximum degree of swelling retarded with the further increasing of β-TCP fraction and leveled off after 30%.

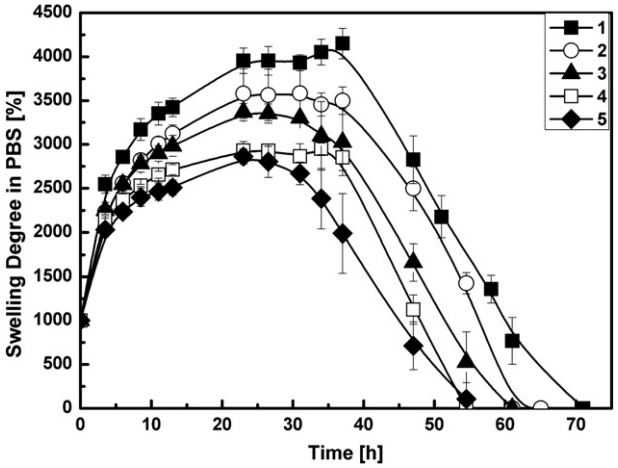

Fig. 3. Swelling isotherm of gelatin/CM-chitosan/β-TCP hydrogels in PBS, where 1–5 represent the samples with β-TCP fractions of 0%, 10%, 20%, 30%, and 40%, respectively.

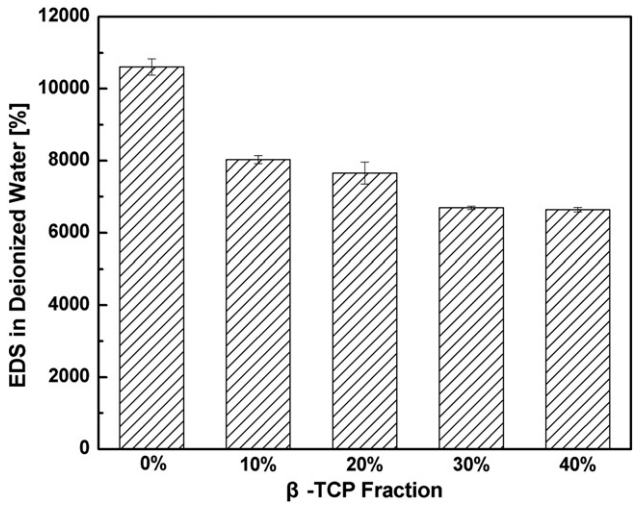

Fig. 4. Maximum degree of swelling of gelatin/CM-chitosan/β-TCP hydrogels in deionized water with β-TCP fractions of 0%, 10%, 20%, 30%, and 40%. Samples were immersed in deionized water for 24 h. The ultrasonic time was 20 min.

The decrease of swelling degree may be attributed to the interaction between gelatin/CM-chitosan network and β-TCP particles, which will interrupt the swelling of polymeric network.

3.2.2. Porosity

The porosity of a scaffold is important for successful cell adhesion and proliferation on implanted biomaterials. The porosities of scaffolds (%) with 0, 5, 10, 20, 30 and 40% β-TCP fractions were 98.4 ± 2.3, 96.9 ± 3.9, 96.4 ± 3.3, 97.9 ± 3.0, 98.7 ± 1.3 and 99.9 ± 0.8, respectively. No significant difference was found among various β-TCP content scaffolds, which could be supported by the SEM images in Fig. 1. The porosity of trabecular bone is 50–90% [23], while more tissue ingrowth and new bone formation occurred in areas with higher porosity after implantation [24]. Therefore, porosity of scaffold prepared in this work was sufficient for cell adhesion and proliferation.

