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Functionalized alginate/chitosan biocomposites consisted of cylindrical struts and biologically designed for chitosan release

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ABSTRACT

A novel alginate/chitosan composite scaffold was developed. The composite scaffolds were fabricated at low temperature using a three-axis robot system connected to a micro-dispenser and a core/shell nozzle. The structure of the composite scaffolds included hollow struts; deposited chitosan on the inner walls (core region) of the struts reacted electrostatically with the alginate layer (shell region). The fabricated, highly porous composite scaffolds exhibited excellent mechanical properties and controllable chitosan release, which was closely dependent on the weight fraction of the alginate in the shell region. The tensile strength in the dry state was ~1.8-fold greater than that of pure alginate scaffold due to the ionic interaction between alginate and chitosan. To determine the feasibility of using the developed scaffold in tissue regeneration applications, *in vitro* cellular responses were evaluated using osteoblast-like-cells (MG63). The cell proliferation on the composite scaffold was ~3.4-fold greater than that on the pure alginate scaffold. Alkaline phosphate activity and calcium deposition of the composite scaffold after 14 and 21 days of cell culture were significantly enhanced (1.6- and 1.8-fold greater, respectively) compared with those of the pure alginate scaffold. These results suggested that the alginate/chitosan composite scaffolds with a controlled chitosan release have great potential for use in regenerating various tissues.

1. Introduction

In the human body, bones perform a mainly integrated locomotion role, ensuring that the skeleton sustains appropriate load-bearing ability while also protecting internal organs [1]. In general, bone extracellular matrix (ECM) consists of type-I collagen, 4-nm-thick plate-like carbonated apatite minerals, and various non-collagenous matrix proteins (glycoproteins and proteoglycans) [2]. Type-I collagen and various inorganic materials (tri-calcium phosphate, hydroxyapatite, bioactive glasses, etc.) have been evaluated as materials for regenerating bone tissues [3–5]. However, although the use of collagen exhibited various positive features, including favourable cell attachment, promotion of chemotactic responses, and outstanding bioceramic compositional similarity to bone, the protein is not free of immunogenicity (the remnant risk of disease transmission) and exhibits difficult process-controllability and insufficient mechanical performance; additionally, collagen bioceramics exhibit low fracture toughness and low processability [2].

As alternatives to these biomaterials, polysaccharide-based hydrogels (*e.g.* chitosan, dextran, agarose, alginate, fucoidan, and pectin) can be used to fabricate scaffolds. These hydrogels exhibited relatively low immunogenic responses and provided three-dimensional (3D) cellular microenvironments with high water content and good cell encapsulation [2,6,7]. Of these hydrogels, chitosan (a natural cationic polymer) exhibited various positive bioactive properties, including hydrophilicity, outstanding biological properties (biodegradability, biocompatibility, non-antigenicity, and non-toxicity), and minimal foreign body reaction in *in vivo* [8,9]. Also, due to its structural similarity to glycosaminoglycans, chitosan has been used widely for cartilage regeneration [10,11]. In particular, in bone tissue regeneration, chitosan scaffolds displayed osteoconductive properties, which accelerate bone formation both *in vitro* and *in vivo* [12]. However, despite its potential as a biomaterial for regenerating bone tissue, it has several shortcomings, including insufficient mechanical properties and processability for fabrication of 3D structures [13]. Alginate, an anionic polymer, is another typical hydrogel used for biomedical scaffolds. Alginate

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exhibits good biological properties, such as biocompatibility, hydrophilicity, biodegradability in physiological conditions, and rapid gelation in the presence of Ca^{2+} ions. For these reasons, it has been applied widely as a scaffold and drug-releasing and cell-delivering material for bone tissue regeneration applications [14,15].

In this study, we developed polysaccharide-based scaffolds from chitosan and alginate. Complementary effects were observed: the formation of a stable bond between the alginate and the chitosan increased the mechanical strength of the scaffold [16]; the structural integrity of the chitosan was reinforced *via* rapid gelation of the alginate; and the alginate component imparted release-control to the chitosan. With these components, we fabricated a layer-by-layer (LBL) scaffold with a chitosan core and alginate shell microscale struts. The mixture of high-molecular-weight chitosan and alginate solution gelled instantly due to the strong electrostatic interaction between the cationic chitosan and the anionic alginate, thereby forming the core–shell structure. The core–shell scaffold structure also enabled the control of chitosan release from the alginate shell region; we observed controlled release of chitosan by varying the weight fractions (3, 5, and 7 wt%) of alginate in the shell and fixing the concentration of chitosan (5 wt%) in the core.

As reported by Gelinsky et al., vascularisation in the scaffolds is a key challenge in scaffold-based tissue engineering [17]. For this reason, they suggested the use of an LBL 3D alginate/polyvinyl alcohol (PVA)-based scaffold with controllable hollow struts. To fabricate hollow struts in our structure, we employed a mixture of poly(ethylene oxide) (PEO) and chitosan in the core region. The PEO was voluntarily leached during the cross-linking and washing process, after which the chitosan came into contact with the alginate shell, and the two materials electrostatically gelled. This process resulted in the fabrication of a highly porous scaffold comprised of hollow alginate struts internally lined with chitosan.

