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# A facile approach to construct three-dimensional oriented chitosan scaffolds with in-situ precipitation method

Yinzhe Li [a,b], Youxiang Wang [a,b,*], Dao Wu [a], Kai Zhang [c], Qiaoling Hu [a,b,*]

[a] Department of Polymer Science and Engineering, Zhejiang University, People's Republic of China
[b] Key Laboratory of Macromolecular Synthesis and Functionalization, Ministry of Education, Zhejiang University, People's Republic of China
[c] Affiliated Dental Hospital of Zhejiang University, Hangzhou 310026, People's Republic of China

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ABSTRACT

How to construct three-dimensional oriented chitosan scaffolds with improved mechanical property is essential to further design bone-regenerative scaffolds. Materials with multi-layer structures involve excellent mechanical properties. In this research, a facile approach was adopted to construct three-dimensional oriented chitosan scaffolds. The chitosan gel with multi-layer structure was first prepared with in-situ precipitation, and then lyophilization was applied to obtain porous scaffolds. SEM images indicated that the porous scaffolds had spoke-like framework in cross-section and multi-layer structure in vertical-section. Compared with the disordered scaffolds prepared with lyophilization method, the scaffolds prepared with in-situ precipitation method showed a significantly improved compressive strength. With chitosan concentration of 4–5% and drying time of 60 min, the scaffold showed the best comprehensive property with a suitable porosity and a high compressive strength. This novel porous scaffold with three-dimensional oriented structure might have potential application in bone tissue engineering.



## 1. Introduction

Tissue engineering is potential to create functional and viable tissue constructs for patients in need of organ replacement. The three-dimensional (3-D) scaffold is served as an artificial extra cellular matrix (ECM) to guide the cell attachment, growth and tissue regeneration. The ideal scaffolds should be biocompatible, biodegradable, and have interconnected pores with a proper size to favor the cell infiltration and vascularization (Agrawal & Ray, 2001; Hutmacher, 2000; Santos, Unge, Sousa, Reis, & Kirkpatrick, 2009). In bone tissue engineering, adequate mechanical properties were very important to maintain the structure and function during the implantation.

Numerous biodegradable polymers have been investigated with scaffolds prepared through phase separation, particles foaming, electrostatic spinning, sintering, etc., including PCL (Kim & Son, 2009; Porter, Henson, & Popat, 2009), PLGA, polyester (Bil, Ryszkowska, & Kurzydlowski, 2009; Hu, Shen, Yang, Bei, & Wang, 2008), chitosan (CS) and composite of these materials (Chaudhuri, Davidson, Ellis, Jones, & Wu, 2008; Dong, Li, & Zou, 2009; Gupta, Venugopal, Mitra, Giri Dev, & Ramakrishna, 2009; Jiang et al., 2008; Lien, Chien, & Huang, 2009; Liu, Han, & Czernuszka, 2009; Nejati, Firouzdor, Eslaminejad, & Bagheri, 2009; Sawyer et al., 2009; Zhang, Hong, Yu, Chen, & Jing, 2009). Among them, chitosan has been extensively applied as scaffolds with its characteristics of biodegradability, biocompatibility and easy acquisition from nature (Kim et al., 2008; Muzzarelli, 2009). Thein-Han and Misra (2009) fabricated chitosan/HA composite scaffold via lyophilization method and studied the influence of chitosan molecular and HA content on the scaffold physico-chemical properties and biological compatibility. Cooney, Petermann, Lau, and Minteer (2009) prepared chitosan scaffold through thermally induced phase separation and the result indicated that the average pore size decreased with lowered freezing temperature. However, the microarchitectures of porous scaffolds mentioned above were disordered, which influenced their mechanical properties and limited their wide use in bone tissue engineering. How to construct three-dimensional oriented chitosan scaffolds with improved mechanical property is the key to designing the bone-regenerative scaffolds.

Multi-layer structure showed excellent mechanical properties, such as bamboo and conch. Recently, Domard (Ladet, Dacid, & Domard, 2008; Montembault, Viton, & Domard, 2005) and Elisseeff (2008) fabricated the onion-like multi-membrane structured hydrogels, and reported the application of this gel in the tissue engineering (Boucard et al., 2007). In our previous research (Hu, Li, Wang, & Shen, 2004a, 2004b), three-dimensional ordered chitosan rods with multi-layer structure were successfully prepared via in-situ precipitation method. The rods showed excellent mechanical property. In this research the chitosan gel with multi-layer

* Corresponding authors. Tel./fax: +86 571 87953726 (Y. Wang).
*E-mail addresses:* yx_wang@zju.edu.cn (Y. Wang), huql@zju.edu.cn (Q. Hu).

