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A honeycomb composite of mollusca shell matrix and calcium alginate

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ABSTRACT

A honeycomb composite is useful to carry cells for application in bone, cartilage, skin, and soft tissue regenerative therapies. To fabricate a composite, and expand the application of mollusca shells as well as improve preparing methods of calcium alginate in tissue engineering research, *Anodonta woodiana* shell powder was mixed with sodium alginate at varying mass ratios to obtain a gel mixture. The mixture was frozen and treated with dilute hydrochloric acid to generate a shell matrix/calcium alginate composite. Calcium carbonate served as the control. The composite was transplanted subcutaneously into rats. At 7, 14, 42, and 70 days after transplantation, frozen sections were stained with hematoxylin and eosin, followed by DAPI, β-actin, and collagen type-I immunofluorescence staining, and observed using laser confocal microscopy. The composite featured a honeycomb structure. The control and composite samples displayed significantly different mechanical properties. The water absorption rate of the composite and control group were respectively 205–496% and 417–586%. The composite (mass ratio of 5:5) showed good biological safety over a 70-day period; the subcutaneous structure of the samples was maintained and the degradation rate was lower than that of the control samples. Freezing the gel mixture afforded control over chemical reaction rates. Given these results, the composite is a promising honeycomb scaffold for tissue engineering.

1. Introduction

A honeycomb composite is useful to carry cells for application in bone [1], cartilage [2], skin, and soft tissue regenerative therapies [3]. *Pinctada fucata* shells have been used to repair bone defects in sheep and obtained good results [4]. These findings suggest that shells are a possible material for tissue engineering research. Comprising three layers, namely, corneous, prismatic, and nacreous [5], the inorganic component in shells is calcium carbonate, and the organic component is shell matrix (mainly polysaccharides). The organic components of *Biomphalaria glabrata* shells have been analyzed [6]. The results showed that the organic components account for 0.9026% of the total shell weight. The organic components include water-insoluble material (0.8688%) that accounts for 96% of the total organic component, and proteins (0.116%) that account for 12.86% of the total organic component. Furthermore, water-insoluble proteins account for 49.612% of the total proteins. In the prismatic layer, the shell matrix and calcium carbonate are arranged such that the shell matrix coats the calcium carbonate crystals, equipping the shell with excellent mechanical properties. *Helix pomatia* shells have been co-cultured with human osteoblasts [7], and the nacreous layer was more suitable than the corneous layer for adhesion and amplification of human osteoblasts.

Sodium alginate has a good biocompatibility [8–13], and has been used as a tissue engineering biomaterial [14–17] in applications such as carriers for drug delivery [18–22], cell encapsulation [23], a matrix for bone regeneration [24], beads for use in embolisation [25], and microcapsules for soft tissue regeneration [26]. Because it is water soluble, the structure of sodium alginate cannot be maintained for long periods *in vivo*. The chemical reaction between calcium salt and sodium alginate produces calcium alginate with poor water solubility and good biocompatibility [27]. However, it is difficult to obtain a larger biomaterials than calcium alginate microspheres [28–32] because the production of calcium alginate may prohibit more calcium salt such as calcium chloride to enter the interior of sodium alginate to which produces more calcium alginate. Therefore, it is important to improve preparing methods of calcium alginate. Shell powder consists of the shell matrix and calcium carbonate. After the shell powder

