

Synthesis and characterization of collagen/hyaluronan/chitosan composite sponges for potential biomedical applications

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Abstract

Cells, scaffolds and growth factors are three main components of a tissue-engineered construct. Collagen type I, a major protein of the extracellular matrix (ECM) in mammals, is a suitable scaffold material for regeneration. Another important constituent of the ECM, hyaluronic acid (hyaluronan, HA), has been used for medical purposes due to its hydrogel properties and biodegradability. Chitosan is a linear polysaccharide comprised of β 1- to β 4-linked D-glucosamine residues, and its potential as a biomaterial is based on its cationic nature and high charge density in solution. This study was conducted to evaluate the characteristics of scaffolds composed of different ratios of type I comb collagen and chitosan with added HA in order to obtain the optimum conditions for the manufacture of collagen–hyaluronan–chitosan (Col–HA–Ch; comprising collagen, HA and chitosan mixed in different ratios: 10:1:0, Col10HACH0; 9:1:1, Col9HACH1; 8:1:2, Col8HACH2; 7:1:3, Col7HACH3; 6:1:4, Col6HACH4; and 5:1:5, Col5HACH5) composite porous scaffolds. Microstructural observation of the composite scaffolds was performed using scanning electron microscopy. The mean pore diameters ranged from 120 to 182 μ m and decreased as the chitosan composition increased. All scaffolds showed high pore interconnectivity. Swelling ratio measurements showed that all specimens could bind 35- to 40-fold of physiological fluid and still maintain their form and stability. For tensile strength, the optimal ratio of collagen and chitosan was 9:1. Thermal stability was investigated using a differential scanning calorimeter and showed that Col5HACH5 and Col6HACH4 were significantly more stable than the other groups. In enzymatic sensitivity, a steady increase in the biostability of the scaffolds was achieved as the chitosan concentration was increased. In biocompatibility testing, the proliferation of the fibroblasts cultured in Co-HA-Ch tri-copolymer scaffolds was high. Overall, we observed the 9:1:1 mixing ratio of collagen, hyaluronan and chitosan to be optimal for the manufacture of complex scaffolds. Furthermore, Col–HA–Ch tri-polymer scaffolds, especially Col9HACH1, could be developed as a suitable scaffold material for tissue engineering applications.

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1. Introduction

Tissue engineering is a field of research which is aimed at regenerating tissues and organs [1]. Cells, scaffolds and growth factors are the three main components for creating a tissue-engineered construct. The principle aim of a scaffold

design should be to mimic the native extracellular matrix (ECM) of the target tissue as much as possible [2]. The development of biodegradable polymers to perform the role of a temporary matrix is an important factor in the success of cell transplantation.

Type I collagen, a main protein of ECM in mammals, is a suitable scaffold material for generating artificial substitutes for diseased or damaged tissue and organs [3,4]. This is due to the fact that collagen strongly promotes cell proliferation [4–6]. In our previous study, atelocollagen

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derived from pepsin-digested chicken combs showed excellent thermal and enzymatic stability and could be used as an excellent biomaterial for tissue engineering [7].

Hyaluronic acid (hyaluronan, HA), an important constituent of ECM, has been used as a viscoelastic biomaterial for medical purposes, such as in cosmetics, because of its high water-holding capacity, and in drug delivery systems due to its biodegradability. HA exists in high concentrations during fetal skin development, is involved in cell migration and differentiation, and is the first macromolecule to appear in the ECM during tissue engineering repair [8]. It has also been reported that the addition of HA promotes cell division in human fibroblasts by enhancing their passage through cell cycles when incorporated within a collagen matrix.

Chitosan is a linear polysaccharide comprising β 1- to β 4-linked D-glucosamine residues, and its potential as a biomaterial is based on its cationic nature and high charge density in solution [9]. Chitosan also possesses characteristics which are similar to various glycosaminoglycans (GAGs), such as chondroitin sulfate and keratin sulfate, which are found in the ECM of articular cartilage. Moreover, the primary monomer of chitosan has a framework similar to the predominant GAGs in cartilage. Because of this similarity, chitosan has been tested as a scaffold for cartilage repair [10].