To demonstrate the feasibility of our approach to fabrication of a tissue regeneration scaffold, we measured the physical properties (water absorption, biodegradability, chitosan-release, and wet/dry mechanical properties) and biological activities [live/dead count after various culture periods, cell proliferation, alkaline phosphatase (ALP) activity, and alizarin red-staining] of scaffolds after various culture periods.

2. Experimental

2.1. Materials

Low-viscosity, high-G-content LF10/60 alginate (FMC BioPolymer, Drammen, Norway) was used at 3, 5, and 7 wt% in PBS. In this study, our extrusion processing capability limited us to the use of alginate solutions with concentrations no greater than 7 wt%. The human osteosarcoma cell line MG63 (CRL-1427) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were obtained from Gibco-BRL, Life Technologies (Grand Island, NY). DMEM, trypsin–ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin/amphotericin (10,000 U mL^{-1}, 10,000 μg mL^{-1}, and 2500 μg mL^{-1}, respectively), and FBS were also obtained from Gibco BRL, Life Technologies. Alizarin Red-S (ARS), *p*-nitrophenyl phosphate (*p*-NPP), and cetylpyridinium chloride monohydrate, were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents used in this study were of analytical grade.

2.2. Preparation of chitooligosaccharide

Chitooligosaccharide was prepared according to the method reported previously [18]. Concisely, 1% (w/v) solution was prepared by dispersing 100 g of chitosan in 1.0 L of distilled water, stirring in 550 mL of 1.0 M lactic acid, and then diluting with distilled water to a final volume of 10 L. The pH of the solution was adjusted to 5.5 using saturated sodium hydrogen bicarbonate solution. Ninety-three percent deacetylated chitosan (93% of the total chitosan polymer) was hydrolysed by an endotype chitosanase derived from *Bacillus* sp. (35,000 U g^{-1} protein; Amicosen Co., Jinju, South Korea) using a substrate to enzyme ratio of 1:1.5 units for 18 h in a batch reactor and then heated at 98 °C for 10 min to inactivate the enzyme. Thereafter, hydrolysates were separated using an ultrafiltration (UF) membrane reactor system (Minitani; Millipore Co., Bedford, MA). The UF membrane used in the system had molecular weight cut-off of 10 kDa. After fractionation, chitooligosaccharide (M_w > 10 kDa) was dialysed against distilled water for 3 days and then lyophilised for 5 days.

2.3. Scaffold fabrication

We used a low temperature process supplemented with a core/shell nozzle (Fig. 1) to fabricate alginate/chitosan composite scaffolds from 3, 5, and 7 wt% alginate solutions and a mixture of chitosan (5 wt%) and PEO (5 wt%) [mixing ratio = 3 (chitosan):7 (PEO)]. All processes were conducted using a 3D dispensing system with a core–shell nozzle (diameter of the core nozzle = 250 μm; diameter of the shell nozzle = 550 μm) connected to a three-axis robot (DTR3-331S-EX; Bucheon, South Korea) and a low-temperature plate to control the temperature from room temperature to −50 °C. The solutions in the core/shell region were extruded in the nozzle, and the microscale struts were then immediately frozen on the low-temperature plate (−30 °C). All fabrication processes were conducted on a clean bench. In this study, the nozzle speed was set at 7.5 mm s^{-1}. The plotted scaffold was immediately placed in a freeze-dryer (SFDSM06; Samwon, South Korea) at −75 °C for 12 h.

As shown in the optical images in Fig. 1, the fabricated scaffold consisted of core (red colour (in the web version))/shell struts. To

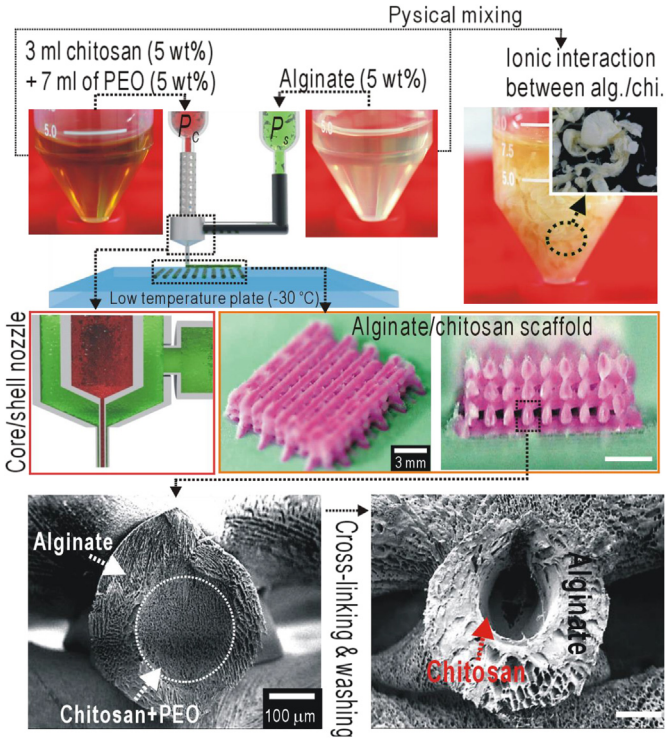

Fig. 1. Schematic diagram of the core/shell scaffold fabrication method; nozzle, optical and SEM images of fabricated alginate/chitosan scaffolds and struts.