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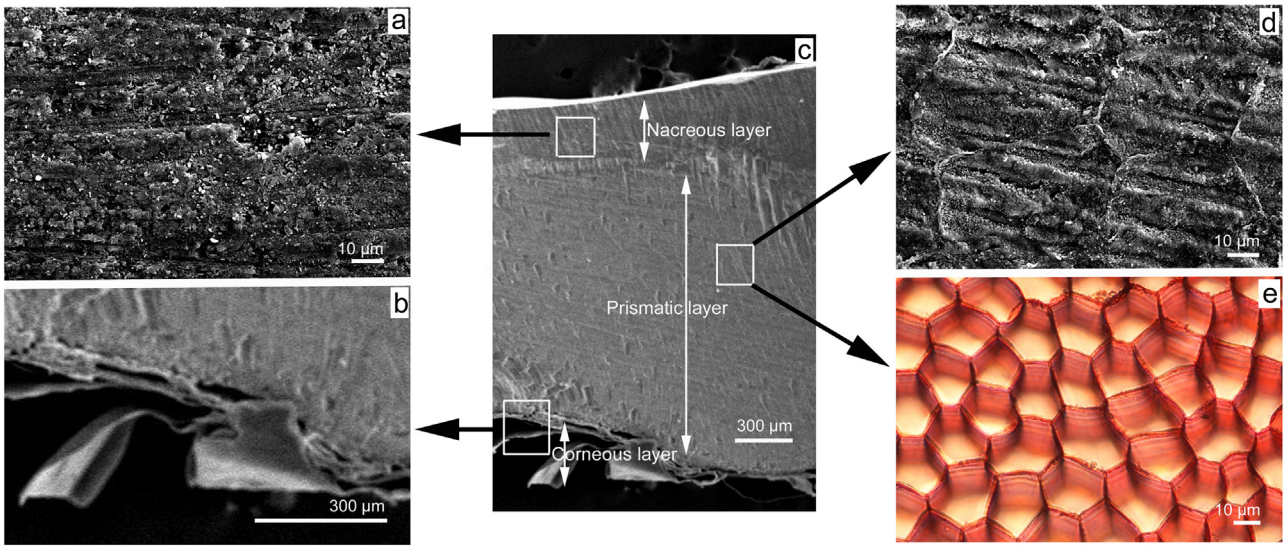

Fig. 1. Structure of *A. woodiana* shells. (a) Nacreous layer (SEM). (b) Corneous layer (SEM). (C) Cross section of the shell (SEM). (d) Prismatic layer (SEM). (e) Shell matrix (Optical microscope).

mixes with sodium alginate, the mixture is treated with excess dilute hydrochloric acid; thus, generating a composite comprising calcium alginate and shell matrix.

Anodonta woodiana (Lea l834) is a mollusca (Lamellibranchia: Unionidae). It mainly lives in freshwater such as rivers and lakes. It is commonly known as a mussel, and is abundant in rivers and lakes. Because the corneous and nacreous layers of the mussel shell are easily contaminated from long-term contact with water, we selected the prismatic layer as the raw material to prepare the shell powder. The shell powder from the prismatic layer was mixed with sodium alginate to form an emulsion. Then, the mixture was frozen, molded, and treated with excess dilute hydrochloric acid solution. After the frozen emulsion gradually melted and reacted with hydrochloric acid, calcium salt in the shell powder allowed the conversion of sodium alginate to calcium alginate. The shell matrix in the shell powder was wrapped in calcium alginate, forming a shell matrix/calcium alginate composite. This study aims to explore the application of shell powder for the preparation of tissue engineering biomaterials.

2. Materials and methods

2.1. Materials

A. woodiana shell was rinsed with water and dried. The nacreous (Fig. 1a) and corneous layers (Fig. 1b) were then removed, and the prismatic layer (Fig. 1d and e) was retained. Specimens of each layer were collected and mechanically ground and sieved at 400 mesh for further use. Sodium alginate (medical grade, 99%) was provided by Beijing Jinluhong Biotechnology Co., Ltd. (Beijing, China).

2.2. Determination of organic content in the shells

For organic content determination, 2 g of the shell powder of the corneous, prismatic, and nacreous layers were collected and placed in a 50-mL plastic centrifuge tube. The tube was then weighed and denoted as W1. Then, 30 mL (5% (v/v)) hydrochloric acid was slowly added dropwise to the tube and left to stand overnight. The solution was diluted to 50 mL using triple-distilled water and centrifuged at 5000 rpm for 30 min. After the supernatant was removed, the solution and tube was freeze-dried in a lyophilizer (EYELA FDU-2200) and weighed again (*W*2). The organic content in the shells was calculated with the following formula:

$$\text{Organic content}\,(\%) = 1 - \left(\frac{(W1 - W2)}{2}\right) \times 100\%$$

2.3. Composite preparation

The composite materials were prepared using sodium alginate/shell powder (prismatic layer) at different mass ratios (75/25, 60/40, 50/50, 45/55, 40/60, and 35/65). Equivalent volumes of calcium carbonate that served as the control were used. The preparation steps are listed below.

