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A novel method for the fabrication of homogeneous hydroxyapatite/collagen nanocomposite and nanocomposite scaffold with hierarchical porosity

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Abstract Homogeneous nanocomposites composed of hydroxyapatite (HAp) and collagen were synthesized using a novel in situ precipitation method through dual template-driven. The morphological and componential properties of nanocomposites were investigated. The HAp particulates, in sizes of about 50–100 nm, were distributed homogeneously in the organic collagen hydrogel. Highly magnified TEM observation showed that HAp inorganic particles were composed of fine sub-particles (2–5 nm) without regular crystallographic orientation. Based on these homogeneous nanocomposites, a novel HAp/collagen nanocomposite scaffold with hierarchical porosity was prepared by multi-level freeze-drying technique. Compared to other conventional scaffolds for tissue engineering, this novel in situ method endows synthesized composite scaffolds with unique morphology—ultrafine HAp particles dispersed homogenously in collagen at nano level and the foam scaffold with hierarchical pore structures. The mechanical performance increased obviously compared with neat collagen. These results provided an efficient approach toward new biomimetic tissue scaffold for the biomedical applications with enhanced intensity/bioactivity and controlled resorption rates. This novel method, we expect, will lead to a wide application in many other hydrogel systems and may be useful for fabrication of various homogeneous inorganic/organic nanocomposites.

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1 Introduction

Nowadays, because of various bone diseases and injures, the needs for the use of biomimetic synthetic biomaterials in the bone grafting field have increased significantly, especially with the limited accessibility of autografts as well as the immune response and disease problems associated with conventional allografts [1–3]. Bone is a complex and highly specialized composite composed of hydroxyapatite and collagen. Following the principle of componential and structural similarity to the natural bone, especially with increasing knowledge of bone mineralization process occurring in vivo, most current researches emphasized the use of inorganic/organic nanocomposites applying biomimetic approaches. Numerous in vitro studies have been devoted to the precipitation of HAp nanocrystals within natural collagenous proteins. The composites were fabricated in a hierarchical structure consisted of particulates, fibers and porous scaffolds, which were very efficient in providing large surface area and space for blood circulation and cell ingrowth [4]. Most conventional composite methods, however, employed mechanical mixing and coprecipitation [5–10], which suffer two common limitations: inorganic particles can not be distributed well in the organic matrices at nano level; the scaffold synthesized from these methods can not meet the desirable demands of varied functions because of their single-sized porous structure. These disadvantages limit their applications for hard tissue repair and replacement.

Differ from other current techniques [11–14], we here developed a novel biomimetic method for the fabrication of homogeneous HAp/collagen nanocomposite scaffold. The in situ precipitation was employed through dual template-driven reaction and freeze-drying technique. In this work, we firstly synthesized the homogeneous HAp/collagen nanocomposite. The collagen hydrogel used as organic template not only provide an aqueous 3D compartment for the precipitation reaction of calcium cations and phosphate anions, but also provide template-driving force under the regulation of pH. The as-synthesized homogeneous HAp/collagen nanocomposites show a scaffold with hierarchical pore structure. Hierarchical pore structure is technologically important for medical and clinical applications. The homogeneous combination of HAp and collagen at nano level could provide the porous structure with mechanical reinforcement, enhanced bioactivity and controlled degradation rate. The mechanical performance of nanocomposite scaffolds did increased obviously compared with pure collagen. Moreover, the pore size of scaffold can also be controlled by varying the cross-linking degree.

2 Materials and methods

All the reagents used in this work were of analytical grade (AR) and used without any further purification. A 0.03 g soluble type-I collagen from porcine dermis (purchased from National Engineering Research Center for Biomaterials of Sichuan university, China) was dissolved in 5 ml of acetic acid (1.5%, v/v). A 0.08 g $(NH)_2HPO_4$ and 0.24 g $Ca(NO)_2$ together with 0.1, 0.2, and 0.3 ml glutaraldehyde were added to the collagen solution. The resulting mixture was stirred for 60 min at room temperature until a transparent hydrogel formed. The hydrogel was then added with NH_4OH solution to adjust the pH to 10 and kept for 24 h under 38°C. Under this condition, hydroxyapatite particles precipitated within the collagen hydrogel in situ, and the transparent gel became opaque gradually. The composite was subsequently washed with distilled water until the pH of eluate was about 7. The composite was first processed by liquid nitrogen for 10 min, and then kept in refrigerator at −20°C for 12 h, The hierarchical porous 3D scaffold was prepared by lyophilizing it for 24 h (−40°C, 0.03–0.05 mBar).

