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Fabrication and Characterization of Biomimetic Collagen-Apatite Scaffolds with Tunable Structures for Bone Tissue Engineering

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Abstract

The objective of the current study is to prepare a biomimetic collagen-apatite (Col-Ap) scaffold for improved bone repair and regeneration. A novel bottom-up approach has been developed, which combines a biomimetic self-assembly method with a controllable freeze casting technology. In this study, the mineralized collagen fibers were generated using a simple one-step co-precipitation method which involved collagen self-assembly and in situ apatite precipitation in a collagen-containing modified simulated body fluid (m-SBF). The precipitates were subjected to controllable freeze casting, forming scaffolds with either an isotropic equiaxed structure or a unidirectional lamellar structure. These scaffolds were comprised of collagen fibers and poorly crystalline bone-like carbonated apatite nanoparticles. The mineral content in the scaffold could be tailored in a range 0–54 wt% by simply adjusting the collagen content in the m-SBF. Further, the mechanisms of the formation of both the equiaxed and the lamellar scaffolds were investigated, and freezing regimes for equiaxed and lamellar solidification were established. Finally, bone forming capability of such prepared scaffolds was evaluated *in vivo* in a mouse calvarial defect model. It was confirmed that the scaffolds well support new bone formation.

Keywords

collagen; apatite; freeze-drying; biomimetic; bone regeneration

1. Introduction

Collagen-apatite (Col-Ap) composite resembling the composition of natural bone has been studied extensively and considered as a promising bone tissue engineering material [1–16]. Apatite exhibits good biocompatibility, osteoconductivity and bone-bonding ability, but its brittleness, rigidity, and slow degradation rate have limited its usefulness in broader biomedical applications [2, 17]. Collagen, the most abundant protein in extracellular matrix, is chemotactic to fibroblasts. It shows high affinity to cells and good resorbability *in vivo*.

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Nevertheless, its poor mechanical properties have restricted its usage in load-bearing applications [18–21]. By adding apatite to collagen, the mechanical properties of the resulting composite could increase substantially [7]. It was also reported that Col-Ap composite scaffolds demonstrate better osteoconductivity and osteogenicity than non-mineralized collagen scaffolds [14].

In recent years, many research groups have worked on the preparation of Col-Ap scaffolds with optimum structures and chemical compositions for bone and cartilage repair [5–16]. Col-Ap scaffolds were synthesized by either physically mixing pre-synthesized apatite particles with collagen fibers or applying a biomimetic apatite coating onto lyophilized collagen scaffolds [5–8]. In the light of all these successes in preparing Col-Ap scaffolds, further efforts have been made to fabricate scaffolds consisting of mineralized collagen fibers mimicking the building block in natural bone [8]. In the last decade, Kikuchi *et al.* developed biomimetic Col-Ap composite scaffolds by mineralizing type I collagen in a solution of calcium phosphate [9]. Since then, the co-precipitation approach has been adopted by many researchers [10–15]. Especially, in situ self-assembly to mimic the biomineralization process found in nature has attracted much of the attention. This process is environmental friendly, and allows a high degree of control over apatite content and crystal growth. Recently, our research group has developed a biomimetic coating process, which successfully formed a layer of calcium phosphate coating onto the surface of tissue engineering scaffolds, ceramics and metals within 24 h of immersion in a modified simulated body fluid (m-SBF) [22–24]. Furthermore, collagen has been added to the m-SBF to allow biomineralization of self-assembled collagen fibers to form a dense Col-Ap composite or a biomimetic composite coating [25, 26]. Using the same approach, a 3-D porous Col-Ap composite scaffold has also been prepared by carefully adjusting the collagen concentration and pH value of the collagen-containing m-SBF in the current study.

In tissue engineering, the function of a scaffold is to provide a 3D spatial and temporal structure to direct cell attachment, proliferation and differentiation, and guide tissue formation. An open porous architecture with suitable pore size is required to facilitate mass transportation of nutrients and vascularization, while maintaining a certain mechanical strength for handling and providing necessary mechanical support [21, 27]. Freeze casting, a method based on physical properties of ice formation, has been used widely to generate scaffolds with distinct structures for tissue engineering [22–44]. This technique does not involve any chemical reaction, thus avoiding potential complications associated with purification procedures [28]. Currently, there are two main porous structures fabricated by controlling freezing regimes: equiaxed structure and anisotropic lamellar structure. In general, the scaffold fabricated under a constant and slow cooling rate demonstrates an anisotropic, equiaxed porous structure. The pore size can be tailored by controlling the final freezing temperature (T_f) in the chamber of freeze dryer, and the distribution of the pore size is determined by the cooling rate [29–38]. In contrast, scaffolds with preferential orientation and open porosity can be prepared using unidirectional solidification to control the direction of ice growth [39–44]. In most of the freeze casting studies, pure ceramic or pure polymeric systems are investigated. The freezing behavior of a Col-Ap composite system has hardly been explored in depth, and the degree of control for scaffold microstructure is still lacking.