Sodium alginate was dissolved in triple-distilled water, divided into equal portions, and placed in a beaker. A certain amount of the shell powder of the prismatic layer or calcium carbonate was weighed and added to the sodium alginate solution. The resulting solution was stirred and placed in a 130-mm-diameter-petri dish for 10 min. Subsequently, the solution-containing petri dish was placed in a freezer at −70 °C for 8 h, and then transferred to a water bath. Specimens were gradually added to 3% (v/v) hydrochloric acid until no carbon dioxide foam was generated. The specimens were allowed to stand at room temperature for 12 h. The solution was decanted and hydrochloric acid was added. The specimens were treated for 12 h. The hydrochloric acid treatment was repeated thrice, and the specimens were rinsed with tap water for 12 h and with triple-distilled water thrice for 4 h each. After washing, the specimens were lyophilized for use.

2.4. Morphological observation

Specimen sheets, and the cross sections were observed under a scanning electron microscope (SEM) (JSM-6510LV, Japan).

2.5. Mechanical test

Specimens were cut into 5 × 15 mm sheets and tension tests were performed on a mechanical testing machine (Instron 1011, USA) at a tensile speed of 10 mm min^{-1}. The breaking strength, breaking extensibility rate, and *Young's* modulus were determined. Specimens of another group were soaked in distilled water for 1 h and tested.

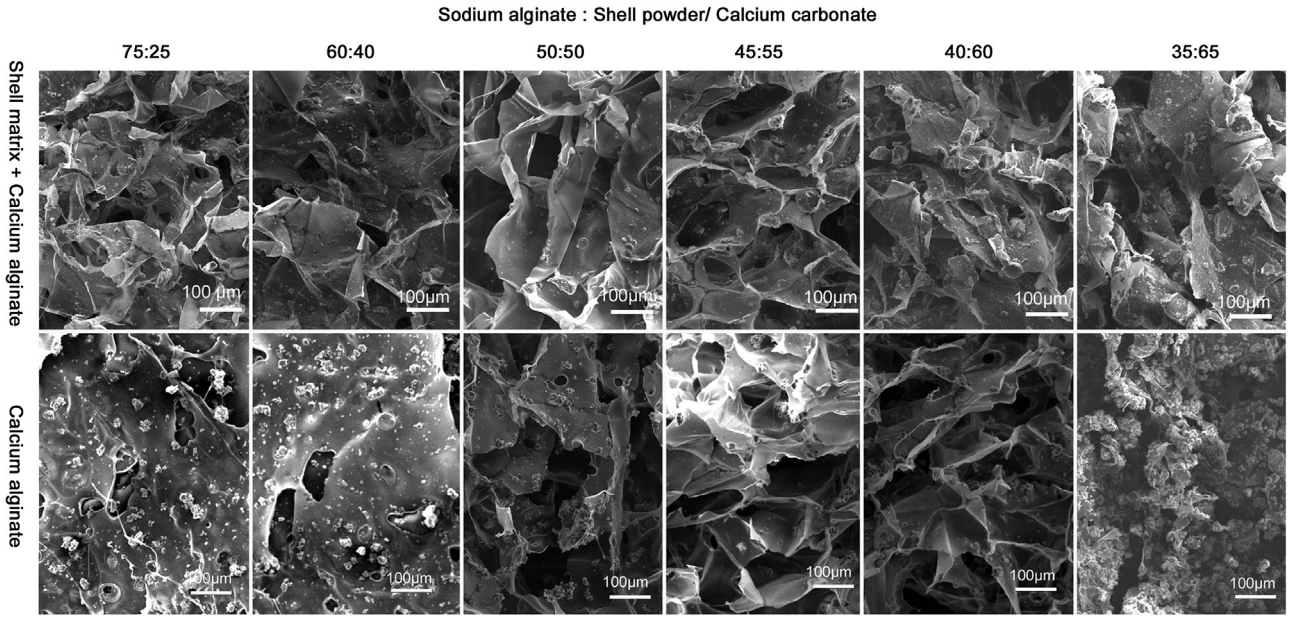

Fig. 2. Cross section of the shell matrix/calcium alginate and calcium alginate hydrogel (SEM).