Morphological investigations were carried out using an environmental scanning electron microscope (ESEM) (Quanta200, FEI, Holland) and a field emission transmission electron microscope (FE-TEM) (2010FEF, JOEL, Japan). Compositional determination of the products was performed by Fourier-transform infrared spectrometer (Nicolet, 5700, America) and energy dispersive X-ray analysis (EDAX) for ESEM. Compression strength tests were employed by universal testing machine (AGS-J, Japan) at a crosshead speed of 1 mm/min.

3 Results and discussion

3.1 Homogeneous HAp/collagen nanocomposite

Figure 1a and b are the SEM images of HAp/collagen nanocomposites. The morphological features revealed that inorganic crystals of HAp had high-affinity with collagen matrices. The apatite particles with size ranging from 30 to 100 nm were distributed in collagen matrices homogenously. Distribution image of calcium element and phosphorus elements from EDAX (Fig. 1c, d) proved this homogeneous extent. It is difficult to obtain this decentralization effect by conventional mechanical mixing or co-precipitation. In our work, however, collagen hydrogel played an important role in the superfine interaction of inorganic and organic phases through the compartment-effect of its interior. The decentralization of inorganic nano-particles within organic matrices was improved significantly.

Figure 2 illuminated this specific process [15]. With the increase of pH after the addition of ammonia solution, residue groups of amino acid in collagen, such as carboxyl and amino groups, may begin to act as nucleation center for calcium phosphate formation. These negatively charged residue groups in the reaction system can bond Ca^{2+} strongly and thus form a large scale of local supersaturation microenvironment, moreover, strong electric field resulted from the high concentration of negatively charged carboxyl groups are in favor of the interaction with the most positively charged crystalline plane. Thus, many nucleation sites formed in the interior network of hydrogel matrix, and each point of nucleation sites can result in microcrystal. Great number of nucleation sites favors the formation of fine crystallites. Furthermore, the compartment effect of cross-linking collagen hydrogel which was provided with three-dimensional network microstructure limited the excessive growth of HAp particles, so the inorganic nano-particles were limited to aggregate in the compartment of the collagen hydrogel template according to orientation of preferential growth of crystal plane. This dual template effect, which was based on multiple-point nucleation of collagen and compartment of hydrogel network, had a very obvious mediation in the formation process of homogeneous composites.

As shown in the SEM micrograph (Fig. 3b, c and d), the composites mostly took on jagged structures with the decrease of cross-linking degree. The change of morphology may be relative to the sizes of collagen template network, which resulted in differences of shrinking velocity between inorganic phase and organic phase in the process