In the current study, we have designed a fabrication process combining a novel biomimetic strategy with controllable freeze casting. This method is simple but capable of fabricating bone-like composites with a range of Col-Ap ratios and structures to meet versatile needs for tissue regeneration. Two freezing regimes have been established to prepare scaffolds with equiaxed structure and unidirectional lamellar structure, respectively. The mechanisms of freeze casting have been explored in depth. Finally, a preliminary *in vivo* evaluation on the Col-Ap scaffold was conducted using a two-hole mouse calvarial defect model.

2. Materials and Methods

2.1 Preparation of Col-Ap suspensions

Biomimetic Col-Ap composites were synthesized using a collagen containing modified simulated body fluid (m-SBF) as shown in Fig. 1. Based on the protocol by Rajan *et al.* [45], type I collagen was extracted from rat tails, purified and freeze-dried. Such obtained collagen was dissolved in 0.02 M acetic acid at 4 °C, and its concentration in m-SBF was adjusted to 1, 2, 3 g/L, to achieve scaffolds with varied apatite content. Such prepared Col-Ap scaffold was named as Col-Ap-x, and x represents the apatite content in the scaffold (wt %). The m-SBF was prepared as reported previously [22–26]. The pH of the m-SBF solution was adjusted to 7 by addition of HEPES (4-(2-hydroxyapatiteethyl)-1-piperazineethanesulfonic acid) and NaOH. After soaking in m-SBF in a sealed vial under constant stirring at 40 °C for 24 h, the precipitates were collected by centrifuging at 10000 rpm for 12 min.

2.2 Freeze casting of Col-Ap suspensions

Two freezing regimes were applied to freeze casting of Col-Ap suspensions (Fig. 1). In the first regime, Col-Ap suspensions made from collagen-containing m-SBF with three different concentrations, 1, 2 and 3 g/L, were placed in the chamber of a freeze-dryer (Free Zone®, Labconco, USA) at room temperature. The chamber was cooled at a constant rate of 0.4 °C/min to a final freezing temperature ($T_f = -25$ °C) and held constant until the whole sample was frozen. To investigate the effect of T_f on the resulting pore structure of the scaffold, Col-Ap suspension prepared from m-SBF with a collagen concentration of 2 g/L was cooled to $T_f = -10$, -25 and -40 °C at a constant cooling rate of 0.4 °C/min. The temperatures within the Col-Ap suspension and in the freeze dryer were monitored at every 10 s interval using thermocouples placed at the center of the suspension and the chamber of the freeze dryer, respectively. The liquid-solid transition time is defined as the time that the solid and the liquid co-exist in the suspension [36]. In this study, the suspension temperature at the transition time remained between 0 and -1 °C following the initial undercooling required for initiation of ice crystal nucleation. In the second regime, the Col-Ap suspension was frozen in a petri-dish covered by polyurethane foam to reduce heat transfer from surrounding environment to the slurry.

The bottom of the petri-dish was placed directly on a thick or thin aluminium plate on liquid nitrogen or dry ice to create a uniaxial thermal gradient at different cooling rates. The as-frozen precipitates were then lyophilized in the freeze dryer. The freeze-dried scaffolds were subsequently cross-linked with 1 wt% N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) hydrochloride for 24 h. Then scaffolds were rinsed thoroughly in distilled water, followed by 5% glycine solution, rinsed again with water, and finally freeze-dried for the second time.

2.3 Characterization

The surface morphology of Col-Ap scaffold was observed using field emission scanning electron microscopy (FESEM, JEOL JSM-6335F) at 5 kV. Pure collagen and Col-Ap composite scaffolds were examined using Fourier-transform infrared spectroscopy (FTIR, Perkin Elmer) in absorption mode in the range 4000 – 500 cm^{-1} at a resolution of 4 cm^{-1} with an average of 32 scans. X-ray diffraction (XRD, BRUKER AXS D5005) was also performed on collagen and Col-Ap scaffolds at a step size of 0.02° and a scan rate of $1^\circ/\text{min}$ with $\text{CuK}\alpha$ radiation ($\lambda = 1.54056$ nm). Thermogravimetric analysis (TGA) was carried out to determine the amount of apatite in the Col-Ap scaffolds. The scaffolds with a weight of 20 mg were studied using a TG analyzer (TGA-1000, Rheometric Scientific, UK) and the measurements were recorded from 30 to 1000 °C with a heating rate of 10 °C/min in air.

2.4 Pore size and porosity measurement

The pore size and lamellar spacing of the scaffold were determined using a histological technique as described by O'Brien *et al.* [34–36]. From each scaffold variant, four sets of cylindrical samples (8 mm in diameter, 4 mm in height) were cut using a punch. The samples were transferred into 30% sucrose/PBS solution and soaked for approximately 1 h. Then, the samples were embedded using embedding media (Cryomatrix, Thermo-Shandon, USA) and sectioned into 7 μm sections using a frozen section station (Leica D-69226, Germany). The cross-sections of the scaffolds were then observed using a Zeiss Axio Imager Z1 microscope with a differential interference contrast (DIC) channel under a dark field. A total of 4 digital images were taken from each sample to give a total of 16 images for each scaffold variant. The mean pore size and pore size distribution of the equiaxed scaffold were calculated according to the average of the pore surface area from optical image data using Image J software. The lamellar spacing was determined from 16 images of cross sections in the plane parallel to the direction of freezing. Twenty-five measurements were taken randomly in each image and then the mean lamellar spacing was calculated. The porosity of the scaffolds was calculated by gravimetric method according to the following equation [7].