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structure was prepared via in-situ precipitation method as illustrated in Fig. 1. Then the chitosan gel was freeze-dried to obtain porous scaffold. Its structure and mechanical properties were investigated as follows.

## 2. Experimental

### 2.1. Materials

Biomedical grade CS (viscosity-average molecular weight ($M_\eta$) $5.63 \times 10^5$) with the degree of N-deacetylation (DD) of 85% was supplied by Zhejiang Golden-Shell Biochemical Co., Ltd., Taizhou, China. Acetic acid and NaOH of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd., China.

### 2.2. Preparation of chitosan scaffolds via in-situ precipitation method (Hu et al., 2004a, 2004b)

Transparent and yellow chitosan solution with concentration of 4–6% (w/v) was prepared by dissolving 20 g chitosan powders in 2% (v/v) acetic acid solution and then the solution was transferred to the beaker to remove the air bubbles. The CS solution was precipitated by 5% (w/v) NaOH aqueous solution in the internal surface of a mold to form a membrane as the template. Then, the above-mentioned CS solution was filled in the mold and put into 5% (w/v) NaOH aqueous solution. After 8 h, the CS gel-rod was constructed via in-situ precipitation. The obtained gel-rod was first rinsed with distilled water until the pH of rinsed water turned to neutral, and then dried in oven at 60 °C for specified time (30, 60, 90 min, respectively). The porous cylindrical chitosan scaffolds could be obtained by treating lyophilization on the chitosan gel-rod. The porous scaffolds prepared only via lyophilization were taken as a control. CS solution (4% w/v) was poured into a cylindrical mold and frozen at −60 °C for 24 h and lyophilized for 48 h. After being neutralized by NaOH solution, the scaffolds were washed with deionized water and lyophilized again (Duarte, Mano, Rui, & Reis, 2009).

### 2.3. Microstructural characterization

The scanning electron microscopy (Japan 2570) was used to observe the microstructures of the vertical and cross-sections of the chitosan scaffolds after gold-spraying.

### 2.4. Testing of mechanical properties

The compressive strength of sample was tested with Shenzhen Reger Company's universal materials testing machine having the maximum compression ratio of 50% and the loading rate of 2 mm/min.

### 2.5. Measurement of the porosity of CS scaffold

The porosity of scaffolds was measured with liquid displacement method (Zhao, Yin, Song, et al., 2001). Ethanol was adopted for the measurement of porosity as it was easy to permeate into the interior of materials without the induction of shrinking and swelling. Materials with certain mass were first placed in the ethanol with certain volume ($V_1$), and then treated by cycled vacuum pumping until no bubbles escaped. The total volume of materials and ethanol was marked as $V_2$, thus the solid volume of CS scaffolds could be expressed as $V_2 - V_1$. After the scaffolds containing ethanol were removed, the volume of remaining ethanol was recorded as $V_3$. Then the volume of the ethanol ($V_1 - V_3$) contained in the scaffolds could be viewed as the volume occupied by the pores of scaffolds and the total volume of the scaffolds was $V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3$. So the porosity of chitosan scaffolds could be expressed as $P = (V_1 - V_3)/(V_2 - V_3)$.