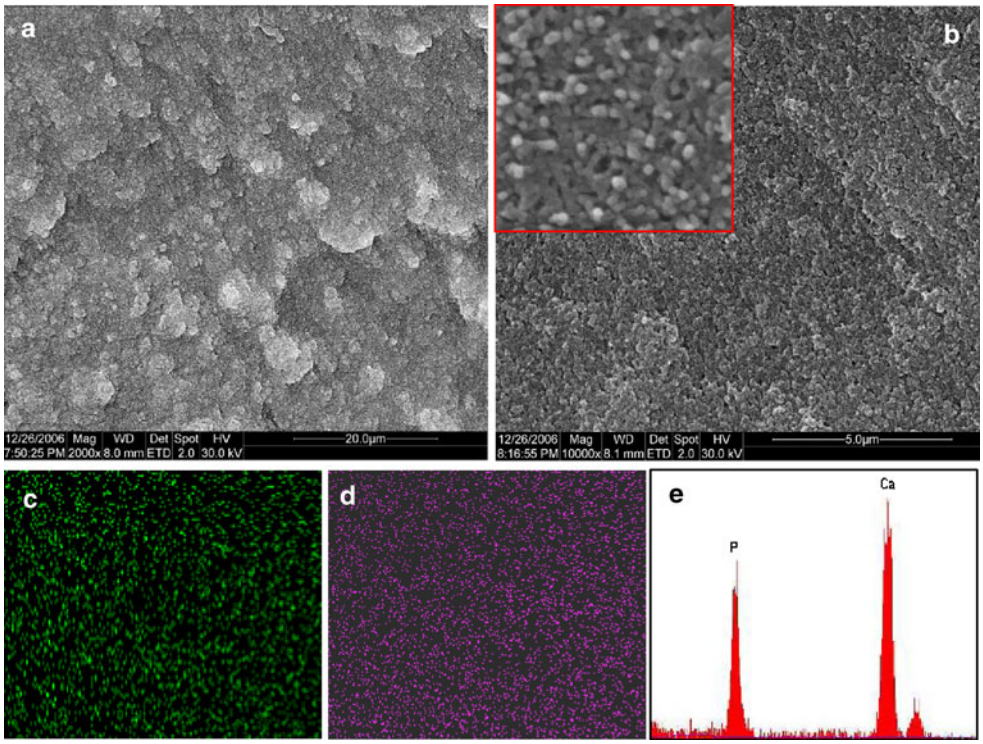

Fig. 1 Characteristics of the homogeneous HAp/collagen nanocomposite. **a** and **b** SEM morphology of HAp/collagen nanocomposite, **c** distribution image of calcium element from EDAX, **d** distribution image of phosphorus element from EDAX, **e** calibrated EDS area analysis of inorganic component shown in micrograph **a**

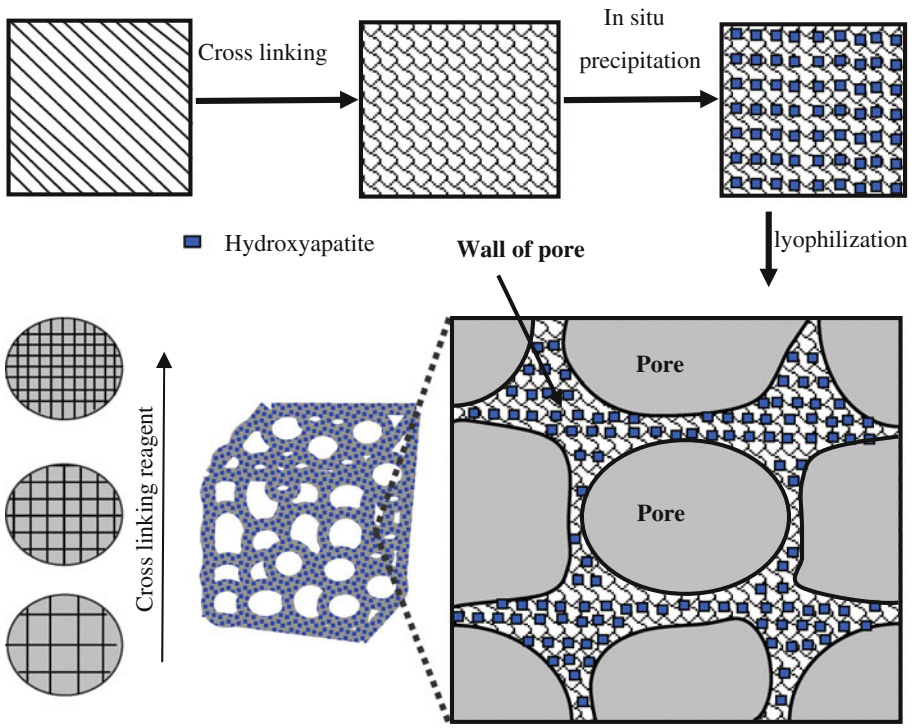

Fig. 2 The scheme of formation of homogeneous HAp/collagen nanocomposite and 3D nanocomposite scaffold

of thermal drying. The composites became smooth and compact with the increase of crosslink reagents added. Nano-sized inorganic particles were combined with organic matrices in nano-scale domain. The pure collagen (Fig. 3a) showed some regular corrugated structure of organic polymer.