$$\text{Porosity} = 1 - \frac{\rho_{\text{scaffold}}}{\rho_{\text{material}}} = 1 - \frac{m_{\text{scaffold}}}{V_{\text{scaffold}}} * \left(\frac{\omega_{\text{collagen}}}{\rho_{\text{collagen}}} + \frac{\omega_{\text{apatite}}}{\rho_{\text{apatite}}} \right)$$

where ρ_{material} is the density of the Col-Ap scaffold and ρ_{scaffold} is the apparent density of the scaffold that is determined by scaffold weight against scaffold volume. The densities for collagen and apatite are 1.32 (ρ_{collagen}) and 3.16 g/cm^3 (ρ_{apatite}), respectively. The weight percent ratio of collagen (ω_{collagen}) and apatite (ω_{apatite}) were measured from TGA measurements. Five sets of cylindrical samples were cut using a punch with a diameter of 8 mm. The mass of each sample (m_{scaffold}) was then measured. The volume of each sample (V_{scaffold}) was calculated based on the dimension of the scaffold.

2.5 In vivo evaluation

2.5.1 Transgenic mice—Transgenic mice carrying pOBCol3.6GFP transgene associated with osteoblast differentiation were used for *in vivo* evaluation of scaffolds. The pOBCol3.6GFPtpz and pOBCol3.6GFPcyan transgenic mice were maintained as homozygous breeders on a CD1 background. To distinguish contribution of the donor cells from the host cell, cells derived from Col3.6GFPcyan mice that express blue fluorescence were used as donor cells, while Col3.6tpz mice with a green fluorescence were used as the recipients. The transgenes remain stable and consistent in their expression for multiple generations [46].

2.5.2 Donor cells isolation and expansion *in vitro*—Calvarial osteoblasts (COBs) cells were derived from pOBCol3.6GFPcyan transgenic mice. Neonatal calvarial cells were isolated from 4–6 day-old mice using a modified sequential digestion method [46]. Briefly, after removal of sutures and adherent mesenchymal tissues, transgenic mouse calvariae was subjected to four sequential 15 min enzyme digestion at 37°C in a solution containing 0.05% trypsin-EDTA and 0.1% collagenase P (Roche Diagnostics, USA). Cells released from the second to the fourth digestions were collected, centrifuged, re-suspended and plated at a density of 1.0×10^6 cells per 100 mm cell culture dish (Falcon, Fisher Scientific) in DMEM medium containing 10% FCS for 5–6 days. Cells at pre-confluent stage were harvested in 0.25% trypsin/EDTA (Roche Diagnostics, USA) after they reached subconfluence and re-suspended in DMEM containing 10% FBS at a concentration of 1.0×10^6 cells/mL.

2.5.3 Animal surgery—A two-hole mouse calvarial defect model was employed where the scaffold loaded with mouse calvarial cells (mCOBs) carrying pOBCol3.6cyan (blue) reporter was implanted into a host mouse carrying pOBCol3.6tpz (green) reporter. A house-made equiaxed Col-Ap scaffold, Col-Ap-35, was employed as the test, which was prepared using m-SBF containing 2g/L collagen and then frozen at $T_f = -25^\circ\text{C}$ at a cooling rate of $0.4^\circ\text{C}/\text{min}$. All surgeries were performed under a protocol approved by the University of Connecticut Health Center Animal Care Committee. A total of 4 transgenic mice were tested and 8 calvarial defects (double-hole model) were created. All animals were irradiated with a lethal dose of 900 cGy total body gamma irradiation using a ^{137}Cs source (Nordion Gammacell 40 Irradiator; Canada), with a dose rate of 66.5 cGy/min [47]. Rescue of the hematopoietic system of irradiated mice was performed by systemic injection of at least 5.0×10^6 total bone marrow cells from a non-transgenic CD-1 mouse into retro-orbital sinus of each anesthetized mouse. The animals were anesthetized with a ketamine/xylazine mixture (135 mg/kg and 15 mg/kg) prior to surgery. Two circular defects, 3.5 mm in diameter, were created at the cranium on each mouse. Col-Ap-35 equiaxed scaffolds were loaded with mCOBs at a density of 1.0×10^6 cells per scaffold and implanted into the left and right side of the defect, respectively. The mice were given an intraperitoneal injection of freshly prepared XO (30 mg/mL, 90 mg/kg) 24 h prior to the sacrifice. Xylenol orange (XO) is a fluorochrome chelating reagent that labels newly calcified tissue *in vivo*. After 4 weeks, all mice were sacrificed and the defect area together with the scaffold was dissected from the surrounding tissue.