2.6. Determination of water absorption properties

Specimens were cut into 20 × 20 mm sheets, dried, and weighed ($W1$). After soaking in triple-distilled water for 1 h, the specimens were weighed again ($W2$). Water absorption rates were calculated with the following formula:

$$\text{Water absorption}\,(\%) = \left[\frac{(W2 - W1)}{W1}\right] \times 100\%$$

2.7. Subcutaneous implantation

The shell matrix/calcium alginate and calcium alginate dried specimens were placed in PBS containing double antibodies (250 IU mL^{-1} penicillin and streptomycin). Twenty adult Sprague–Dawley rats (Third Military Medical University of Chinese PLA; half male and half female, weighing at 200 ± 10 g) were randomly divided into two groups. The animals were anesthetized with 3% sodium pentobarbital at 40–50 mg kg^{-1} *via* intramuscular injection. A 2-cm incision was made. Then the blunt end of a knife handle was inserted into the wound to isolate the muscle tissue and create a 3-cm opening where the specimens were implanted. The wounds were stitched up, and the animals were housed in separate cages. At 7, 14, and 42 days after implantation, specimens were collected and sliced using a freezing microtome (Leica CM 1850), then stained with hematoxylin–eosin and observed under a biological microscope (Nikon 80i, Image-pro plus 5.1). At 70 days, the frozen sections were subjected to DAPI staining (Beijing Biosynthesis Biotechnology Co., Ltd.) of the nuclei, β-actin staining (Gene Tax Co.) of the cytoskeleton, and collagen type-I (Gene Tax Co.) immunofluorescence staining. The specimens were observed using laser confocal microscopy (LCM; ZEISS LSM200; ZEISS Observer Z1; Lumen Dynamics X-cite 120Q) at 555, 488, and 405 nm.

3. Results

3.1. Organic content in the shells

The contents of organic components in the corneous, prismatic, and nacreous layers were respectively 56.32%, 6.03%, and 4.15%. Our results are different from previous reports [6] because of the differences in the species.

3.2. Morphology

Both the shell matrix/calcium alginate composite and calcium alginate formed a gel after hydration, and featured the same surface morphology. The cross section of the specimens reveals a honeycomb structure (Fig. 2). The observed pores are the results of the escaped carbon dioxide gas generated during the chemical reaction between the dilute hydrochloric acid and calcium carbonate. During the preparation of the shell matrix/calcium alginate composites (experimental group), excessive or insufficient amounts of sodium alginate may decrease the homogeneity of the resulting specimen structure. A similar trend was observed in the calcium alginate samples (control group).

3.3. Water absorption studies

The prepared shell matrix/calcium alginate composite and calcium alginate hydrogel exhibited strong water absorption properties, and their water absorption rates were respectively 205–496% and 417–586% (Fig. 3a). However, the water absorption rate of the shell matrix/calcium alginate composite was considerably lower than that of calcium alginate. As the amount of sodium alginate was gradually decreased, the water absorption rate of the resulting shell matrix/calcium alginate composite was substantially decreased, whereas that of calcium alginate was increased except that when the amount of sodium alginate was 75%. This evidence indicates that the strong water absorption capabilities of calcium alginate may be significantly lowered by the shell matrix.

3.4. Mechanical properties

The mechanical properties of the shell matrix/calcium alginate and calcium alginate hydrogels, and the shell matrix/calcium alginate and calcium alginate dry specimens were tested. However, the calcium alginate hydrogel could not be tested because of its poor mechanical properties.

Breaking strength properties mainly reflect the strength of the specimens (Fig. 3b). As the amount of shell powder of the prismatic

