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In vivo evaluation of porous hydroxyapatite/chitosan–alginate composite scaffolds for bone tissue engineering

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ABSTRACT

Porous hydroxyapatite (HAp)/chitosan–alginate composite scaffolds were prepared through *in situ* co-precipitation and freeze-drying for bone tissue engineering. The composite scaffolds were highly porous and interconnected with a pore size of around 50–220 μm at low concentrations of HAp. As the HAp content increased, the porosity of the scaffolds decreased from 84.98 to 74.54%. An MTT assay indicates that the obtained scaffolds have no cytotoxic effects on MG-63 cells, and that they have good biocompatibility. An implantation experiment in mouse skulls revealed that the composite scaffold provides a strong positive effect on bone formation *in vivo* in mice. Furthermore, that HAp/chitosan–alginate composite scaffold has been shown to be more effective for new bone generation than chitosan–alginate scaffold.

1. Introduction

Recently, tissue engineering has emerged as one such promising approach for bone repair and reconstruction [1]. Tissue engineering involves the expansion of cells from a small biopsy, followed by the culturing of the cells in temporary porous scaffolds to form the new organ or tissue [2–4]. With this approach, the porous scaffold serves an important role in the manipulation of the functions of osteoblasts and a central role in the guidance of new bone formation into desired shapes. Therefore, the scaffold materials must be biocompatible, osteoconductive, and osteointegrative, and have enough mechanical strength to provide structural support.

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) has been widely used in medicine and dentistry because it is biocompatible, osteoconductive, and has excellent chemical and biological affinity with bony tissue [5]. As a result, HAp is accepted as a bioactive scaffold material for guided bone regeneration. In addition to the requirements for chemical composition of the scaffold material, an interconnected porous structure is necessary to allow cell attachment, proliferation, and differentiation, and to provide pathways for biofluids. Chitosan is a natural cationic polymer that is biologically renewable, biodegradable, biocompatible, non-antigenic, non-toxic, and biofunctional. It has been studied as a useful biomaterial in diverse tissue engineering applications because of its hydrophilic surface promoting cell adhesion, proliferation and differentiation, good biocompatibility and good host response, high biochemical significance in hemostasis, angiogenesis and macrophage activation, biodegradability by lysozyme and other enzymes, bactericidal/bacteriostatic activity, and capacity to maintain a predefined shape after cross-linking [3,6–8]. Alginate is a biocompatible, hydrophilic, and biodegradable anionic polymer under normal physiological conditions and is widely used as an instant gel for bone tissue engineering [9–11].

Chitosan–alginate scaffolds have been tested in the regeneration of various tissues and bone with promising results [12,13]. However, many biopolymers are fragile and do not exhibit undoubtedly biocompatible behavior. Some polymer and bioactive ceramics composites have been developed for bone tissue engineering in order to increase the bioactivity and mechanical properties of the materials [14–17]. Hydroxyapatite (HAp)/polymer composites have attracted a great deal of attention because they exhibit osteoconductivity because of the presence of HAp [18,19], which has a similar chemical composition and structure as the mineral phase of human bones and hard tissues.

In this study, porous HAp/chitosan–alginate composite scaffolds were prepared through *in situ* co-precipitation and freeze-drying for bone tissue engineering. With the aim of fabricating HAp/chitosan–alginate composite scaffolds and *in vivo* culture of bone tissue engineered constructs.

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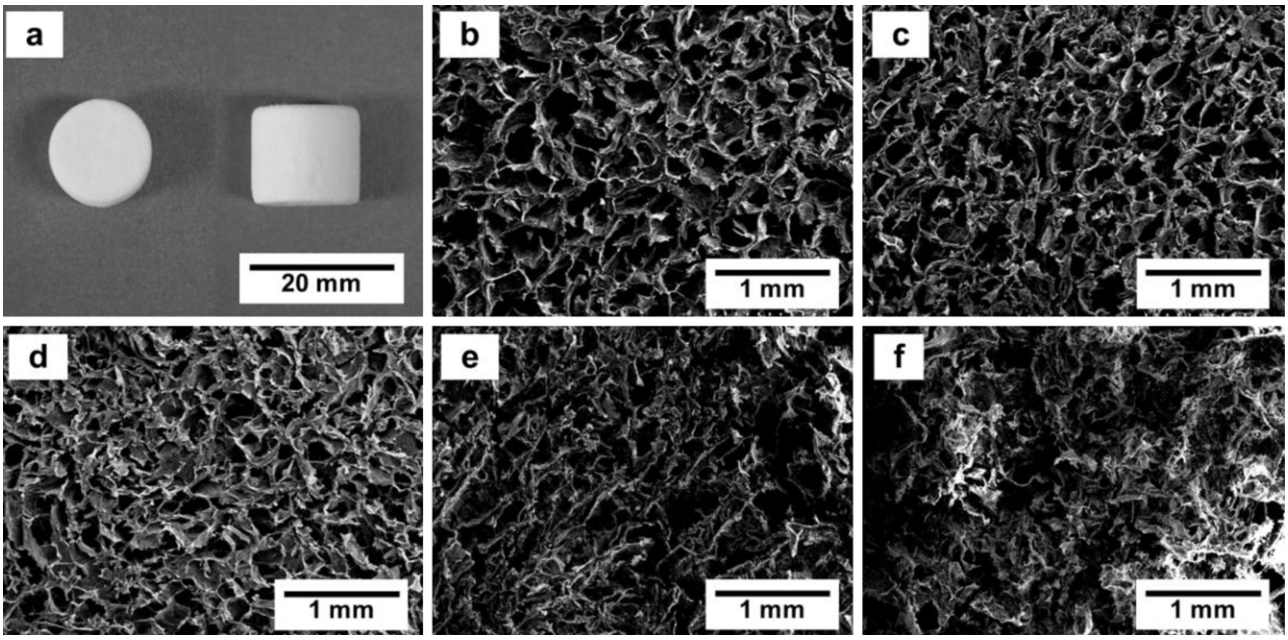