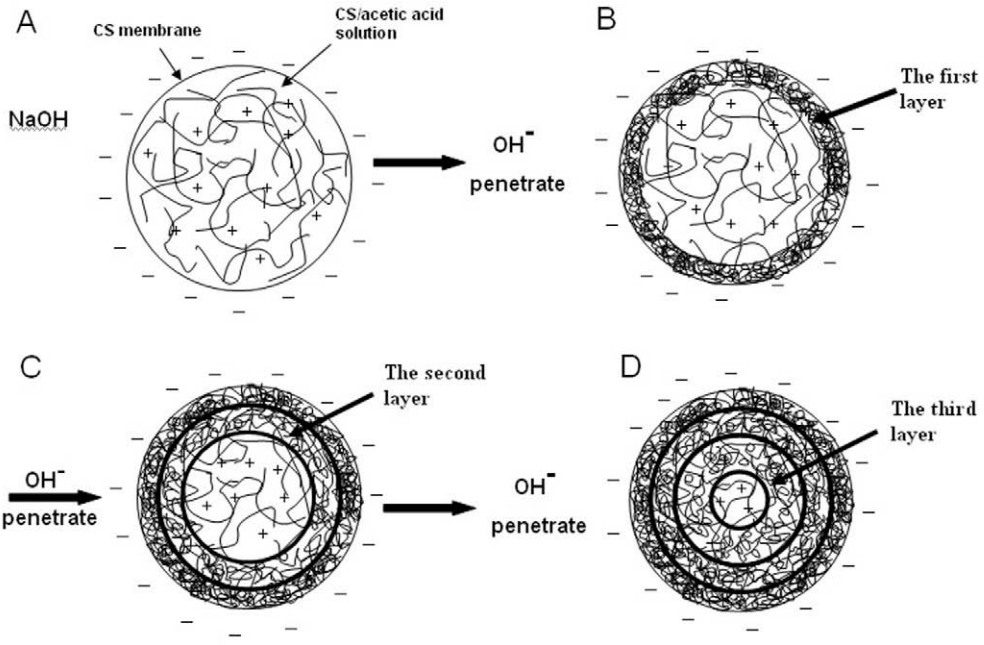


**Fig. 1.** Schematic representation of formation process of chitosan gel with multi-layer structure: (A) The mold filled with CS/acetic acid solution was put into the NaOH solution; (B) the first layer of the gel was formed with the $OH^-$ penetration through the CS membrane; (C) the second layer of the gel formed with the $OH^-$ penetration through the first layer; (D) the step C was ceaselessly repeated until all of the CS solution turned into chitosan gel.

## 3. Results and discussion

### 3.1. Morphology of the chitosan hydrogel prepared via in-situ precipitation

The chitosan gel was prepared via in-situ precipitation method as mentioned above. Layered structure of the gel was clearly observed in the cross-section of chitosan hydrogel (Fig. 2). The possible mechanism was discussed in our previous research (Hu et al., 2004a, 2004b). CS was positively charged because of the protonation of the amino groups in the acid solution. The CS solution and 5% NaOH aqueous solution were separated with a CS membrane so that small ions like $OH^-$ could permeate. When $OH^-$ was diffused into CS solution, CS was precipitated and formed into the first layer. As the time passed, $OH^-$ continued to penetrate through the formed gel layers and diffused into the solution, and then the second layer was shaped. The above-mentioned process was repeated until the multi-layer structure was finally formed (Fig. 1). Meanwhile, due to the inductive effect of the $OH^-$ concentration gradient, CS gel showed an orderly structure resembling a spoke, which together with the layer-form process constituted the unique three-dimensional oriented gel-rod (Fig. 3).

### 3.2. Morphology of porous chitosan scaffolds

High porosity and good pore-connectivity were essential to ensure sufficient nutrient diffusion through the scaffold, such as transporting oxygen and nutrients towards the cells while allowing metabolic products to be removed (Hutmacher, 2000). The morphology of the porous CS scaffolds was observed by SEM.

The scaffold with CS concentration of 4% (w/v) was prepared via lyophilization method and was taken as a control. The images in Fig. 4(a) indicated that the morphology in both cross-section and vertical-section had disorderly structures with staggered pores of 100–300 μm. However, the morphology of scaffolds prepared via in-situ precipitation showed regular structure in some extent. From the images of cross-sections, the pore diameter was about 180–300 μm and gradually increased from the center to the corners, exhibiting a spoke-like distribution. In the vertical-section, layer-oriented structure could be observed. All the results indicated that during the process of lyophilization, the three-dimensional oriented structure could be reserved to obtain the porous scaffolds. The potential mechanism was that during the freezing process of CS hydrogel, the phase separation between water and CS occurred, then the ice sublimated and pores were formed during the vacuum pumping process, while the CS remained in the original place. This kind of three-dimensional oriented structure might benefit the improvement of the mechanical property of the scaffolds.

**Fig. 2.** The photo of the cross-section of chitosan hydrogel via in-situ precipitation.

### 3.3. Compressive strength

In bone tissue engineering, adequate mechanical property was a very important factor. With the CS concentration of 4% (w/t) and the drying time of 60 min, the compressive strength of scaffold was prepared via lyophilization and in-situ precipitation was 0.45 MPa and 0.71 MPa, respectively. The difference of compressive strength suggested different structures. The scaffolds prepared via in-situ precipitation method had three-dimensional oriented structures, which contributed to the uniform dispersion of loads to the surface and interior of scaffolds under the stress. As a result, the compressive strength with three-dimensional oriented structure could be remarkably improved.