2.5.4 Radiological examination and histological evaluation of GFP expression and bone formation—After sacrifice, the calvarial tissues were harvested and fixed in 10% Buffered formalin (Sigma, HT501320.9) for 2 days and transferred into 30% sucrose/PBS solution and soaked overnight. The samples were subsequently embedded and cryosectioned as described previously. After rehydration in PBS, the slides were mounted using 50% glycerol with 1:1000 dilution of Hoechst solution (Mol Probes #H-3570). The GFP expression in the calvarial sections was examined using Zeiss Axio Imager Z1 equipped for fluorescence imaging and computer-controlled mechanical stage [48]. A set of filter combinations was used to distinguish the GFP signal and XO label from the autofluorescent background of bone, bone marrow and scaffold. The GFP filter was used to identify the Col3.6tpz green fluorescent signal, while the Cyan filter was employed for the Col3.6cyan blue fluorescent signal. Cell nucleus stained by Hoechst was taken by DAPI channel with a pseudo yellow color to distinguish it from cyan, tpz and red. All images were taken in air and recorded using a Zeiss Axio digital camera. Image processing was performed using Axia Vision 4.7 (Zeiss, Germany) and Adobe Photoshop (Adobe systems, San Jose, CA).

Following detection and imaging of GFP expression, all the slides were soaked in PBS for 60 min to remove the cover slides. They were then decalcified in 15% ethylenediamine tetraacetic acid (EDTA) overnight at room temperature. The sections were stained with hematoxylin after decalcification. Briefly, the slides were first stained in hematoxylin for 1 min and then washed thoroughly with distilled water. The slides were mounted with 50% glycerin in PBS and covered by a coverslip. Images of H staining were recorded by Zeiss Axio Imager Z1 microscope with Zeiss Axio color camera.

2.6 Statistical analysis

Results are presented as mean $\pm$ standard deviation. Statistically significant differences were determined by one- or two-way analysis of variance (ANOVA). Statistical significance was accepted at $p < 0.05$.

3. Results

3.1 Microstructure of Col-Ap scaffolds

TGA tests demonstrate that Col-Ap precipitates prepared at a collagen concentration of 1.0, 2.0 and 3.0 g/L had an apatite content of 54 ± 3.8 , 35 ± 2.3 and $18 \pm 2.9\%$, respectively (Fig. 2 a). Fig. 2 b shows the FTIR spectra obtained from collagen and Col-Ap scaffolds. The broad band at 3500 cm^{-1} is associated with absorption of water in the scaffolds. The peak around 1670 cm^{-1} arises from the C=O stretch of amide I, while those at 1540 cm^{-1} and 1200 cm^{-1} arise from N-H deformation of amide II and C-N stretch of amide III, respectively. The band at 1630 cm^{-1} corresponds to the O-H vibration overlapping with the peak of amide I. In Col-Ap scaffolds, the bands between 500 and 600 cm^{-1} are antisymmetric bending vibration of the PO_4^{3-} groups. Bands in the region 1200 – 965 cm^{-1} are stretching vibrations of the PO_4^{3-} groups. The carbonate peak at 875 cm^{-1} suggests the formation of carbonated apatite, which is similar to the mineral composition in natural bone [11].

Fig. 2 c shows XRD patterns of pure collagen and Col-Ap scaffolds. A broad diffuse peak at about 20° is attributed to be collagen. In Col-Ap scaffolds, the peak at 26.2° is attributed to the (002) plane of apatite. The major peak at $2\theta = 31.8$ – 32.2° corresponds to an overlap of three major planes (211), (112) and (300) of apatite. In addition, three weak peaks at 28.1° , 48.2° and 53.1° were detected in both Col-Ap-35 and Col-Ap-54. These peaks correspond to the (102), (312) and (004) planes of apatite, respectively [41]. All diffraction peaks were broad, implying that the apatite in Col-Ap scaffolds was poorly crystallized. The apatite peaks are the broadest in Col-Ap-18, suggesting that the apatite crystals is the least crystallized and may with the smallest size comparing to those in either Col-Ap-35 or Col-Ap-54.

3.2 Col-Ap scaffolds with tunable structures

3.2.1 Equiaxed structured Col-Ap scaffold—Under the same freezing regime ($T_f = -25^\circ\text{C}$, cooling rate $= 0.4^\circ\text{C}/\text{min}$), suspensions with different Col-Ap weight ratios were frozen and lyophilized. As shown in Fig. 3, both of collagen and Col-Ap scaffolds with different apatite contents exhibited an isotropic equiaxed structure. Macro-pores with diameters ranging from 40 to $160 \mu\text{m}$ were observed in both collagen and Col-Ap scaffolds, as shown in Fig. 3 left panel. FESEM images at a higher magnification (right panel) revealed that surface of collagen scaffold exhibited a smooth morphology (Fig. 3 a2). In contrast, the surface of Col-Ap scaffolds consisted of self-assembled collagen fibers and apatite clusters. As shown in Fig. 3 b2, apatite crystals with a size of $\sim 100 \text{ nm}$ dispersed uniformly on the surface and embedded within collagen fibers with a diameter of 100 – 200 nm in Col-Ap-18 scaffold. Increased amount of apatite clusters were observed for Col-Ap-35 (Fig. 3 c2) and Col-Ap-54 scaffolds (Fig. 3 d2). EDX mapping of calcium (Fig. 3 e1) and phosphate (Fig. 3 e2) distributions in Col-Ap-54 scaffold confirms that the calcium phosphate mineral phase distributed uniformly throughout the scaffold.