3.2.3. Mechanical properties

Mechanical properties, especially compressive strength, are important for bone tissue engineering porous scaffolds [20,25]. To evaluate the mechanical properties of the scaffolds utilized in various circumstances, compressive test was performed on scaffolds both in dry status and hydrogels. Hydrogels with a β-TCP fraction of 5% had higher compressive modulus and compressive strength than hydrogels without β-TCP as displayed in Table 2. However, the mechanical properties declined when the fraction of β-TCP exceeded 10%. Kuo et al. revealed that the modulus and mechanical strength of chitosan/β-TCP/poly (methyl methacrylate) cement composites decreased with increasing in chitosan/β-TCP microspheres content [26]. Venugopal et al. also reported that with the addition of 1% HA to collagen, the tensile strength and Young's modulus decreased significantly, which was attributed to the particle defects within the polymer matrix [27]. In our study, the higher fraction of β-TCP would lead to more significant agglomeration and more serious stress concentrations, and thus reduced the compressive modulus and compressive strength of the composite scaffolds. A similar profile had been observed in the test of scaffolds in dry status. The results also showed an initial increase and a further decrease in mechanical properties; while the maximum occurred at 10% fraction of β-TCP.

3.2.4. X-ray diffraction

The XRD spectra of composite scaffolds with different fractions of β-TCP are shown in Fig. 5. The XRD patterns indicated that with an increase of β-TCP fraction, the characteristic peaks of β-TCP at 26.7°, 30.6° and 32.8° [28] became incisive. However, the diffraction peak at 22.3°, which was assigned to the polymer chains aligned through intermolecular interaction [20], became wider and flatter. Especially when the β-TCP fraction reached 40%, the peak nearly complied with the datum line. It was implied that β-TCP binding to the polymer chain of gelatin/CM-chitosan matrix, in turn, affected the intermolecular interactions.

Table 2
Mechanical properties of gelatin/CM-chitosan/β-TCP composite hydrogels and scaffolds in dry status as a function of β-TCP fractions.

β-TCP fraction in the solid part	Hydrogels		Scaffolds in dry status	
	Compressive modulus (KPa)	Compressive strength (KPa)	Compressive modulus (MPa)	Compressive strength (MPa)
0%	16.8 ± 1.2	82.4 ± 17.2	36.3 ± 5.1	2.5 ± 0.2
5%	24.2 ± 2.2	116.0 ± 35.6	36.7 ± 3.3	1.8 ± 0.2
10%	9.7 ± 0.9	70.5 ± 11.0	41.1 ± 6.3	1.9 ± 0.2
20%	5.5 ± 0.9	59.7 ± 14.8	36.7 ± 5.5	1.8 ± 0.4
30%	2.6 ± 0.7	31.2 ± 10.5	31.3 ± 4.3	1.5 ± 0.1
40%	1.6 ± 0.4	34.9 ± 10.3	21.9 ± 3.4	1.2 ± 0.1

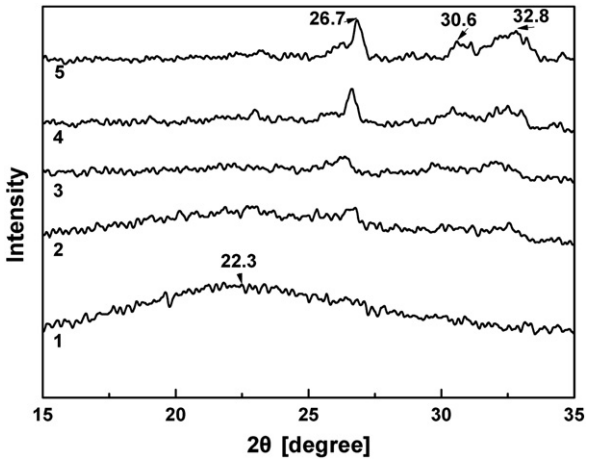

Fig. 5. XRD spectra of gelatin/CM-chitosan/β-TCP composite scaffolds: (1) β-TCP fraction 0%, (2) β-TCP fraction 10%, (3) β-TCP fraction 20%, (4) β-TCP fraction 30%, and (5) β-TCP fraction 40%. The ultrasonic time was 20 min.