The concentration of CS solution and drying time also had great effect on the compressive strengths of porous chitosan scaffolds. The results in Fig. 5 indicated that with the same CS concentration, the compressive strengths of scaffolds increased as the drying time increased. With CS concentration of 6% (w/t) and the drying time of 90 min, the compressive strength of scaffold even reached 1.8 MPa.

### 3.4. Porosity

The porosity of CS scaffolds was also influenced by the CS concentration and drying time of the hydrogel. Fig. 6 indicated that when the concentration of CS solution was consistent, the porosity of scaffold decreased as the drying time increased. On the other hand, when the drying time of gel-rod remained consistent, the porosity increased as the concentration of solution decreased. With the same concentration of solution, as the drying time increased, the water content of CS gel reduced, so did the ice granule formed during the lyophilization, which led to smaller porosity. With consistent drying time, when the concentration of CS solution dropped off, the water content of gel-rod increased, leading to a larger porosity. In our experiment, when the drying time reached 90 min, the porous scaffold showed poor pore-connectivity. However, when the concentration of CS solution was above 5%, the porosity deceased too much to satisfy the demand of tissue engineering (data not shown in Fig. 5).

The compressive strengths of scaffolds had some contradiction with the porosity. As the results have, when the concentration of the CS solution was lower than 4% or the drying time was

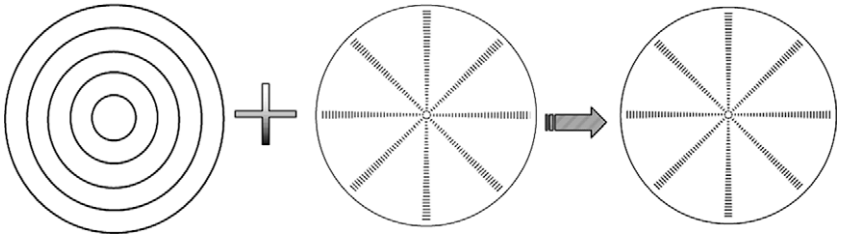

**Fig. 3.** Hierarchal architecture of chitosan hydrogel via in-situ precipitation.

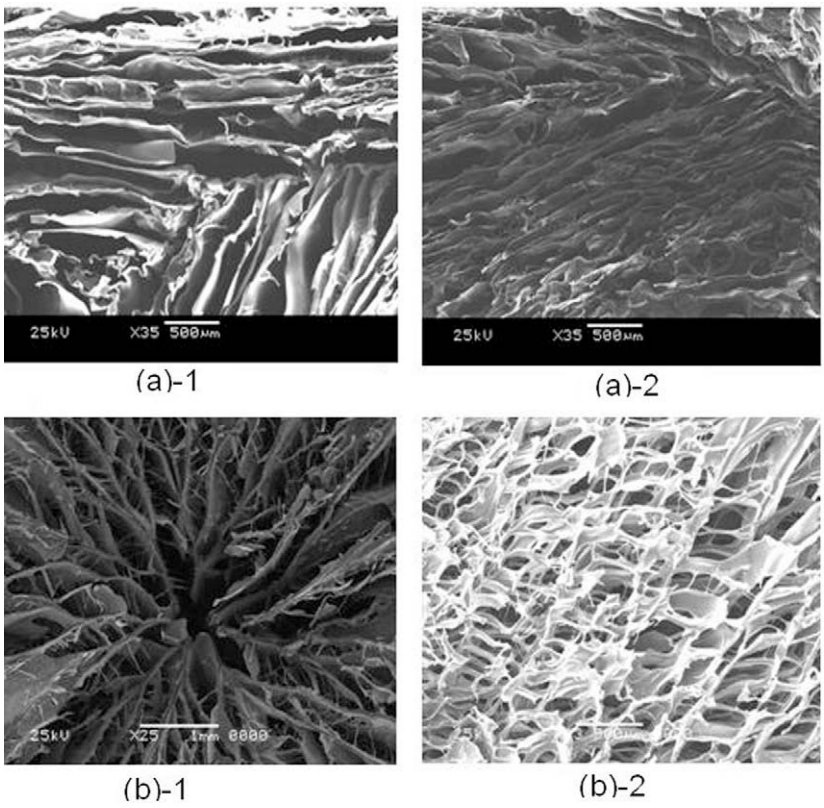


**Fig. 4.** The SEM images of cross-section ((a)-1) and vertical-section ((a)-2) of the porous chitosan scaffold prepared by lyophilization method; and the SEM images of cross-section ((b)-1) and vertical-section ((b)-2) of the porous chitosan scaffold prepared by in-situ precipitation method.