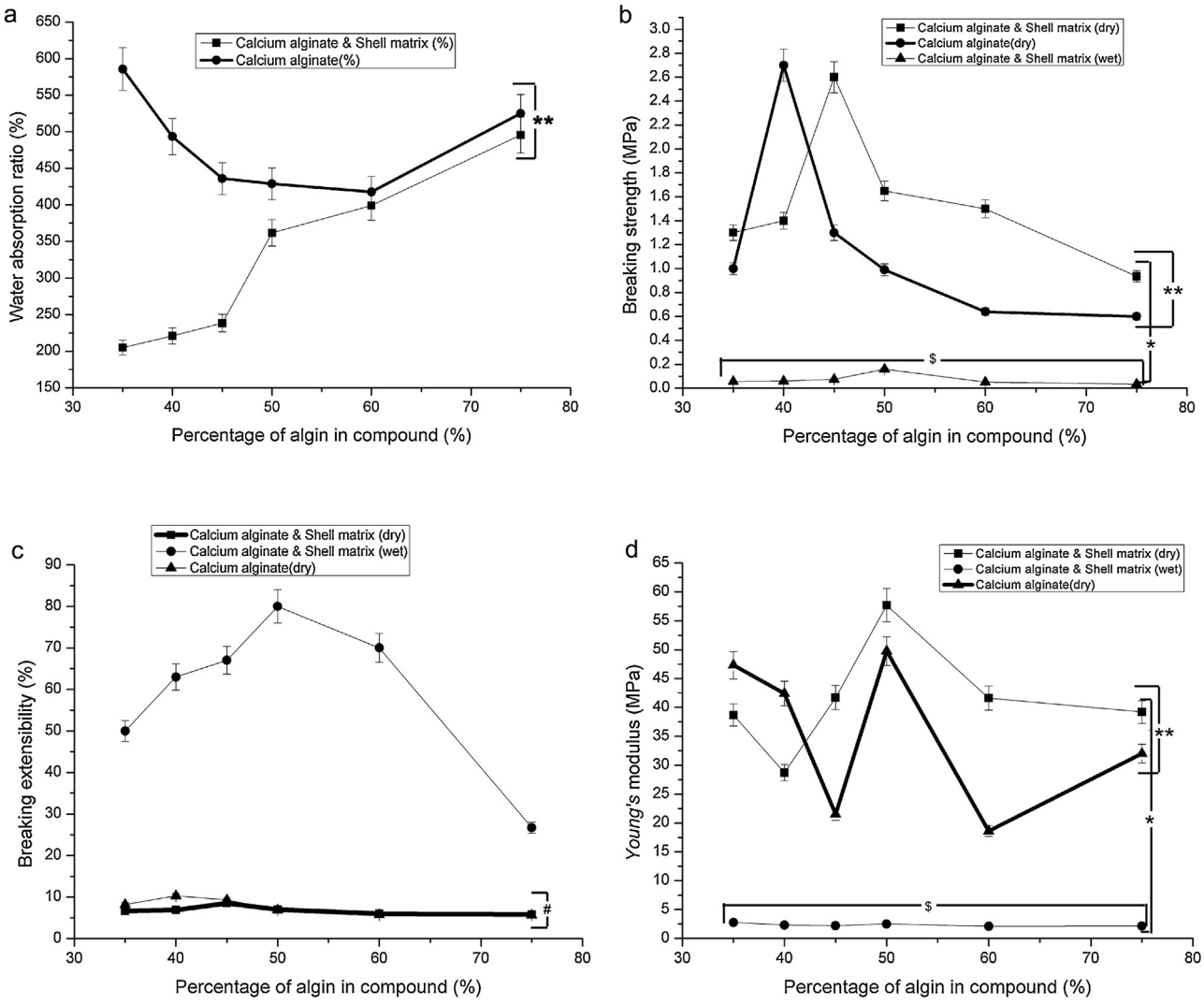

Fig. 3. Physical properties of the shell matrix/calcium alginate and calcium alginate hydrogel (Value = mean ± SD, $n = 3$). (a) Water absorption of the specimens. (b) Rupture strength of the specimens. (c) Breaking extensibility of the specimens. (d) Young's modulus of the specimens. *: The statistics analysis indicated that the comparison among groups showed significant difference, $p < 0.05$ (t-test). **: The statistics analysis indicated that the comparison between groups and the components in each group showed significant difference, $p < 0.05$ (t-test). $: The statistics analysis indicated that the comparison among the different percentage in the same group showed no significant difference, $p > 0.05$ (t-test). #: The statistics analysis indicated that the comparison between groups and the components in each group showed no significant difference, $p > 0.05$ (t-test).

layer or calcium carbonate was increased, the breaking strength of the composite initially increased and reached a peak before gradually decreasing. The shell matrix/calcium alginate composite showed a considerably higher breaking strength than that of the calcium alginate dry specimens. The breaking strengths of the hydrogels following water absorption were low.

Breaking extensibility mainly reflects the flexibility of the specimens (Fig. 3c). As the amount of shell powder of the prismatic layer or calcium carbonate increased, the breaking extensibility of the composite initially increased, then reached a peak before gradually decreasing. The shell matrix/calcium alginate dry composite showed a similar breaking extensibility to that of the calcium alginate dry specimens. The breaking extensibility rate of the hydrogels doubled after water absorption.