Fig. 1. (a) Photograph of HAp/chitosan–alginate composite scaffolds, and SEM morphology of the composite scaffolds with different HAp contents; (b) 0, (c) 10, (d) 30, (e) 50 and (f) 70 wt%.

2. Experimental

2.1. Preparation of HAp/chitosan–alginate composite scaffolds

The HAp/chitosan–alginate composite scaffolds were synthesized through *in situ* co-precipitation. A chitosan aqueous solution was prepared by dissolving 3.84 g of the chitosan powder (viscosity > 200 cP: 1 wt% solution in the 1 wt% acetic acid solution in Brookfield, Sigma–Aldrich) in 64 ml 1 M acetic acid. To prepare the alginate solution, 3.84 g of the sodium alginate powder (viscosity 200–400 cP for 1 wt% solution at 20 °C, Sigma–Aldrich) was dissolved in 96 ml of 1 M NaOH. H_3PO_4 and $Ca(OH)_2$ were added to the chitosan aqueous and alginate solutions, respectively, to form the HAp/chitosan–alginate composite scaffolds. The ratios of chitosan to H_3PO_4 and alginate to $Ca(OH)_2$ were adjusted so that the final HAp/chitosan–alginate weight ratios were 10/90 and 70/30, respectively. The resulting suspension was mixed under constant stirring in a blender for 1 h. Acetic acid was gradually added drop-wise to the suspension until a pH of 7.4 was obtained. The slurry was placed into 24-well cell culture plates and stored in a freezer at −15 °C until frozen. Subsequently, the samples were lyophilized in a freeze dryer at −80 °C for 24 h. The dried samples were cross-linked with a 1% (w/v) $CaCl_2$ solution for 15 min and then immersed in distilled water for 24 h to remove any residual sodium acetate and unbound $CaCl_2$ [12]. After immersion, the samples were washed three times and then freeze-dried at −80 °C for 24 h to obtain the HAp/chitosan–alginate composite scaffolds.

The morphologies, pore configuration, and pore size of the HAp/chitosan–alginate composite scaffolds were investigated using scanning electron microscopy (SEM, S-4300, Hitachi, Japan). The porosity and density were measured by the liquid displacement method [18].

2.2. Cell culture

The cytotoxicity of the HAp/chitosan–alginate composite scaffolds was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [20]. MG-63 cells

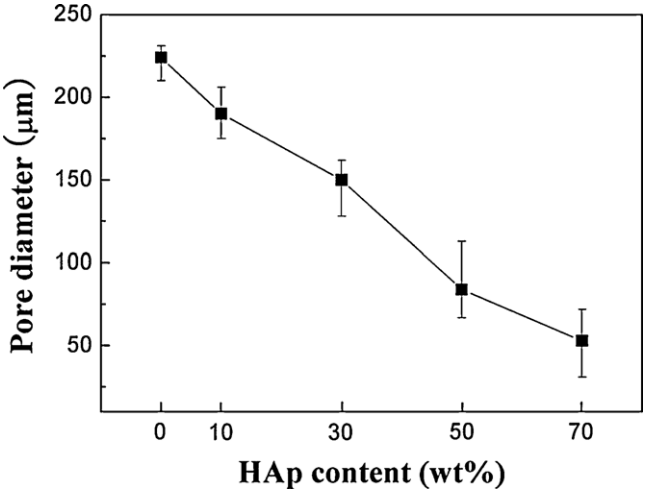

Fig. 2. Pore diameter of HAp/chitosan–alginate composite scaffolds with different HAp contents.

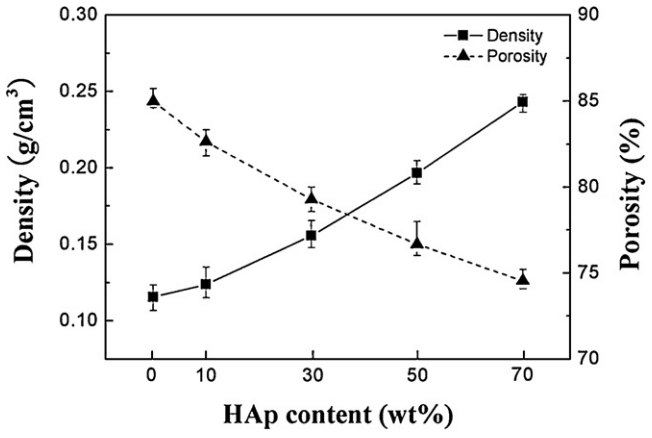

Fig. 3. Density and porosity of HAp/chitosan–alginate composite scaffolds with different HAp contents.