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A facile method for the preparation of chitosan-based scaffolds with anisotropic pores for tissue engineering applications

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ABSTRACT

To date, great efforts have been made to prepare different kinds of isotropic tissue engineering (TE) scaffolds. However, little attention has been paid to anisotropic porous scaffolds in spite of many examples of their excellent performances. In this work, a facile method termed "ammonia-induced method" (AIM) was proposed and applied to generate anisotropic pores in chitosan (CS)-based scaffolds. The pore structures of these scaffolds were studied in detail. In order to clarify the rationale behind this process, a speculative explanation was provided on basis of the experimental results and the theory of Uras (Uras & Devlin, 2000). Compression tests indicated that the mechanical strengths of these scaffolds were sufficient for TE applications. *In vitro* cell culture showed that MC3T3-E1 cells cultivated in the pores of these scaffolds had positive proliferation potential. We anticipated that this novel AIM could inspire research not only in TE but also in other fields.

1. Introduction

In the field of tissue engineering (TE), any anisotropy in the properties of a desired tissue (*e.g.,* bone and cardiac muscle) must be replicated in the pore structures in order to ensure appropriate tissue regrowth (Engelmayr et al., 2008; Pawelec, Husmann, Best, & Cameron, 2014). For instance, trabecular bone has an optimized structural anisotropy because of the trabecular orientation along the principal stress trajectories (Butscher, Bohner, Hofmann, Gauckler, & Müller, 2011). Thus, in bone tissue engineering (BTE), oriented porous structure is needed to mimic the high anisotropy of bone tissue.

It has been reported that scaffolds with parallelly orientated tube-like pores may support homogeneous cell seeding as well as sufficient nutrient supply and could facilitate neo-vascularization (Bernhardt et al., 2009). Choi et al. validated the importance of uniformity in pore size and pore structure of a TE scaffold. It was claimed that uniform pore size and pore structure facilitated diffusion of macromolecules, spatial distribution of fibroblasts, and differentiation of preosteoblasts (Choi, Zhang, & Xia, 2010). In addition, there was also evidence showing that tissue synthesized in a scaffold with non-uniform pore architecture had inferior biomechanical strength compared with tissue synthesized in a scaffold with a more uniform pore structure (Hollister, Maddox, & Taboas, 2002; O'Brien, Harley, Yannas, & Gibson, 2004). In some other researches, researchers also discovered that scaffolds with axially oriented pores could facilitate the regeneration of conjunctiva, peripheral nerve, central nervous system, and the skin of burn patients (Moore et al., 2006; Stokols & Tuszynski, 2006; Yannas, Lee, Orgill, Skrabut, & Murphy, 1989).

To date, though much attention has been paid to porous scaffolds in TE field (Bognitzki et al., 2001; Ho et al., 2004; Intranuovo et al., 2014; Kucharska, Butruk, Walenko, Brynk, & Ciach, 2012; Tayalia, Mendonca, Baldacchini, Mooney, & Mazur, 2008), only a few researchers have focused on scaffolds with anisotropic arrangements and longitudinal pore alignments (Davidenko et al., 2012; Deville, Saiz, & Tomsia, 2006; Madaghiele, Sannino, Yannas, & Spector, 2008; Mathieu, Mueller, Bourban, Pioletti, Müller, & Månson, 2006). Furthermore, though scaffolds developed by those researchers demonstrated high anisotropy in pore structure, additional heating and/or use of harsh chemicals were required. In addition, the inherent defects of these scaffolds (*e.g.,* poor mechanical strength, non-uniform pore structure, non-biodegradability, and poor biocompatibility) limited their clinical applications.

For instance, Deville et al. invented a novel ice-template method to mediate the growth of ice crystals. By combining this

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method with a freeze-drying process, they prepared a hydroxyapatite scaffold with anisotropic pores and high mechanical strength. However, the lamellar structure of this scaffold limited its widespread applications in TE field (Deville, Saiz, Nalla, & Tomsia, 2006). Besides, Davidenko et al. prepared a collagen scaffold with anisotropic pores. In addition, by introducing multiple temperature gradients, the pore orientation, the anisotropy in pore size and alignment became adjustable. However, this scaffold suffered from poor mechanical strength (Davidenko et al., 2012). Huang et al. synthesized a novel CS-coated nano-hydroxyapatite/polyamide66 BTE scaffold utilizing a thermally induced phase inversion technique (Huang et al., 2012). Inter-connective pores can be observed on the cross-section of the scaffold and some of the pores are tube-like and parallel ranged along the pore axis. However, polyamide66 is non-degradable *in vivo*.

The objective of this work was the implementation of a facile method termed "ammonia-induced method" (AIM) to generate anisotropic pores in CS-based scaffolds, which included chitosan/polyvinyl alcohol (CS/PVA), chitosan/gelatin (CS/GEL), chitosan/silk fibroin (CS/SF), chitosan/hydroxyapatite (CS/HAp), and chitosan-silk fibroin/hydroxyapatite (CS-SF/HAp). Briefly, this process requires three basic elements: (I) CS-based hydrogels; (II) ammonia; (III) low crosslinking degree. Compared to conventional methods, this novel AIM has many advantages. And scaffolds prepared by using the AIM showed great potential for TE applications.

