Preparation and characterization of bioactive collagen/wollastonite composite scaffolds

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A novel biodegradable collagen/wollastonite composite was prepared as three-dimensional scaffolds by freeze-drying method. Scanning electron microscope (SEM) micrographs of scaffolds showed a continuous structure of interconnected pores, and pore size was about 100 μm. The tensile strength of the scaffolds was improved by incorporation of wollastonite and the in vitro bioactivity of the scaffolds was evaluated by examining the hydroxyapatite (HA) deposition on their surface in simulated body fluid (SBF). After soaking in SBF for 7 days, collagen reconstituted to fibers and HA nodules formed on collagen fibers. The result suggests that the incorporation of wollastonite could improve the mechanical strength and the *in vitro* bioactivity of the composite. The scaffolds could be a potential biomaterial for bone tissue engineering.
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1. Introduction

The shortcoming of autografting and allografting has inspired the development of tissue engineering which aims to produce biological substitutes that may overcome the limitations of conventional clinical treatments for damaged tissues or organs. One of the principle methods behind tissue engineering involves growing the relevant cells *in vitro* to form the required tissues or organs before planting into the body [1]. The ideal scaffold should be biocompatible, bioactive, biodegradable, highly porous with a large surface to volume ratio, mechanically strong, and capable of being formed into desired shapes [2].

As the major component of extracellular matrix, collagen has native surface which favor cell attachment [3, 4]. Furthermore, collagen substrates can modify the morphology, migration and in certain case the differentiation of cells [4]. These properties of collagen made it the commonly used biomaterial in tissue engineering. However, recent research showed that it lack both mechanical strength and bioactivity, and the nucleation of hydroxyapatite (HA) on collagen was very slow in SBF [5].

For combination of bioresorbability and bioactivity, the most efficient way is to combine resorbable polymers and bioactive materials in a composite scaffold. Wollastonite (β-$CaSiO_3$) as reinforcement additives for polymer materials has been widely investigated [6]. Recently, wollastonite has attracted more attention for its excellent bioactivity and biocompatibility, suggesting that it might be used as bone repair biomaterials [7–11]. Considering the bioactivity and reinforcement effect of the wollastonite, it is assumed that the incorporation of wollastonite into collagen matrix may improve the bioactivity and mechanical strength of the composite.

Therefore, in this study, highly porous collagen/wollastonite composite scaffolds were fabricated by freeze-drying method, and the bioactivity of the composite was evaluated by soaking in simulated body fluid (SBF) for different periods.

2. Materials and methods

2.1. Preparation of collagen and wollastonite

Collagen was prepared from calf derma according to the procedure described previously [12]. Briefly, finely grounded calf derma was suspended in 0.05 M acetic acid for 24 h and stirred at 4 °C. Pepsin was added subsequently, and the solution was incubated at 4 °C for 24 h. After incubation, saturated tris–(hydroxymethyl)-aminomethane ([$(CH_2OH)_3CNH_2$], Tris) solution was added to adjust the pH to 7.4 to disable the activity of pepsin. The insoluble material was removed from the preparation by centrifugation at 12000 rpm (Jouan, KR22, France) for 1 h at 4 °C. Then, solid NaCl was added to the pepsin-soluble portion with 4 M in final concentration. The precipitated collagen was separated by centrifugation and the collected collagen was dispersed in 0.05% (v/v) acetic acid. Finally, the collagen solution was dialyzed in 0.05% (v/v) acetic acid solution at 4 °C to remove the ions such as Na^+ and Cl^-.

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The main content of final collagen solution was type I collagen (identified by Sodium dodecyl sulfate polyacrylamide gel electrophoresis). The collagen solution was freeze-dried and stored at 4 °C until further use.

Wollastonite was prepared as described previously [13]. Briefly, 0.4 M Na_2SiO_3 solution was slowly droped into 0.4 M $Ca(NO_3)_2$ solution and stirred continuously. The precipitation was filtrated and washed in distilled water and ethanol for three times respectively. After air dried, the powders were calcined at 800 °C for 2 h. The prepared β-$CaSiO_3$ was characterized by XRD.