Young's modulus reflects the hardness of the specimens (Fig. 3d). The hardness of the hydrated specimens was significantly lower than those of the dry specimens. The addition of 45–75% sodium alginate considerably increased the hardness of the shell matrix/calcium alginate dry composite, whereas that of 35–40% decreased the hardness of the dry composite when compared with those of the calcium alginate specimens.

3.5. Subcutaneous implantation experiment

Based on the morphology and mechanical analyses, the optimal concentration of the shell powder in the preparation of the specimens is 40–60%. For the subcutaneous implantation experiment, to prepare the composite material, the shell powder (or calcium carbonate) and sodium alginate were added at a mass ratio of 5:5.

At 7 days after transplantation (Fig. 4), in the experimental group, the specimen structure was loose and contained few cells, and the morphology of the specimens remained intact (blue arrows in Fig.). In the control group, there were a larger number of cells within the specimens, and the morphology was maintained; however, to a lower extent than those in the experimental group. At 14 and 42 days after transplantation, similar results were observed (Fig. 4).

At 70 days after implantation, the shell matrix/calcium alginate sample was harvested and frozen sections were subjected to DAPI

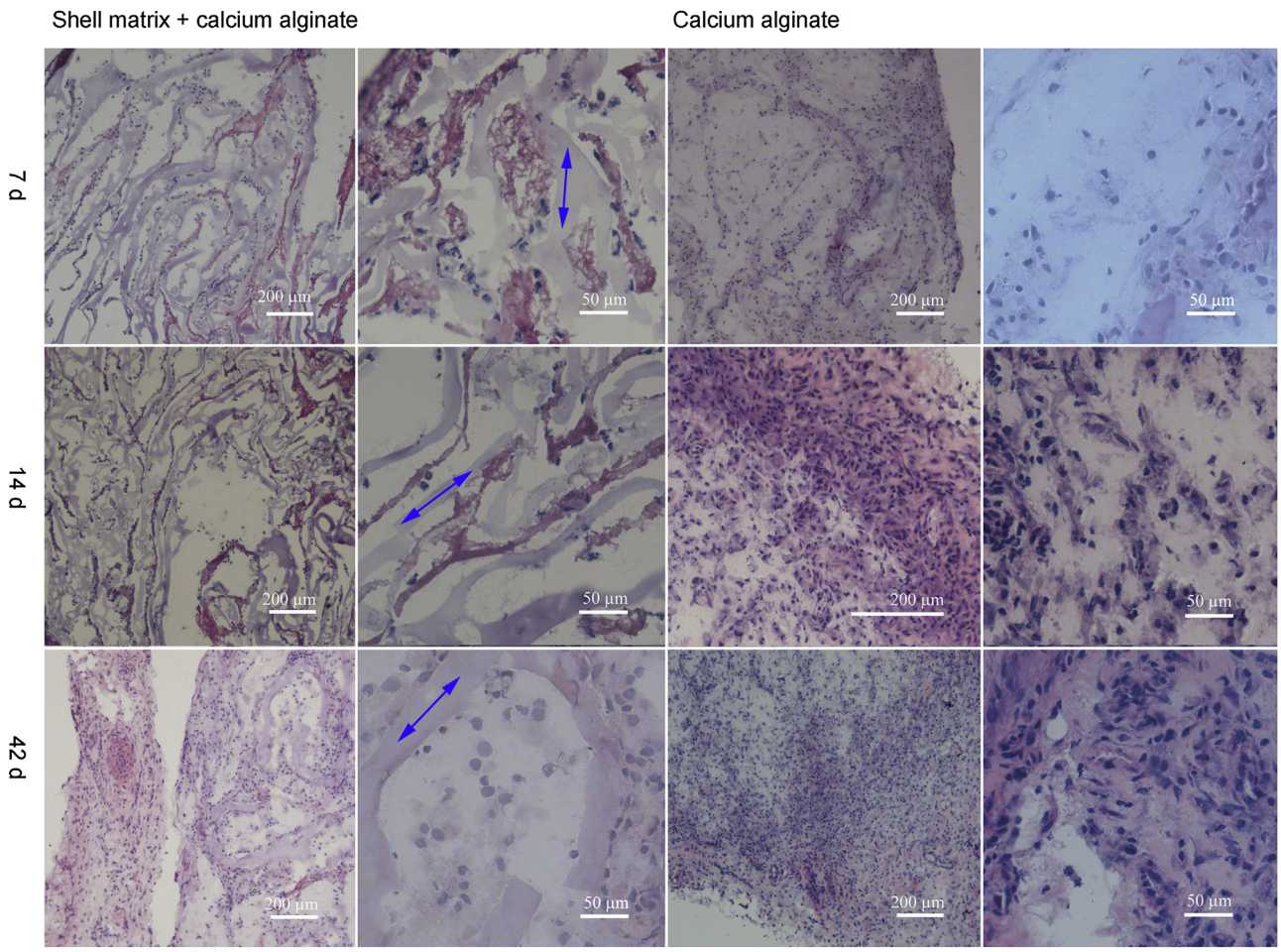