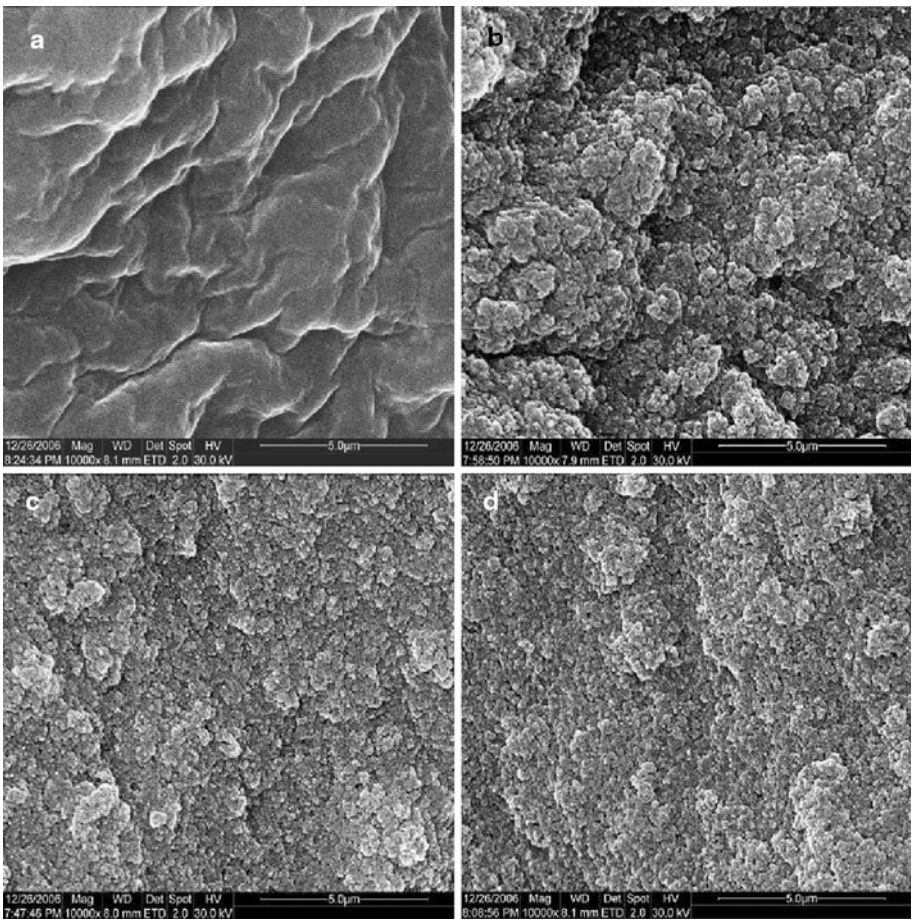

Fig. 3 SEM morphology of pure collagen and HAp/collagen nanocomposite. **a** Pure collagen; **b–d** composite, cross-linking reagent: **b** 0.1 ml, **c** 0.2 ml, **d** 0.3 ml

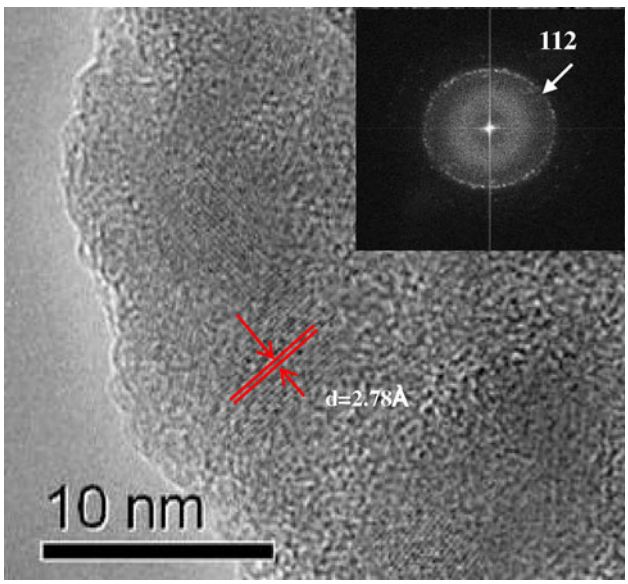

Fig. 4 TEM analysis of HAp/collagen nanocomposite; the *inset* shows polycrystal diffraction ring and amorphous spots