Under the same freezing regime, the pore size and porosity were down regulated by apatite content in Col-Ap scaffolds as shown in Fig. 4 and Table 1. Based on pore size distribution analysis (Fig. 4), the amount of pores with a size larger than $100 \mu\text{m}$ decreased when apatite content increased from 0 to $54 \text{ wt}\%$. The collagen scaffold exhibited largest pore size and highest porosity compared with Col-Ap scaffolds. When apatite content increased from 18 to $54 \text{ wt}\%$, the average pore size decreased from $106.4 \mu\text{m}$ to $69.1 \mu\text{m}$ and porosity decreased from 93.2% to 91.5% . Pore size of Col-Ap-18 is larger compared to that of Col-Ap-35 and Col-Ap-54.

Fig. 5 a shows FESEM images of scaffolds obtained by freezing Col-Ap-35 suspension at a constant cooling rate of 0.4 °C/min, but at different final freeze dryer temperatures. A homogeneous porous structure with interconnected, equiaxed pores was achieved throughout the entire scaffold. Fig. 5 b clearly shows Col-Ap scaffold prepared at a final freezing temperature of -10 °C exhibited a higher number of large pores (pore size > 80 µm) compared with those prepared at a final freezing temperature of -25 °C and -40 °C, indicating larger pore size formed at an elevated final freezing temperature.

The temperature-time curves during the cooling process of Col-Ap-35 suspension at different final freezing temperatures are shown in Fig. 5 c. Initially, the temperature decreased at a rate of 0.4 °C/min until it reached the nucleation temperature at ~-8 °C. A sudden increase of the temperature was observed at t=70 min from the nucleation temperature toward the equilibrium crystallization temperature for ice. The temperature then decreased gradually until it reached the final freezing temperature. A longer liquid-solid transition time was observed for scaffolds with a final freezing temperature of -10 °C (31.3 min) than those of -25 °C (22.5 min) and -40 °C (20.1 min). Table 2 summaries the correlation between freezing time and pore size.

3.2.2 Lamellar structured scaffold—As shown in Fig. 6 a, the Col-Ap scaffolds prepared by unidirectional freezing exhibit anisotropic pore structure in which pores were aligned along the direction of ice growth. In Fig. 6 b, the lamellar spacing was measured parallel to the c-axis of the plate-like ice crystals. It was affected by the cooling rate and final freezing temperature. With an increase of cooling rate from 1.8 to 9.1 °C/min, corresponding to the decrease of final freezing temperature from -80 to -180 °C, a significant decrease of lamellar spacing from 110.4 to 7.2 µm was observed.

3.3 *In vivo* evaluation

The Col-Ap-35 scaffold has a composition most closely mimicking that of natural bone, so it was selected for *in vivo* test. Prior to the *in vivo* test, the scaffold was optimized based on its handling property and mechanical strength. Fig. 7 shows images of the calvarial defect model in which Col-Ap-35 scaffold with an equiaxed structure was loaded into the defects with mCOBs cells (pOBCol3.6cyan). One million calvarial osteoblast progenitor cells were loaded into each scaffold. Due to the high number of cells comparing to the low volume of the scaffold, some of the cells died prior to contributing to new bone formation. However, the survival cells are sufficient to heal the critical-sized defect. After four weeks of implantation, more than 90% of the defect is filled with new bone. The pOBCol3.6cyan positive osteoblast cells (blue, indicated by blue arrows) were found distributing throughout the entire defect area. The new bone formation was indicated by XO, a fluorescent marker which labels dynamic new bone formation. An overlap of XO label (indicate by red arrow) and blue cells was clearly observed, and they together marked the outer surface of new bone (Fig. 7 d). This suggests the new bone formation was mainly attributed to the blue donor cells. In comparison, although very few host cells (green, pointed by green arrows) were also observed in the defect area, they did not overlay with the mineral label (XO), suggesting the host cells may not contribute to new bone formation in the defect. Hoechst stained nuclei of all cells, indicating cells were well distributed within the defects (Fig. 7 b). Especially, there were nuclei within the area labeled by an overlay of OX (red) and Col3.6cyan osteoblasts (blue), further confirming new bone formation. Besides new bone, blood cells and bone marrow were also formed in the defect area indicated by H staining (Fig. 7 e).

4. Discussion

A series of Col-Ap scaffolds possessing different pore structures were prepared using a biomimetic fabrication technique combined with controllable freeze casting. An isotropic equiaxed structure was created by freezing the Col-Ap suspension at a slow cooling rate in a homogeneous cold environment, whereas an aligned lamellar structure was prepared by unidirectional freezing of Col-Ap suspension. The composition and pore size of the scaffold could be easily adjusted. The scaffolds were evaluated *in vivo* using a two-hole calvarial defect model, which demonstrates that the Col-Ap scaffolds well supported osteoblast activities and new bone formation.