Fig. 4. Subcutaneous implantation of the shell matrix/calcium alginate composite and calcium alginate specimens (hematoxylin–eosin staining). Arrows: Shell matrix/calcium alginate composite remained.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and β-actin immunofluorescence staining for observation under LCM. The results (Fig. 5) showed that the internal structure of the compound was preferentially maintained, and there were a few cells in the specimens. Frozen sections were subjected to DAPI and collagen type-I immunofluorescence staining, and imaged *via* LCM (Fig. 5). The results showed that the structure of the compound

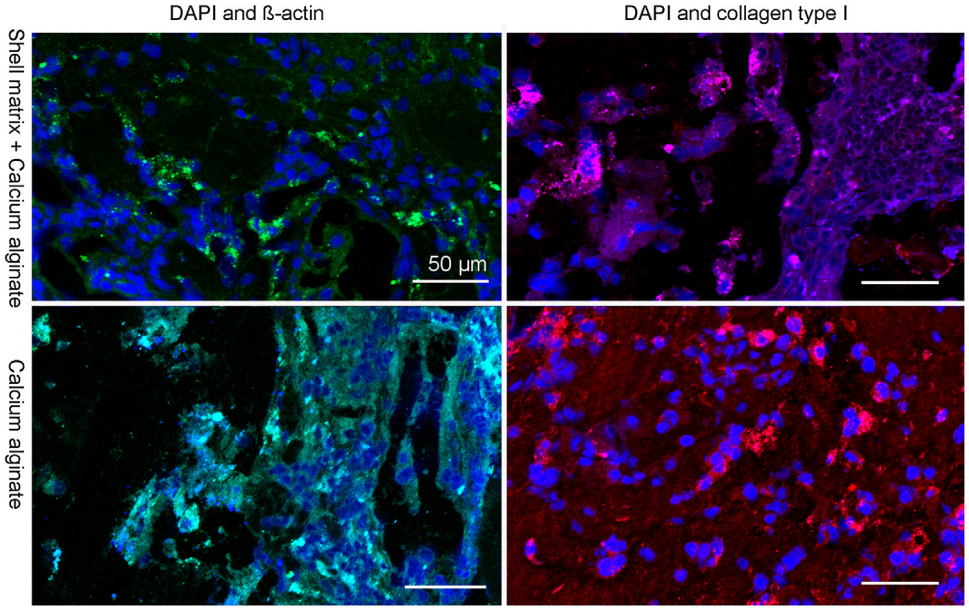

Fig. 5. LCM analysis of the shell matrix/calcium alginate composite at 70 days after implantation using immunofluorescence staining.

remained intact, and that there was a small amount of cells and collagen type-I in the compound. In the control group (calcium alginate specimens), at 70 days after implantation, frozen sections were harvested and subjected to DAPI and β-actin immunofluorescence staining, and imaged *via* LCM (Fig. 5). The results showed that the specimens were not completely degraded, and that there were a large number of cells within the specimens. Frozen sections were harvested and subjected to DAPI and collagen type-I immunofluorescence staining, and imaged under LCM (Fig. 5). The results showed that a large number of cells and collagen type I were present in the specimens. At 70 days after implantation, both the shell matrix/calcium alginate composite and calcium alginate underwent significant degradation but did not completely degrade. During the degradation process, the structure of the shell matrix/calcium alginate composite was maintained more than that of the calcium alginate sample.