Furthermore, as shown in the high-magnification TEM image of crystal lattice (Fig. 4), the internal structure of the uniform nanocomposite was observed clearly. Evidences proved that composites had more precise bonding at 2–5 nm level, this distinctly indicated that nano-scale sub-crystallites in organic matrices had no uniform crystallographic orientation. In this case, rapid and strong chemical cross-linking between collagen molecules and glutaraldehyde occurred before promotion of HAp and collagen self-organization and caused missing of long-range orientation [16]. The polycrystalline diffraction ring and amorphous spots shown in the selected area electron diffraction pattern (SAED) (Fig. 4, inset) also were consistent with the characteristic of the typical apatite structure and collagen fibril shown in Fig. 1a.

Compositional determination of the products was performed using Fourier-transform infrared spectrometer and energy-dispersive X-ray analysis (EDAX) for ESEM. As shown in the FT-IR spectra, compared with amide vibrational band ($\sim$1652, 1544 and 1237 cm^{-1}) derived from pure collagen (Fig. 5A), the additional PO_4^{3-} vibrational bands ($\sim$1051, 603 and 561 cm^{-1}) were observed after the in situ precipitation of inorganic particles in collagen hydrogel (Fig. 5B, C and D). The result of EDAX (Fig. 1e) revealed a Ca/P (1.63) similar to that of apatite. It suggested that inorganic component of composite mainly consisted of hydroxyapatite, However, FT-IR spectra revealed

the existence of C–O bands ($\sim$1453 and 868 cm^{-1}). This proved the high level of carbonate substitution into the HAp lattice during the precipitation process [17]. No significant change in band structure was observed with the increase of the amount of cross-linking reagent. It indicated that there was little chemical reaction between HAp and collagen in the composites.

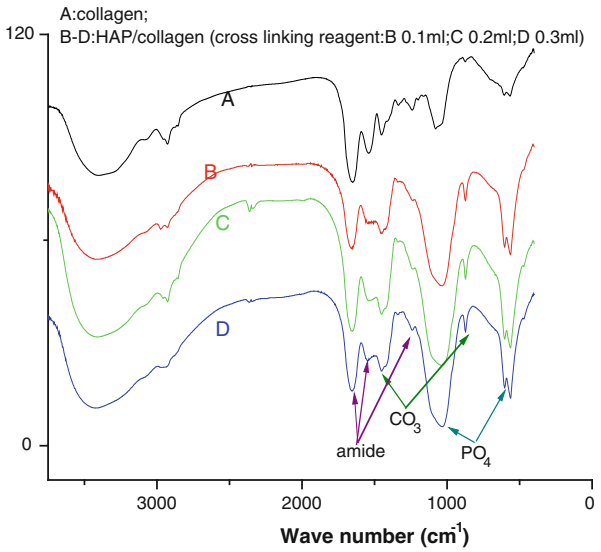

Fig. 5 FT-IR spectra of collagen and HAp/collagen nanocomposite with different cross-linking degree

3.2 Hierarchical porous nanocomposites scaffold

Based on above homogeneous nanocomposite, freeze-drying technique was employed to transform these nanocomposites into hierarchical porous scaffold. As shown in SEM photographs (Fig. 6), the product was a hierarchical porous three-dimensional scaffold with a well controlled interconnected porosity. The foam showed a well-developed macropore structure with a pore size of about 50–100 μm. The higher magnification SEM photographs reveal that the stem surface was composed of sub micropore with a pore size of 1–5 μm. On this nanoscale, HAp particles were still distributed homogeneously throughout the collagen fibers. These macropores and micropores were formed during the lyophilization process. Studies revealed that macroporosity (pores $>$ 50 μm) is thought to contribute to osteogenesis by facilitating cell and ion transport, and microporosity (pores $<$ 20 μm) can improve bone growth into scaffolds by increasing surface area for protein adsorption and providing attachment points for osteoblasts [18]. We believe that this hierarchical configuration from primary macropores to sub micropores, and finally to inorganic/organic nanocomposite was quite ideal for bone tissue engineering scaffolds.