2.2. Preparation of collagen/wollastonite scaffolds

Collagen/wollastonite sponges were prepared by freeze-drying method. Briefly, collagen was dispersed in 0.012 M HCl with final concentration of 15 mg/ml. Then the wollastonite powders were added to the collagen solution and dispersed homogeneously, wollastonite/collagen ratio of the composite was varied from 0 to 6 (sample W0C1 to W6C1, see Table I). After homogeneous dispersion, the suspension was poured into a petri dish and quickly removed to refrigerator to solidify the solvent at −81 °C. The solidified mixture was freeze-dried for 24 h, resulting in a sponge approximately 3 mm in thickness. A light microscope (Leica, Germany) and field emission scanning electron microscope (SEM; JSM-6700F, JEOL Co., Japan) was used to observe the morphologies of the scaffolds and evaluate the pore structure. The porosities of the composite scaffolds were determined with a liquid displacement method as described previously [14]. For mechanical testing, composite with 25 mm in width and 3 mm in thickness were prepared, and the measurement of the tensile strength was conducted using an AG-1 Shimadzu mechanical tester (Shimadzu Co., Japan) with crosshead speed of 2 mm/min.

2.3. Evaluation of *in vitro* bioactivity

The bioactivity of the composite was evaluated by examining the HA deposition on the surface of the scaffolds in SBF with ion concentrations similar to those in human blood plasma (BP), according to Kokubo solution [15]. The scaffolds were soaked in the SBF solution at 37.5 °C for 7 and 15 days at a solid/liquid ratio of 15 mg/ml. After soaking, the scaffolds were removed, washed in distilled water for three times, and finally freeze-dried. The morphologies of the soaked scaffolds were observed by SEM and the composition of scaffolds after soaking in SBF was determined by Energy dispersive spectrometer (EDS; Oxford Instruments Co., UK). In order to determined the structure of the inorganic component, the SBF soaked scaffolds were calcined at 700 °C for 2 h to remove all the collagen components and the morphology was observed by SEM.

TABLE I Porosity and tensile strength of the scaffolds with different wollastionite/collagen ratio

Composition	Porosity (%)	Tensile strength (KPa)
W0C1	99.1	28.76
W1/3C1	98.9	28.91
W1C1	98.6	29.79
W3C1	97.3	39.90
W6C1	96.2	71.87

3. Results

3.1. Characterization of collagen/wollastonite scaffolds

Bulk porous collagen/wollastonite scaffolds were prepared by freeze-drying method. The achieved collagen/wollastonite sponge had a uniform structure and could be fabricated into the scaffolds of desired shapes. The porosities and tensile strength of the scaffolds with different wollastonite/collagen ratio are showed in Table I. It is clear to see that, when the wollastonite/collagen ratio was lower than 1, the porosity and the tensile strength of the scaffolds were not much changed. In contrast, when the wollastonite/collagen ratio further increased from 1 to 6, the porosity decreased remarkably from 99 to 96%, accompanied with a sharp increase of the tensile strength from 29 to 71 KPa. It can be seen from Fig. 1 that the porosities linearly decrease with the increase of wollastonite/collagen ratio.

Fig. 2(a) shows a light micrograph of composite scaffolds of W6C1 which revealed homogenous porous structure, and Fig. 2(b)–(d) shows the SEM micrographs of the same scaffolds at different magnifications. It can be seen that the composite scaffolds possessed a continuous structure of interconnected pores, and the pore size was about 100 μm (Fig. 2(b)). The wollastonite particles were dispersed uniformly on the pore walls of the composite scaffolds as showed in Fig. 1(c) and (d), and collagen served as glue sticking the wollastonite particles together.

3.2. Evaluation of the *in vitro* bioactivity

Fig. 3 shows the SEM micrographs of the composite with W6C1 after soaking in SBF for 7 and 15 days. It can be seen that, after soaking in SBF for 7 days,

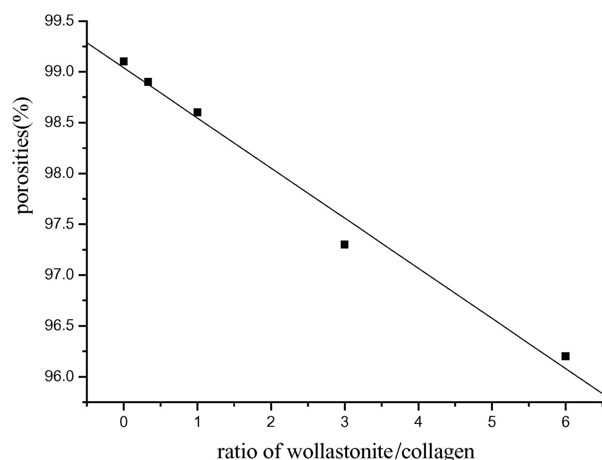

Figure 1 Porosity of the scaffolds with different wollastonite/collagen ratio.