4.1 Control Col-Ap microstructure and composition using a novel biomimetic fabrication technique

Biom mineralization using simulated body fluid (SBF) has attracted the attention of many researchers and proved to be an easy method in developing bone-like apatite on polymer scaffold. Previous studies reported that a biomimetic apatite coating could be deposited throughout porous 3D structures by immersing scaffolds into the SBF solution [7, 8]. Although mechanical strength of the scaffolds was enhanced, a reduction in porosity and pore interconnectivity was also observed. Here a novel one step co-precipitation method was employed to prepare mineralized collagen scaffold in a collagen containing m-SBF for the first time. Compared to conventional co-precipitation methods, the biomimetic technique is simpler and easier to operate. One major disadvantage of conventional co-precipitation method is that the composition of the precipitates is sensitive to pH change, and therefore continuous titration is required to maintain the solution pH constant during chemical reaction. In our biomimetic approach, bone-like apatite was readily achieved by only adjusting the initial pH of the solution. As a result, self-assembly of collagen fibril and precipitation of poorly crystalline hydroxyapatite occurred simultaneously, forming a composite mimicking the composition of natural bone. The kinetics of apatite crystal growth was carefully controlled by monitoring the decomposition of hydrogen carbonate as well as the diffusion rate of calcium and phosphate ions (see Reaction (1) below). On one hand, stirring accelerated the diffusion of calcium and phosphate ions, leading to the precipitation of apatite into the space between collagen fibrils (see Reaction (2) below). On the other hand, the decomposition of hydrogen carbonate in m-SBF was suppressed by sealing the vial containing m-SBF [22–26]. Hydrogen carbonate in m-SBF, known as the apatite crystal growth inhibitor, delays the apatite growth and stabilizes apatite nanoclusters [49]. As a result, collagen self-assembly occurred synchronously with the nucleation and growth of apatite crystals to achieve a bone-like composite.

In addition, with this technique, the mass fraction of apatite could be easily tailored over a wide range (0~54 wt%) by simply varying collagen concentration in m-SBF. The resulting scaffold was poorly crystalline, bone-like Col-Ap composite. Especially, when the apatite content in the scaffold was low (e.g. Co-Ap-15), the apatite particles in the composite scaffold became more amorphous compared to those with a higher apatite content. This can be explained as that apatite crystal growth was inhibited in m-SBF at a high collagen concentration because increased viscosity lowered the diffusion rate of calcium and phosphate ions [49, 50].

4.2 Control scaffold pore structure

During the lyophilization process, the porous structure of scaffold is a replica of ice. Therefore, formation of such a structure depends largely on the physics of ice crystal growth and the interaction between ice front and Col-Ap precipitates in the colloidal suspension [27–44, 50–56]. The growth of ice crystal is kinetically in favor of the direction perpendicular to the c-axis of hexagonal ice. Thus, an isotropic structure is formed when temperature gradient is minimal. In contrast, anisotropic structure with pores aligned along the preferred crystal growth direction is formed by increasing the temperature gradient (Fig. 1). The formation of equiaxed and unidirectional lamellar structures are illustrated in Fig. 8 and Fig. 9, respectively.

Under a slow freezing rate and non-unidirectional freezing condition, an equiaxed, isotropic porous scaffold was obtained as shown in Fig. 8. When the temperature dropped below its freezing point, undercooling occurred in the solution. Consequently, a large number of ice nuclei were formed in the solution when the suspension was undercooled to the nucleation temperature at -8°C (Fig. 8 a). The undercooling further drove rapid crystal growth during recalescence of suspension, which was demonstrated as a sudden temperature rise from -8°C to equilibrium freezing temperature at 0°C in Fig. 5 c. Highly branched ice dendrites were formed during the rapid crystal recalescence, which in turn increased the interfacial energy of the system (Fig. 8 b). The highly curved interface of solid and liquid became unstable during the slow crystallization stage after recalescence. Consequently, the highly branched crystals formed during recalescence became more round and coarsened to reduce interfacial curvature and interfacial energy during the slow crystallization stage. Meanwhile, as the temperature continuously decreased, constant extraction of the latent heat of fusion led to further crystallization and the need to reduce interfacial energy further drove crystal coarsening (Fig. 8 c). As a result, a large final crystal size was achieved by freezing the suspension at a lower rate. When solid and liquid co-existed over a long period of time, coarsening was enhanced during the solid-liquid transformation (Fig. 8 d). This can be acquired by adjusting the final freezing temperature to which the freeze dryer chamber was cooled. Larger pores were observed in scaffold prepared at higher final freezing temperatures where heat extraction rate from the solution was slower and a longer freezing time was needed [34–36]. However, in the current study, the pore size obtained under a final freezing temperature of -25°C was similar to that of -40°C . This discrepancy might due to the fact that the change in freezing rate at those two different final freeze temperatures is smaller comparing to those reported in literature, so it did not result in obvious microstructure differences among the scaffolds studied.

Another intriguing result of our study is that the pore size in the equiaxed foam regime changed dramatically with the Col-Ap ratio in the suspension as shown in Table 1. Furthermore, ice crystal coarsening depends on the transport of mineralized fibers and energy from the interface of one crystal to the interface of another crystal. The porosity decreased when apatite content in the scaffold increased from 18 to 54 wt% due to higher solid loading density in Col-Ap-54 and Col-Ap-35 compared to that in Col-Ap-18. At a higher solid loading density, more transport is required to move solid from the interface of one crystal to the interface of another crystal and coarsening is retarded. Smaller ice crystals were formed in Col-Ap-54, resulting in scaffolds with smaller mean pore size. At a fast cooling rate under unidirectional heat flux, a dendritic structured scaffold with aligned pores was created (Fig. 9). In the lamellar regime, ice nucleated at the bottom wall of the mold and grew along the direction of heat flux. Our experimental setup allowed close control over both the thermal gradient and ice front velocity. In contrast to the equiaxed freezing regime, the lamellar freezing behavior is analyzed based on a steady-state freezing regime. The initial ice crystals demonstrated a random ordered structure in the area close to the cold finger where spontaneous nucleation at multiple sites was followed by rapid growth of ice