Especially, as the concentration of cross-linking reagent increased, the size of scaffold macropores decreased with the formation of cross-linkages. As the SEM photographs

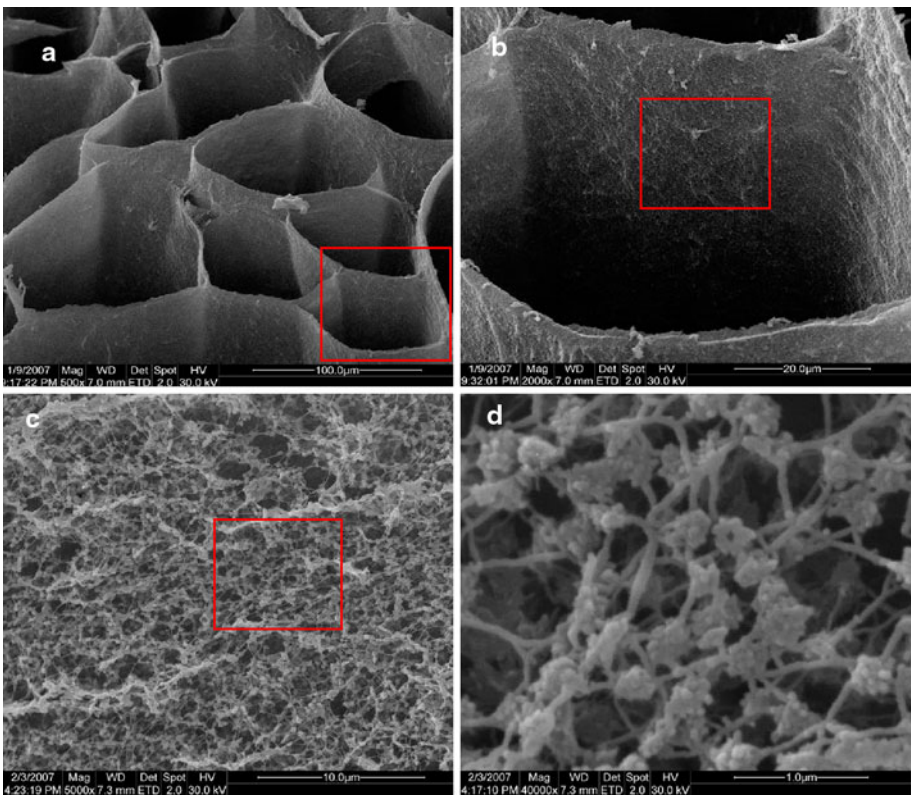

Fig. 6 SEM morphology of HAp/collagen nanocomposite 3D scaffold, *insets* are enlarged in the following figures. **a** 500×, **b** 2000×, **c** 5000×, **d** 40,000×