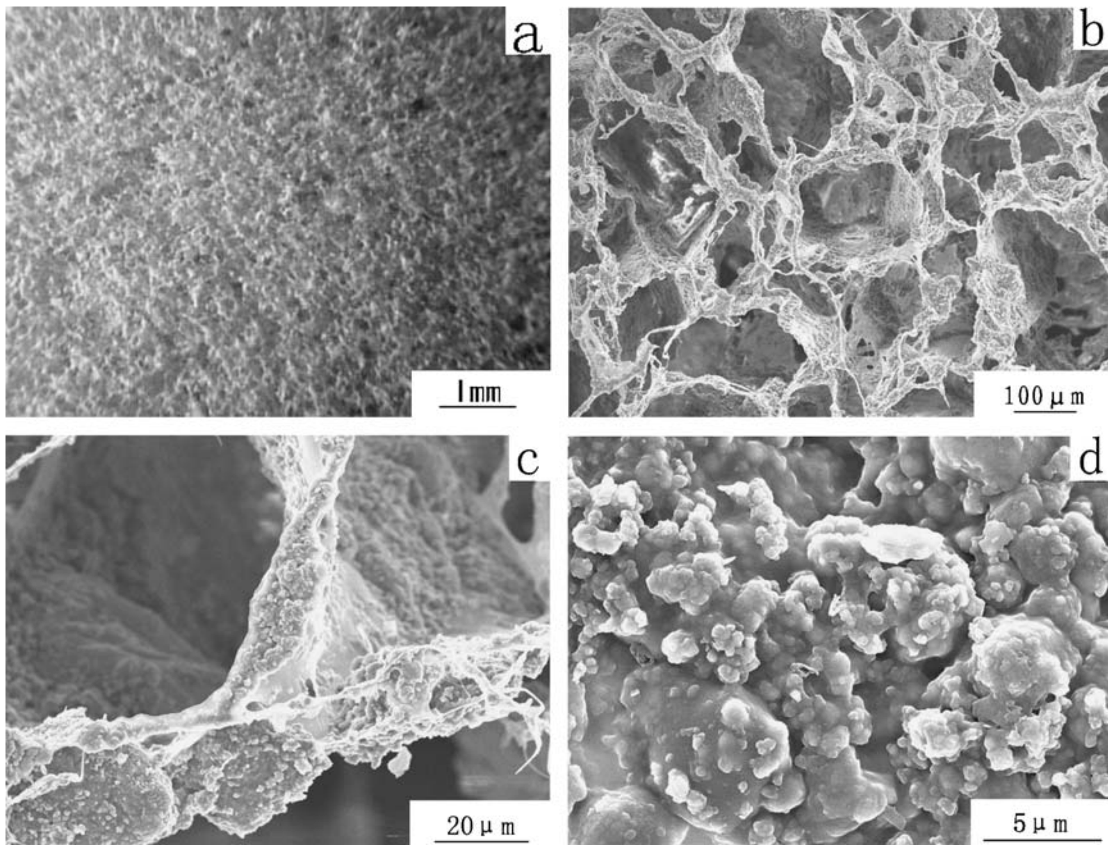

Figure 2 Micrographs of collagen/wollastonite composite scaffolds: (a) light micrograph and (b–c) SEM micrographs.