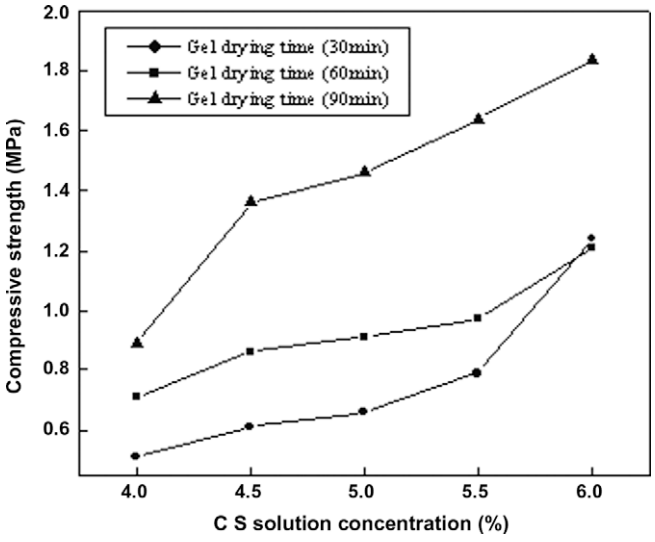


**Fig. 5.** The compressive strengths of porous chitosan scaffolds prepared under different concentration of CS solution and different drying time.

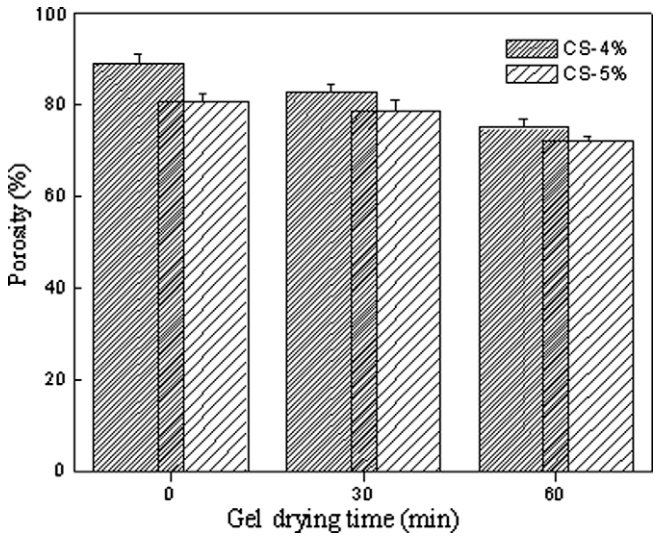


**Fig. 6.** The porosities of chitosan scaffolds prepared under different drying time.

30 min, the compression strength was too low, and the pore-connectivity and the porosity were poor when the concentration was above 5% or the drying time reached 90 min. By selecting the proper preparation condition, the porous scaffold with a suitable porosity and a high compressive strength should be obtained. With chitosan concentration of 4–5% and drying time of 60 min, the scaffold showed the best comprehensive property with a suitable porosity and a high compressive strength.

## 4. Conclusion

Three-dimensional oriented porous chitosan scaffolds have been successfully prepared via in-situ precipitation method. The SEM images indicated that the porous scaffolds had spoke-like framework in cross-section and multi-layer structure in vertical-section. The pores were connective, which was essential to ensure sufficient nutrient diffusion through the scaffold. Compared with the disorderly scaffolds prepared via lyophilization method, the scaffolds prepared via in-situ precipitation method showed a sig-

nificantly improved compressive strength. CS concentration and the drying time had also great influence on the microstructure, porosity and mechanical properties of CS scaffolds. With chitosan concentration of 4–5% and drying time of 60 min, the scaffold showed the best comprehensive property with a suitable porosity and a high compressive strength. This novel porous scaffold with three-dimensional oriented structure might be potentially used to treat cancellous bones.

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