These three biomaterials have been widely studied in any two polymer mixed for biomedical applications. Lin and Liu [17] investigated sponges blended from 80% bird feet, 20% pig skin collagen and hyaluronan and found them to have higher biostability and mechanical strength than sole-component sponges. Yamane et al. [9] also indicated that chitosan-based hyaluronan hybrid polymer fibers show great potential as a desirable biomaterial for cartilaginous tissue scaffolds due to its excellent characters of cell adhesion, proliferation and matrix secretion. Further, Ma et al. [19] showed that collagen/chitosan scaffolds were potential candidates for the dermal equivalent, with enhanced biostability and good biocompatibility. However, there have been few physical–chemical studies investigating the effect of different ratios of protein–GAG scaffolds and the application of tri-polymers. Because the major components of ECM are protein, glycosaminoglycan and proteoglycan, we hypothesize that scaffolds made from these three kinds of polymers could be an excellent environment for cell growth. This study was conducted to evaluate the characteristics of different ratios of comb collagen to chitosan modified by hyaluronan in order to obtain the optimum conditions for the manufacture of collagen–hyaluronan–chitosan (Col–HA–Ch) porous scaffolds.

2. Materials and methods

All chemicals were obtained from Sigma–Aldrich unless otherwise specified. Hen combs were obtained from a local poultry plant and thawed overnight at 4 °C. The degree of deacetylation of chitosan is 90%. A CyQuant cell prolifer-

ation assay kit, Dulbecco's modified Eagle's medium (DMEM)/F12, penicillin/streptomycin and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen Corporation, Carlsbad, CA). An MTS cell viability assay kit was purchased from Promega (Promega Corporation, USA).

2.1. Preparation of Col–HA–Ch scaffolds

Composite scaffolds, mixed in different ratios of collagen, HA and chitosan (10:1:0, 9:1:1, 8:1:2, 7:1:3, 6:1:4 and 5:1:5) were fabricated (Table 1). Native type I collagen and HA were isolated from hen combs using a protocol developed in previous studies in our laboratory [7]. Collagen, HA and chitosan were dissolved in 0.5 M acetic acid (HAc) to prepare a 1.0% (wt./vol.) blend solution. All solutions were slowly mixed at the ratio above and homogenized to obtain a Col–HA–Ch blend. After using a vacuum system to remove entrapped air bubbles, the Col–HA–Ch blend was injected into a mold developed in our laboratory (diameter: 6 mm, depth: 10 mm), then frozen at –20 °C for 4 h and –80 °C for 24 h. After treating by freeze-lyophilization, Col–HA–Ch scaffolds were immersed in 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/95% alcohol cross-linking solution at 4 °C twice for 48 h. The EDC cross-linking takes place by reaction between carboxyl groups of molecules to generate stable amide bonds. The residual EDC of scaffolds were replaced thrice by deionized water. Finally, the cross-linked scaffolds were freeze-dried again under the same condition.

2.2. Microstructure observation

Overall morphology and arrangement of the inner pores of the Col–HA–Ch scaffolds ($n = 5$) were coated with gold–palladium alloy and observed by scanning electron microscopy (SEM; TOPCON ABT-150S).

2.3. Swelling measurements

Three dry scaffolds were weighed (W_d) and placed into phosphate-buffered saline (PBS) at room temperature for 10 h. After removing the unabsorbed solution, the wet weight (W_w) of the specimens was determined. The swelling ratio of the scaffold was defined as the ratio of the weight increase ($W_w - W_d$) to the initial weight (W_d) according to following equation:

$$\text{swelling ratio}(\%) = ((W_w - W_d)/W_d) \times 100$$

2.4. Mechanical testing

The mechanical properties of the scaffolds were assessed by tensile testing (Testomertic, M500-25KN). The specimens were placed in rectangular disks (5 mm \times 1 \times T cm, where T is the thickness of the scaffold). The cross-head speed was 5 mm min⁻¹. The fraction stress elongation at

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