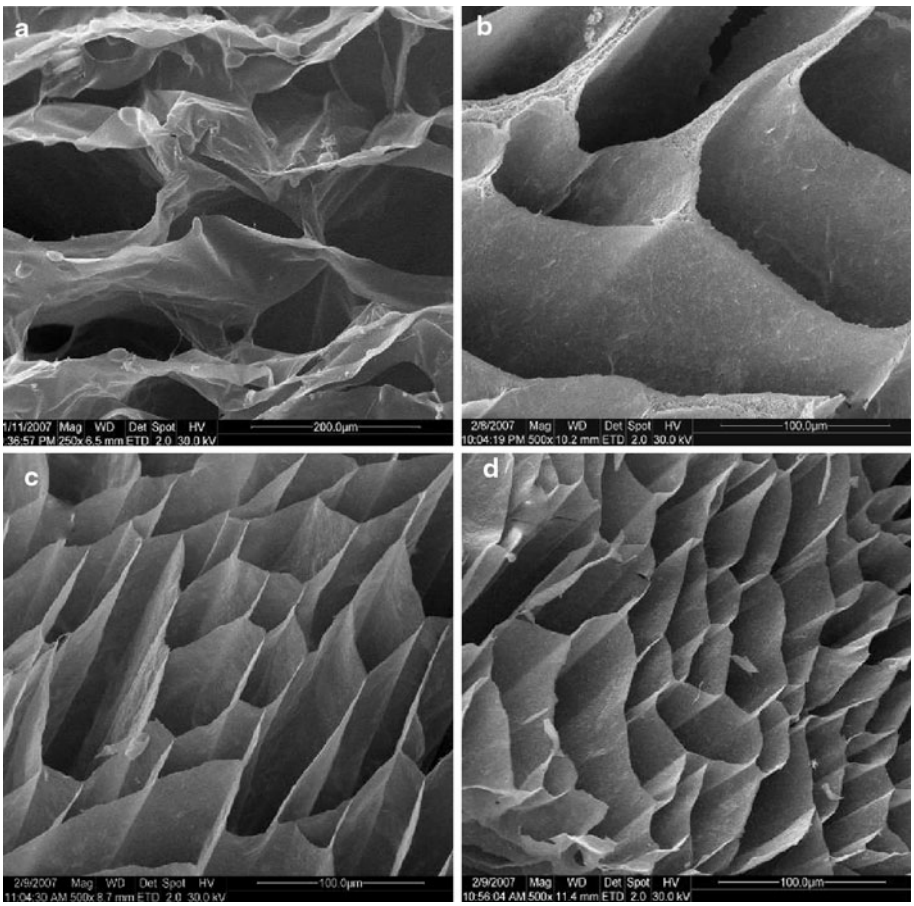

Fig. 7 SEM morphology of pure collagen and HAp/collagen nanocomposite 3D scaffold. **a** Pure collagen; **b–d** nanocomposite, cross-linking reagent: **b** 0.1 ml, **c** 0.2 ml, **d** 0.3 ml

illustrated (Fig. 7), with the increase of crosslink reagent from 0.1 to 0.3 ml, the size of macropores decreased from 150 to 30 μm. These morphological changes are also in good agreement with previous deduction (Fig. 2). The pure collagen scaffold didn't exhibit any hierarchical structure, thought it showed macropore structure.

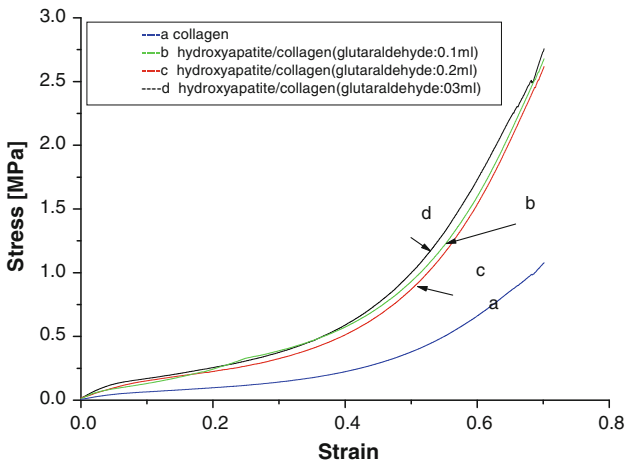

Fig. 8 Compressive stress–strain curves of the collagen and hydroxyapatite/collagen nanocomposites scaffold. **a** collagen; **b–d** nanocomposite, cross-linking reagent: **b** 0.1 ml, **c** 0.2 ml, **d** 0.3 ml

The mechanical properties of the hydrated nanocomposite scaffold in different crosslinking degrees and neat collagen hydrogel were investigated. Figure 8 showed the stress–strain data under a compressive load at a constant speed. As the results shown, all the samples of the nanocomposite scaffold presented similar stress–strain behavior. The stress increased sharply at initial stage, then reduced in slope, and subsequently increased again at the stage of high strain. All the samples didn't show any final fracture. Compared with pure collagen, the composite obviously exhibited higher stress at same strain. All these data indicate that the addition of nano-scale HAp can make homogeneous composite scaffold resist higher stress and is beneficial for real surgical application.

4 Conclusions

In this study, we originally put forward a novel in situ precipitation method for homogeneous nanocomposites of HAp/collagen based on dual template-driven of hydrogel. This method is especially expected to extend to many other hydrogel systems for various inorganic/organic nanocomposites. After freeze-drying process, the products exhibited

hierarchical structure of foams with primary macroporosity and sub microporosity. The nanocomposite scaffolds revealed higher mechanical performance than pure collagen scaffold. With these special characteristics, this novel method is useful for the fabrication of nanocomposite and nanocomposite scaffold with hierarchical porosity and thus regarded as a promising routine for bone regenerative application.

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