3.2.5. Fourier Transform Infrared Spectroscopy

FTIR spectra were recorded to investigate the intermolecular interaction between the components in the scaffolds. Fig. 6 displays the FTIR spectra of gelatin, CM-chitosan, β-TCP, gelatin/CM-chitosan, and gelatin/CM-chitosan/β-TCP composite scaffold. The characteristic peaks of CM-chitosan appeared at 3386 cm^{-1}, 1585 cm^{-1}, 1404 cm^{-1} and 1057 cm^{-1} were assigned to the –OH stretching vibration, N–H bending vibration of amide (II), the symmetric stretching vibrations of COO- groups, and the C–O asymmetric stretching vibration, respectively [29,30]. Gelatin was characterized as its N–H stretching of amide A at 3327 cm^{-1}, C═O stretching of amide I peak at 1653 cm^{-1} and N–H bending vibration of amide (II) at 1540 cm^{-1} [31]. The absorption bands at 1061 cm^{-1} were assigned to stretching and bending of PO_4^{3-} group of β-TCP [32].

In the gelatin/CM-chitosan scaffolds, the C═O stretching peak at 1653 cm^{-1} of gelatin moved to lower wavenumber at 1641 cm^{-1}, and the –OH stretching vibration band of CM-chitosan at 3386 cm^{-1} shifted to 3289 cm^{-1}. Meanwhile, N–H stretching peak of amide A for gelatin at 3327 cm^{-1} and –OH stretching vibration peak of CM-chitosan at 3386 cm^{-1} merged into each other to form a broad band in the range of 3338–3200 cm^{-1}. Besides, the N–H bending vibration of amide (II) at 1585 cm^{-1} of CM-chitosan and 1540 cm^{-1} of gelatin merged into each other and appeared as a broad band from 1588 to 1550 cm^{-1} in the gelatin/CM-chitosan

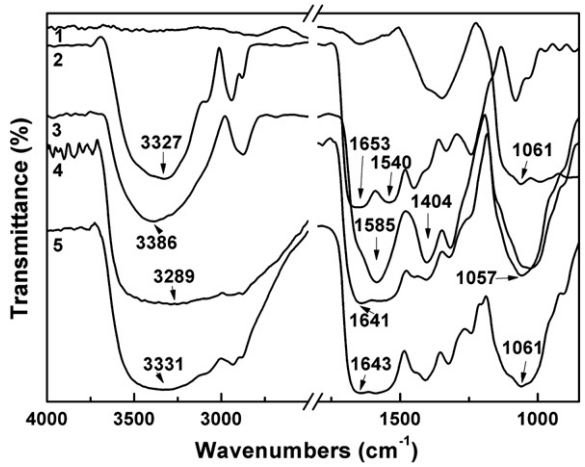

Fig. 6. FTIR spectra of (1) β-TCP, (2) gelatin, (3) CM-chitosan, (4) gelatin/CM-chitosan composite scaffold, and (5) gelatin/CM-chitosan/β-TCP composite scaffold with 30% β-TCP fraction. The ultrasonic time was 20 min.

spectrum. The above mentioned changes revealed that strong hydrogen bond can be formed between amide group and carboxyl groups of gelatin and CM-chitosan [31,33]. With the incorporation of 30% β-TCP, the peak of the C═O stretching peak at 1641 cm^{-1} and –OH stretching vibration at 3289 cm^{-1} moved to higher wavenumber at 1643 and 3331 cm^{-1}, which indicated that β-TCP interrupted with the hydrogen bond within the hydrogel network. The interactions between β-TCP and gelatin/CM-chitosan include: electrostatic interaction between calcium ions and carbonyl bands, and interaction between amine bonds and phosphates [34,35]. Therefore, lead to the decreasing of swelling degree after incorporation with β-TCP. In all, the interactions between gelatin/CM-chitosan and β-TCP brought good miscibility to the components.

3.3. In vivo implantation

In vivo biocompatibility of the scaffold was evaluated by implanting the scaffold in mandible of beagle dogs. All of the beagle dogs survived until being sacrificed at 4 weeks after surgery. Wound infections, dropsies, necrosis, and serious inflammation were not observed in all tissue specimens, which manifested that the scaffolds had excellent biocompatibility. The excised mandible tissue is shown in Fig. 7A, and the hole filled with scaffold for 4 weeks is shown at the arrow.

