Colloids and Surfaces B: Biointerfaces 139 (2016) 100–106

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

A honeycomb composite of mollusca shell matrix and calcium alginate

Hua-jian You[a,c,1], Jin Li[a,c,1], Chan Zhou[b], Bin Liu[a,c,*], Yao-guang Zhang[a,c]

[a] Key Laboratory of Freshwater Fish Reproduction and Development (Southwest University), Ministry of Education, Chongqing 400715, China
[b] Chongqing Academy of Animal Science, Chongqing 402460, China
[c] School of Life Science, Southwest University, Chongqing 400715, China

ARTICLE INFO

Article history:
Received 6 August 2015
Received in revised form
11 November 2015
Accepted 2 December 2015
Available online 4 December 2015

Keywords:
Anodonta woodiana
Shell
Matrix
Calcium alginate
Sodium alginate

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

* Corresponding author at: Key Laboratory of Freshwater Fish Reproduction and Development (Southwest University), Ministry of Education, Chongqing 400715, China. Fax: +86 2368367856.
E-mail addresses: xytom@swu.edu.cn, xytom@163.com (B. Liu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.colsurfb.2015.12.006

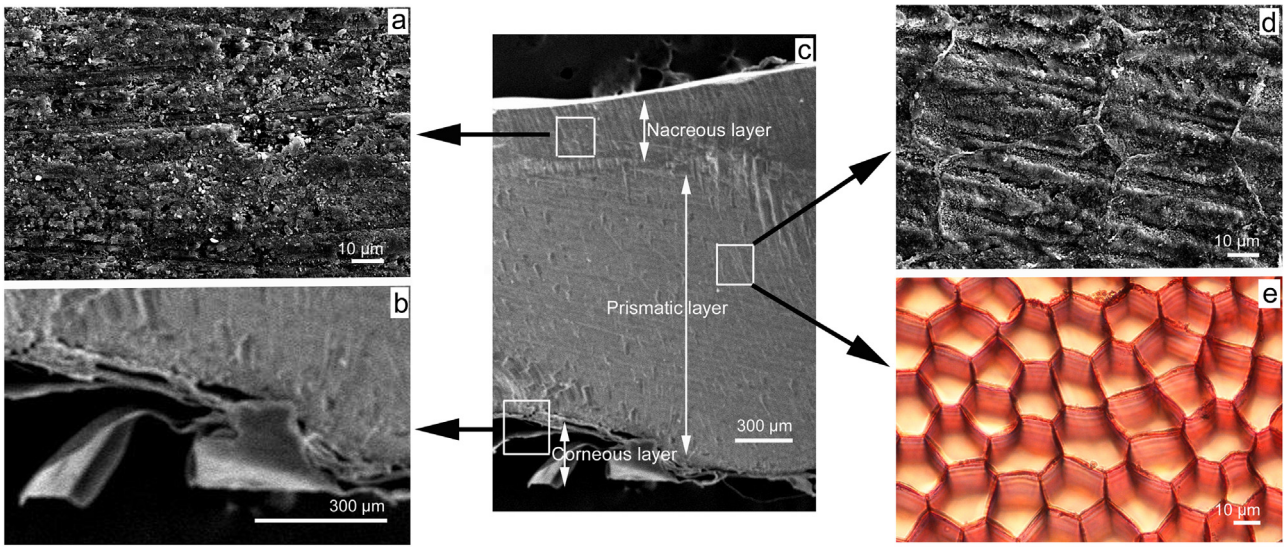

Fig. 1. Structure of *A. woodiana* shells. (a) Nacreous layer (SEM). (b) Corneous layer (SEM). (C) Cross section of the shell (SEM). (d) Prismatic layer (SEM). (e) Shell matrix (Optical microscope).

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.