First, except ammonia, no additional reagents and/or operations are required. Compared to currently available methods (*e.g.,* freeze-drying, thermally induced phase inversion technique, 3D printing), the AIM is cost-effective and easy to operate; Second, inter-connective pores prepared by using the AIM take on high anisotropy, and the pore diameters are sufficient for cell migration and transport of nutrients and metabolic waste products. Furthermore, nano-scale pores were generated on the walls of the micron-scale pores of the CS/HAp and CS-TSF/HAp scaffolds, which are of great significance in facilitating cell-material interaction. In addition, hierarchical porous architectures can perform better than only micron-scale pores in modulating the mechanical strength of scaffolds (Ajalloueian et al., 2014; Holzwarth & Ma, 2011; Karageorgiou & Kaplan, 2005; O'Brien, Harley, Yannas, & Gibson, 2005; Sai et al., 2013; Saracino, Cigognini, Silva, Caprini, & Gelain, 2013; Woodard et al., 2007). Third, scaffolds prepared by using the AIM exhibited high stiffness and strength. The elastic modulus of CS/SF scaffold reached up to 300 MPa, which is in the range of elastic modulus of cancellous bone. In addition, the compressive modulus at strain of 0.7 of the CS-SF/HAp scaffold was as high as 301 MPa, which is even higher than that of cortical bone. Last, CS molecules have excellent biocompatibility, biodegradability, and non-toxicity (Roosen et al., 2016). In addition, genipin (GNP) was utilized as crosslinking reagent in this experiment (Tsai, Huang, Sung, & Liang, 2000). Thus, it was believed these CS-based hydrogels were in low teratogenicity or immunogenicity.

In order to clarify the rationale behind this process, a carefully designed experiment was carried out. We mainly focused on the influencing factors, which regulated the porous structures of the CS hydrogels. Four control variables (*i.e.,* the cross-linker concentration, the pre-treatment temperature, the ammonia concentration, and the type of inducing reagents) were included in this investigation. Furthermore, a speculative explanation was provided and a theoretical model was developed in this article. To make it clear whether this method can be applied to composite hydrogels with multiple phases and multiple components, five types of additives (*i.e.*, HAp, GEL, PVA, SF, and SF/HAp) were incorporated into the CS hydrogel. The results indicated that this method could be applied to prepare diverse composite scaffolds with anisotropic pores. As to CS/HAp and CS-SF/HAp scaffolds, the pore fabrication and the *in situ* precipitation of HAp were integrated into one step by using this AIM. The *in situ* precipitation method has been depicted in our previous work (Cai et al., 2009; Shen, Tong, Zhu, Wan, & Hu, 2007). The pore structures and the mechanical strengths of these scaffolds were investigated in detail. In addition, *in vitro* cell culture was utilized to evaluate the cytocompatibility of these scaffolds. We anticipated that these CS-based anisotropic porous materials could be ideal TE scaffolds. Besides, this AIM was expected to inspire research in pore fabrication in other fields.

2. Experimental section

2.1. Materials

Chitosan (*Mw* 1,000,000) was obtained from Golden-Shell Biochemical Co. (Zhejiang, China) with 95% degree of deacetylation. The *Bombyx mori* silkworms were bought from Nanyang, Henan, China. Genipin was purchased from Chengdu ConBon Bio-tech Co., Ltd. (Chengdu, China). Gelatin with gel strength ~240 g bloom and lithium bromide (LiBr) were obtained from Aladdin Industrial Co., Ltd (Shanghai, China). Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), diammonium hydrogen phosphate ($(NH_4)_2HPO_4$), acetic acid, sodium hydroxide (NaOH), polyvinyl alcohol (PVA), ammonia chloride (NH_4Cl), and ammonia were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and all of analytical grade. Deionized ultrapure water was used throughout the experiment. MC3T3-E1 cells were supplied by the School of Stomatology, Wuhan University (Wuhan, China).

2.2. Preparation of CS-based scaffolds with anisotropic pores

Briefly, a certain quality of CS was dissolved into acetic acid solution (2 vol%) at 45 °C with vigorous agitation for 30 min. Then specific additives (*e.g.,* inorganic salts, PVA, GEL, SF) were added into the CS solution. The preparation process of SF solution has been depicted elsewhere (Ran et al., 2015). Afterwards, GNP was added into the solution above. 20 min' later, the resultant solution was pre-treated at different temperatures (0 °C, 4 °C, 10 °C, and 37 °C) for 12 h. After that, ammonia was poured onto the surface of the solidified hydrogel through the permeation from the top to the bottom. 3 days' later, these hydrogels were rinsed with deionized water thoroughly for observation. To clarify the rationale behind this process, a series of specimens were synthesized. The specific treatments of different specimens were listed in Table 1. Next, these hydrated samples were air-dried in a constant temperature oven (37 °C) for 5 d. The ultimate specimens were utilized for morphology observation, compression tests, and *in vitro* cell culture.

2.3. Compression tests

Four typical scaffolds (*i.e.,* CS, CS/SF, CS/HAp, and CS-SF/HAp) were selected for compression tests by using a universal testing machine (SHIMADZU, AGS-J, Japan). All the specimens were shaped into cylinders (4 mm in height, 2 mm in diameter) with cross-section perpendicular to the pore axis. Samples were compressed in a direction perpendicular to the cross-section of the cylinders at a cross-head speed of 0.5 mm min^{-1}. Elastic modulus was calculated as the slope of the initial linear portion of the stress-strain curves and expressed as means of three replicates.

2.4. Scaffold characterizations

The macroscopic pore structures of the hydrated samples were observed by using a stereoscopic microscope (SM) (XTL-165, Phoenix, China). The morphologies of the air-dried scaffolds were observed by utilizing a field emission scanning electron microscope