crystals. With the movement of ice front in the direction of the maximum temperature gradient, the crystals with preferred growth direction aligned along the direction of heat extraction direction were selected for continued growth. At increasing distance from the chill plate the number of grains in a cross-section decreases and the orientation of the surviving grains was more aligned with the heat extraction direction. The most favorable orientation for ice growth was the preferred dendrite direction. To reduce the surface energy in the system, some of the arms became unstable and melt away while others continued to grow. For a given system, the heat transfer efficiency is determined by the temperature gradient [54]. The temperature gradient is steeper at a faster cooling rate (e.g. 9.1 °C/min). In Fig. 9, $\frac{dT}{dt}$ is the cooling rate, ΔT ($\Delta T = \frac{dT}{dt}(t_2 - t_1)$) is temperature change at the same location of time interval of t_1 and t_2 . At a fixed position, a large ΔT was observed in panel a. $\frac{dZ}{dt}$ is ice front velocity and ΔZ ($\Delta Z = \frac{dZ}{dt}(t_2 - t_1)$) is ice front movement distance at a time interval of t_1 and t_2 . As a result, ice front velocity ($\frac{dZ}{dt}$) and ice front movement distance (ΔZ) are larger in panel a. The inter-lamellae spacing of scaffold (λ) is determined by the magnitude of constitutional supercooling, which is the degree of temperature difference of the suspension ahead of the freezing front below its equilibrium freezing temperature [53–55]. The cooling rate could be monitored to control the freezing rate ($\frac{dZ}{dt}$) and thereby adjust the inter-lamellae spacing [40–44]. At a faster cooling rate, the freezing rate is also higher. Thus, the resulting scaffold has a relatively dense structure with narrow inter-lamellae spacing.

In summary, the present paper is the first report which elaborates the mechanisms of ice nucleation and growth in Col-Ap suspension under both non-unidirectional and unidirectional temperature gradients. At a slow cooling rate, isotropic equiaxed pore structure is formed because the suspension temperature is almost identical throughout the entire suspension during freezing [34]. The crystal growth and coarsening are controlled by the rate of heat extraction and solid diffusion. In contrast, a high cooling rate produces a predominant direction of heat extraction which leads to the formation of an anisotropic columnar structure in the heat flow direction. It has been found that the velocity of ice front movement is proportional to the cooling rate. The rate of ice crystal growth depends on the heat extraction rate, which in turn determines the inter-lamellae spacing of the lamellar scaffold [28, 54, 55].

4.3 Suitability for bone repair

The aim of the present study was to prepare a Col-Ap scaffold mimicking the composition and structure of natural bone for bone repair and regeneration. Natural bone has a complex hierarchical structure comprised of mineralized collagen fibers, which were aligned and assembled to form into a highly ordered structure [10, 56]. Our Col-Ap scaffold was also comprised of hierarchically organized collagen fibers and poorly crystalline apatite crystals mimicking the microstructure of natural bone (Fig. 3).

The osteoblastic response of the scaffold is largely affected by its pore size and pore shape. On one hand, small pores may result in a large surface area, which can facilitate cell adhesion. On the other hand, large pores could improve cell permeability, but may sacrifice mechanical properties of the scaffold [35, 36]. However, there is little consensus about the ideal pore size for bone regeneration. Some researchers reported that the optimal pore size for bone ingrowth is in the range 100–350 μm while others suggested that optimal pore size should be in the range 300–500 μm [57–59]. Nevertheless, it is commonly agreed that the pore size smaller than 50 μm may impede the cell migration and long-term vascular ingrowth, whereas pore size larger than 1000 μm may not provide sufficient surface area for cells to attach to [60–62]. In the equiaxed regime, average pore size of the scaffolds was ranged from 69.1 to 118.9 μm . In the lamellar regime, the scaffolds had an inter-lamellar

spacing ranging from 7.2 to 110.4 μm . The distance of consecutive lamellae in cortical bone is only 1 to 7 μm , which is narrower than that found in the lamellar scaffold [63]. However, relatively large distance between two consecutive lamellae is needed for cell migration and nutrient/waste transportation. In addition, our Col-Ap scaffold possesses interconnected pores with a porosity of more than 90%, which is sufficient for osteoblast cells ingrowth and migration [27].

Our previous results have demonstrated that lamellar scaffold is osteoconductive [39]. In this study, we found that our equiaxed Col-Ap-35 scaffold is capable of repairing the non-union defect within four weeks, indicating that it has great potential in tissue engineering application. A separate manuscript is under preparation, which has investigated the effect of apatite content in Col-Ap scaffolds on new bone formation in a mouse long-bone defect model. It has been found that the presence of apatite in the scaffold remarkably accelerated new bone formation in the defect comparing to the defect loaded with pure collagen scaffold. High apatite content (45 wt% apatite) is slightly more effectively in inducing new bone formation than the low apatite content (25 wt% apatite) scaffold. Nevertheless, the defects loaded with either composite scaffold plus mouse bone marrow stromal cells were healed after 6 weeks of implantation. Further investigations will be carried out to establish the relationship between pore structure and bone formation capability of the scaffold, providing the necessary guidelines to tailor the structure to the actual bone repairing requirements.