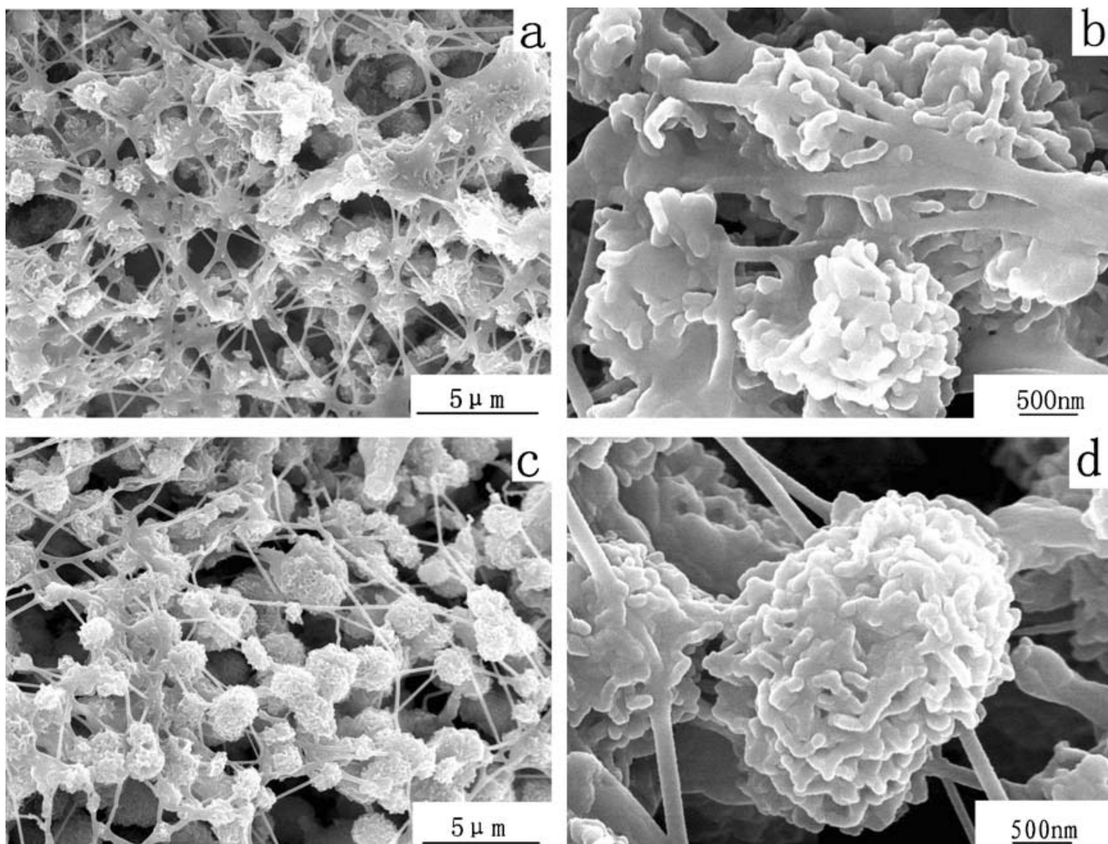

Figure 3 SEM micrographs of collagen/wollastonite scaffolds soaked in SBF for 7 days (a, b) and 15 days (c, d).

nodules formed and interconnected by nanofibers, the diameter of the nodules was about 1 μm (Fig. 3(a)), and consisted of silkworm-like nanocrystals with typical morphology of HA (Fig. 3(b)). With prolonged soaking up to 15 days, the size of nodules increased to 2 μm in diameter and the morphology of the nodules was similar to that after soaking for 7 days (Fig. 3(c) and (d)). Fig. 4 showed the SEM images of the SBF soaked

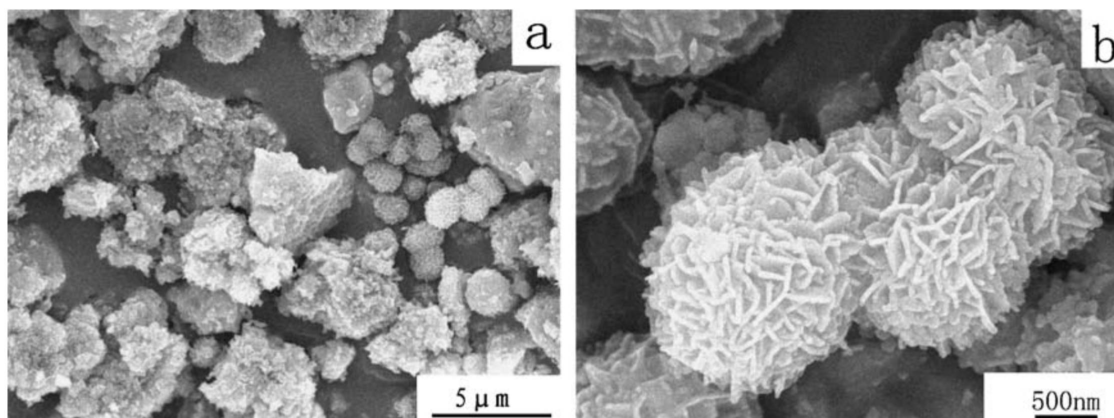

Figure 4 SEM micrograph of the soaked scaffolds after calcination at 700 °C.