Micro-CT is a fast and non-destructive technique to characterize and measure the 3D properties of a scaffold. Therefore, it is frequently used in determining scaffold characteristics and bone ingrowth parameters [36]. Reconstruction images of micro-CT are shown in Fig. 7B. Ellipse area marked in the left and right side represented the area of defect implanted by gelatin/CM-chitosan/β-TCP and gelatin/CM-chitosan, respectively. Sound amount of new bone regenerated was observed on both sides. Moreover, to assess the structural features of bone ingress more clearly, a 3D model of the regenerated bone in the gelatin/CM-chitosan/β-TCP composite scaffold repaired region and the surrounding bone tissue was created by micro-CT images, as shown in Fig. 7C. As shown at the arrow, at 4 weeks after implantation, most of the scaffold had been biodegraded and the osteogenic reaction occurred from the rim of the surgical lesion toward the center. The reason was that the delivery of osteoblast, bone morphogenetic proteins, and nutrient came from surrounding bone tissue. The volumetric density (BV/TV, %), a quantified estimation of the new bone formation in defects, was calculated to be 13.3% and 12.5% for the gelatin/CM-chitosan/β-TCP and the gelatin/CM-chitosan scaffold respectively. The results showed that the scaffold consisted of β-TCP had marginal but definite higher ability on promoting bone regeneration than the scaffold without β-TCP, probably because CM-chitosan and gelatin could also enhance bone regeneration. Besides, there was no significant difference between both compositions in terms of biocompatibility.

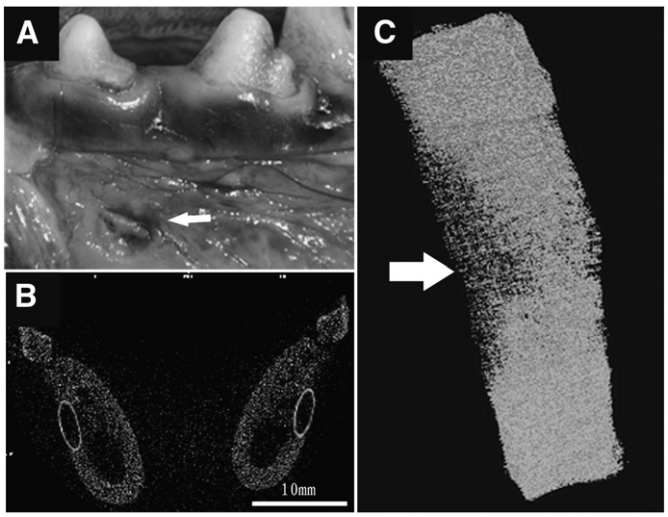

Fig. 7. All the animals were sacrificed at 4 weeks after surgical. (A) The image of excised mandible tissue with defect filled by scaffold. (B) Reconstruction image of micro-CT for the defects repaired. Ellipse area marked in the left and right side represented the area of defect implanted by gelatin/CM-chitosan/β-TCP and gelatin/CM-chitosan, respectively. Sound amount of regenerated new bone was observed on both sides. (C) 3D model of the defect repaired by gelatin/CM-chitosan/β-TCP scaffold, as well as the surrounding bone tissue. The arrow showed the direction of osteogenic reaction, which started from the rim of the surgical lesion toward the center.

In all, besides their biocompatibility, gelatin/CM-chitosan/β-TCP composite scaffolds were completely degradable in PBS. Meanwhile, they possessed biomimetic composition, excellent swelling ability, interconnected porous structures and proper mechanical properties, which supported their prospect for the application as bone tissue engineering materials.

4. Conclusion

Gelatin/CM-chitosan/β-TCP composite scaffolds were prepared using a green fabrication method, i.e. radiation-induced crosslinking. Distribution of β-TCP particles could be controlled by the time of ultrasonic treatment. The fraction of β-TCP particles considerably affected the crosslinking and swelling properties of different scaffolds. Considering their excellent and adjustable water retention capacity, highly interconnected porous network structure, proper compressive strength and high porosity, the scaffolds would meet the criteria for bone tissue regeneration. In addition, *in vivo* implantation experiment confirmed that the scaffolds could accelerate bone regeneration in accompany with the degradation of the scaffolds. The comprehensive results of this study suggested that gelatin/CM-chitosan/β-TCP composite scaffolds have potential as bone tissue engineering materials.