4. Discussion

During the design of the specimens, because of the action of excess dilute hydrochloric acid, calcium carbonate decomposed into carbon dioxide and calcium ion, which consequently converted sodium alginate to calcium alginate [27]. For this process to initiate, the amounts of calcium carbonate and sodium alginate should be equal. Excess amounts of calcium alginate or sodium alginate result in an incomplete chemical reaction. Because the inorganic component of the shell powder is mainly calcium carbonate, the inhomogeneity of the prepared specimens is due to the abundant amount of calcium salt or sodium alginate that influences the water absorption and mechanical properties.

Neither the shell powder nor calcium carbonate is water soluble, whereas sodium alginate is weakly soluble in water, and sodium alginate solution at a high concentration is present as a gel. The shell powder and calcium carbonate dispersed in the sodium alginate gel could aggregate and precipitate under the action of free diffusion and gravity, if they are dispersed for a long period, instigating the formation of an inhomogeneous structure. Furthermore, during the reaction between the shell powder or calcium carbonate and sodium alginate, the shell powder or calcium carbonate may violently react with dilute hydrochloric acid to generate carbon dioxide, which will affect the homogeneity of the structure. In the present study, the mixture of the uniformly dispersed shell powder or calcium carbonate and sodium alginate was frozen and then treated with excess dilute hydrochloric acid solution at room temperature. The frozen mixture gradually melted and reacted with hydrochloric acid, effectively controlling chemical reaction rates.

The mechanical properties of calcium alginate hydrogels are too low to tolerate the minimal requirements for instrument testing, and may cause inconvenience for transplantation in animals. For this reason, the shell matrix/calcium alginate composite is superior to calcium alginate specimens. Tissue engineering materials require different water absorption properties according to the application. However, the strong water absorption capabilities of calcium alginate may be significantly lowered by the shell matrix.

Based on the results of the morphological and mechanical tests, the optimum amount of the shell powder for the preparation of the shell matrix/calcium alginate compound is 40–60%.

Biocompatibility is critical to tissue engineering materials. In the animal experiments, all the studied rats achieved good healing after surgery and the life indicators were normal. This evidence suggests that the specimens prepared in this study are biocompatible.

Cytoskeleton is responsible for maintaining cell morphology and cell movement, and is widely distributed in the cytoplasm of cells. β-Actin is a type of cytoskeleton. As observed from the frozen sections, all cells were stained by β-actin and exhibit green fluorescence. Collagen type I, as secreted by the fibroblasts, is widely present in mammalian dermal and subcutaneous tissues. In this study, collagen type-I immunofluorescence staining revealed red fluorescence. Aside from red blood cells, all the mammalian cells contain nuclei, and DAPI immunofluorescence staining of nuclei exhibits blue fluorescence. Experimental findings (Fig. 5) showed that in the subcutaneous implantation experiment, the degradation of the specimens *in vivo* was followed by cell infiltration and collagen production.

Tissue engineering materials have different requirements pertaining to the inner structure and degradation rate according to the application [33–35]. However, these requirements are based on the preservation of the structure *in vivo* over a long time. The animal experiments (Figs. 4 and 5) showed that the shell matrix/calcium alginate composite degraded slowly when compared with the rates of the calcium alginate specimens, and the ability to maintain the inner structure was better in the composite compared with that of the calcium alginate.

5. Conclusion

Rapid freezing of the gel mixture of shell powder or calcium carbonate and sodium alginate, and control of the concentration of dilute hydrochloric acid solution can achieve a shell matrix/calcium alginate composite material with a homogeneous structure. The optimum amounts of the shell powder and sodium alginate are 40–60% and 60–40%, respectively. Within the required composition range, the mechanical properties of the composite are better than those of calcium alginate. However, the composite features lower water absorption rates. The shell matrix/calcium alginate is a bio-safe material. When the shell powder or calcium carbonate is mixed with sodium alginate (molar ratio of 5:5), the rate of degradation *in vivo* is slightly lower in the shell matrix/calcium alginate composite when compared with that of calcium alginate at 70 days. However, the ability to maintain the inner structure is significantly better than that in calcium alginate. The shell matrix/calcium alginate composite can be used as an auxiliary material in surgery, drug delivery carrier, and an artificial extracellular matrix to carry cells for application in bone, cartilage, skin and soft tissue regenerative therapy.

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