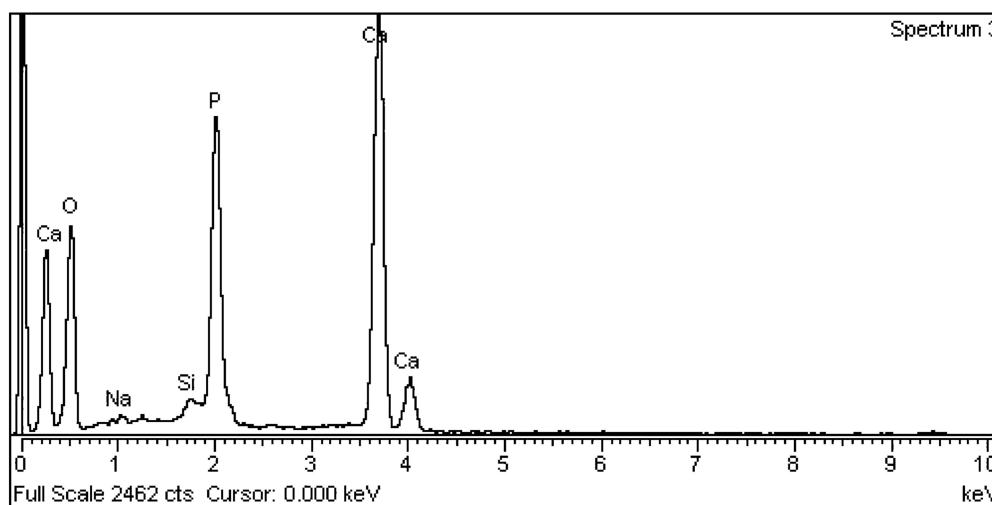

Figure 5 EDS analysis of HA deposited on the collagen/wollastonite scaffolds after soaked in SBF for 15 days.

scaffolds after calcinations at 700 °C. It is clear to see that the nanofibers disappeared after calcinations (Fig. 4(a)) although the morphology of the HA nodules was maintained as shown by the high magnification SEM image (Fig. 4(b)). This result suggests that the nanofibers were consisted of collagen matrix, which was burnt off during the calcination. Fig. 5 shows the EDS spectra of the nodules, which indicate that the nodules were mainly composed of calcium and phosphorus, and the ratio of Ca/P was 1.67, which was the same as the Ca/P ratio for HA. From the morphology and composition of the nodules, it can be seen that HA formed on the surface of the scaffold after soaking in SBF for 7 days.

4. Discussions

The incorporation of wollastonite caused a significant improvement of tensile strength of the composite when the wollastonite/collagen ratio exceeded 1, this could be mainly due to the decrease of porosities which linearly decrease with the increase of wollastonite/collagen ratio. In addition, the interfacial adhesion between the wollastonite and collagen is an important factor affecting the tensile strength of the composite. According to the mechanism of the mechanical reinforcement postulated by some researchers [16], not only the physical incorporation of wollastonite into the collagen matrix occurred, but also the chemical reactions among the collagen and wollastonite might take place because of the high surface charge density of collagen and its ability to form ionic complexes.

SBF immersion results showed that large amount of HA formed on the scaffolds after 7 days of soaking, which suggested that the composite scaffolds had good bioactivity. Previous study have shown that wollastonite has excellent bioactivity, and could induce growth of HA when soaking in SBF [7, 11]. The incorporation of wollastonite into collagen matrix introduced the sites for the growth of HA. The release of Ca^{2+} during the degradation of wollastonite could increase the supersaturation of HA in SBF and also accelerated the growth of HA.

After soaking in SBF, lots of collagen fibers appeared, and HA nodules grew on collagen fibers. This phenomenon was different with other composite scaffolds, such as chitosan/wollastonite scaffolds reported by Zhao in which HA nodules grew on the pore wall of the scaffolds after 3 weeks soaking in SBF [17]. This phenomenon could be due to the dissolution and reconstitution of collagen in SBF. Type I collagen is the primary structural element in many extracellular matrices. It exists in form of fibers that have hierarchical structure including fibrils and fibril bundles. Collagen molecules

and aggregates could spontaneously self-assemble to form fibers at 37 °C, which was first reported by Gross *et al*. approximately 40 years ago [18, 19]. However, the mechanism of the fibril formation was still a subject of debate. Growth of fibers appeared to occur by formation of 'subfibers' that subsequently entwined with other 'subfibers' [20]. When the scaffolds were soaked in SBF, collagen partly dissolved in form of collagen molecules or 'subfibers', the undissolved larger fibers in scaffolds served as nuclei for assembly of dissolved collagen and subsequently grew to form interconnected nanofibers.

5. Conclusion

The present study demonstrated the fabrication of collagen/wollastonite composite in form of three-dimensional porous scaffolds using the freeze-drying method. The obtained scaffolds possessed interconnected pores with a diameter about 100 μm and porosities more than 96%. The SBF soaking result showed the deposition of HA on collagen fibers after soaking for 7 days, which indicated good bioactivity of the scaffolds. Our results suggested that the incorporation of wollastonite into collagen matrix enhanced the bioactivity and mechanical strength of the scaffolds, and might be a useful approach in preparation of scaffolds for tissue engineering.

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