5. Conclusions

A novel biomimetic fabrication technique has been developed successfully to prepare mineralized collagen fibers. Combining the biomimetic approach with controllable freeze casting, a bone-like tissue engineering scaffold with adjustable composition and pore size was achieved. It has been confirmed that such prepared Col-Ap scaffolds well supported bone repair at a mouse calvarial site. This is the first paper that discussed the mechanisms of controllable freeze casting of Col-Ap suspension in-depth, and thereby established freezing regimes for achieving Col-Ap scaffolds with either an isotropic equiaxed structure or a unidirectional lamellar structure suitable for different tissue repair applications.

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Fig. 1. Schematic procedure for preparation of biomimetic Col-Ap scaffolds with different pore structures.

Fig. 2.
(a) TGA, (b) FTIR and (c) XRD of collagen and Col-Ap scaffolds.

Fig. 3. FESEM micrographs of (a) collagen, (b) Col-Ap-18, (c) Col-Ap-35 and (d) Col-Ap-54 scaffolds. Left panel is low magnification images of porous structure of scaffolds at a macro-scale level. Right panel is high magnification images of scaffold surface structures at a micro-scale level. (e) EDX mapping of (e1) calcium and (e2) phosphate distributions within Col-Ap-54 scaffold.

Fig. 4.
Pore size distribution of collagen and Col-Ap scaffolds. *Significantly different ($p < 0.05$).

Fig. 5. (a) FESEM images and (b) pore size distribution of Col-Ap scaffolds obtained at different final freezing temperatures, (c) Temperature-time curves of the center of Col-Ap-35 suspensions (solid line) and the surrounding air (dashed line) in the freeze dryer during the cooling process at different final freezing temperatures. *Significantly different ($p < 0.05$).

Fig. 6. (a) FESEM images of cross-section parallel to the freezing direction for Col-Ap scaffolds fabricated by freezing the suspension at different cooling rates, (b) Influence of the cooling rate and final freezing temperature on the lamellar spacing.

Fig. 7.

GFP fluorescence images of bone formation at the cross-section of the calvarial defect implanted with equiaxed structure Col-Ap-35 loaded with mCOB cells of Col3.6cyan mice at 28 days. Cyan channel shows blue fluorescence signal from Col3.6cyan cells, Trite channel shows red fluorescence signal from xylene orange labeling, YFP channel shows green fluorescence signal from Col3.6tpz cells, and dark field (DF) channel shows mineral tissue. Cell nuclei were stained using Hoechst using DAPI filter with a pseudo yellow color. (a) 10x scanning image with Cyan+TRITC+Tpz+Dark field channel, (b) 10x scanning image with Cyan+TRITC+Tpz+DAPI, (c) Cyan+TRITC+Tpz+DAPI, (d) Cyan+TRITC+Tpz and (e) Homatoxylin staining of defect area. CN: cell nuclei, BC: Blood cells, BM: Bone marrow, NB: Newly formed bone. Scale bars: 500 μm in a&b and 100 μm in c, d&e. The blue arrows indicate blue osteoblast donor cells, the green arrows indicate host cells, while the red arrows point to OX red label.

Fig. 8.
Schematic illustration of how equiaxed structure was formed.

Fig. 9.

Schematic illustration of the growth of dendritic ice at different cooling rates. A_1A_4 and B_1B_4 are the direction of temperature gradient from the bottom of the petri-dish to the top. T_{L-S} is nucleation temperature. ΔT is temperature change at the same location and ΔZ is ice front movement distance at a time interval of t_1 and t_2 . t_1, t_2, t_3 : time points, where $t_1 < t_2 < t_3$. λ is the inter-lamellae spacing of scaffold. Panel a is for the lamellar structure formed at a faster cooling rate compared with panel b.

Table 1

Mean pore size and porosity of collagen and Col-Ap scaffolds.

	Average pore size (μm)	Porosity (%)
Collagen	118.9 $\pm$ 20.3	95.9 $\pm$ 3.9
Col-Ap-18	106.4 $\pm$ 24.1 [*]	93.2 $\pm$ 1.7 [*]
Col-Ap-35	82.1 $\pm$ 16.7 ^{* #}	92.2 $\pm$ 4.7 ^{* #}
Col-Ap-54	69.1 $\pm$ 25.2 ^{* #}	91.5 $\pm$ 1.1 ^{* #}

^{*} Significantly different from Collagen scaffold,

[#] Significantly different from Col-Ap-18 scaffold (p<0.05).

Table 2

Pore size analysis and the time required for the liquid-solid transition during cooling of Col-Ap-35 suspension.

T_f (°C)	Freezing time (min)	Mean pore size (μm)
-10	31.3	87.3 ± 18.3
-25	22.5	82.3 ± 15.1 *
-40	20.1	80.8 ± 17.0 *

* Significantly different from $T_f = -10$ °C ($p < 0.05$).