Acknowledgment

This work was supported by the State Key Development Program for Basic Research of China (Grant 2007CB936103), Peking University Interdisciplinary and Emerging Disciplines Research Foundation (Grant PKUJC2009001), Peking University's 985 Grant. We thank Mr. Xiaomian Wu for assistant in swelling and micro-CT experiment. Professor Anyuan Cao and Mr. Luhui Zhang are acknowledged for offering facilities for the measurement of mechanical properties.

References

[1] H. Maeda, et al., Sci. Technol. Adv. Mater. 6 (1) (2005) 48–53.
[2] X. Ma, et al., J. Bioact. Compat. Polym. 24 (5) (2009) 457–472.
[3] R. Muzzarelli, Carbohydr. Polym. 83 (2011) 1433–1445.
[4] F. Zhao, et al., Biomaterials 23 (15) (2002) 3227–3234.
[5] L.Y. Jiang, Y.B. Li, C.D. Xiong, J. Biomed. Sci. 16 (2009) 65–74.
[6] Y. Marois, et al., Biomaterials 16 (15) (1995) 1131–1139.
[7] Y.J. Seol, et al., Biotechnol. Lett. 26 (13) (2004) 1037–1041.
[8] A. Di Martino, M. Sittinger, M.V. Risbud, Biomaterials 26 (30) (2005) 5983–5990.
[9] N. Shanmugasundaram, et al., Biomaterials 22 (14) (2001) 1943–1951.
[10] X. Chen, et al., Biomaterials 23 (23) (2002) 4609–4614.
[11] L.J. Kong, et al., J. Biomed. Mater. Res. A 75A (2) (2005) 275–282.
[12] A. Ogose, et al., J. Biomed. Mater. Res. B Appl. Biomater. 72B (1) (2005) 94–101.
[13] M.J. Yaszemski, et al., Biomaterials 17 (2) (1996) 175–185.
[14] C. Yang, et al., Carbohydr. Polym. 82 (4) (2010) 1297–1305.
[15] L. Zhao, et al., Carbohydr. Polym. 51 (2) (2003) 169–175.
[16] Y. Zhang, M.Q. Zhang, J. Biomed. Mater. Res. 55 (3) (2001) 304–312.
[17] M. Sous, et al., Biomaterials 19 (23) (1998) 2147–2153.
[18] J. Li, et al., Mater. Sci. Eng., C 29 (4) (2009) 1207–1215.
[19] J.N.J. Pratt, et al., Biomaterials 23 (16) (2002) 3309–3317.
[20] Y.J. Yin, et al., J. Biomed. Mater. Res. A 67A (3) (2003) 844–855.
[21] L. Xu, et al., Carbohydr. Polym. 82 (4) (2010) 1297–1305.
[22] G.M. Eichenbaum, et al., Macromolecules 31 (15) (1998) 5084–5093.
[23] V. Karageorgiou, D. Kaplan, Biomaterials 26 (27) (2005) 5474–5491.
[24] T.D. Roy, et al., J. Biomed. Mater. Res. A 66A (2) (2003) 283–291.
[25] S. Panzavolta, et al., Acta Biomater. 5 (2) (2009) 636–643.
[26] S.M. Kuo, et al., J. Appl. Polym. Sci. 89 (14) (2003) 3897–3904.
[27] J. Venugopal, et al., J. Mater. Sci. - Mater. Med. 19 (5) (2008) 2039–2046.

[28] S.S. Homaeigohar, et al., J. Eur. Ceram. Soc. 26 (3) (2006) 273–278.
[29] S. Kim, et al., J. Biomed. Mater. Res. Part B 75 (2) (2005) 442–450.
[30] J. Brugnerotto, et al., Polymer 42 (8) (2001) 3569–3580.
[31] F.L. Mi, Biomacromolecules 6 (2) (2005) 975–987.
[32] A. Dupraz, et al., Biomaterials 20 (7) (1999) 663–673.
[33] K.D. Yao, et al., Polym. Int. 48 (6) (1999) 429–432.
[34] K.D. Yao, et al., Biomaterials 28 (5) (2007) 781–790.
[35] M.Q. Zhang, Y. Zhang, J. Biomed. Mater. Res. 62 (3) (2002) 378–386.
[36] A.C. Jones, et al., Biomaterials 28 (15) (2007) 2491–2504.