Materials Science and Engineering C 45 (2014) 343–347

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Development of keratin–chitosan–gelatin composite scaffold for soft tissue engineering

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ARTICLE INFO

Article history:
Received 25 June 2014
Received in revised form 10 August 2014
Accepted 11 September 2014
Available online 16 September 2014

Keywords:
Keratin
Biomaterial
Wound healing
Gelatin
Chitosan
Tissue engineering

ABSTRACT

Keratin has gained much attention in the recent past as a biomaterial for wound healing owing to its biocompatibility, biodegradability, intrinsic biological activity and presence of cellular binding motifs. In this paper, a novel biomimetic scaffold containing keratin, chitosan and gelatin was prepared by freeze drying method. The prepared keratin composite scaffold had good structural integrity. Fourier Transform Infrared (FTIR) spectroscopy showed the retention of the native structure of individual biopolymers (keratin, chitosan, and gelatin) used in the scaffold. Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) results revealed a high thermal denaturation temperature of the scaffold (200–250 °C). The keratin composite scaffold exhibited tensile strength (96 kPa), compression strength (8.5 kPa) and water uptake capacity (>1700%) comparable to that of a collagen scaffold, which was used as control. The morphology of the keratin composite scaffold observed using a Scanning Electron Microscope (SEM) exhibited good porosity and interconnectivity of pores. MTT assay using NIH 3T3 fibroblast cells demonstrated that the cell viability of the keratin composite scaffold was good. These observations suggest that the keratin–chitosan–gelatin composite scaffold is a promising alternative biomaterial for tissue engineering applications.

1. Introduction

There is an imminent need for the development of new and improved materials for soft tissue engineering applications. Over the last few decades, more importance has been given to materials, which are biologically active and that show better biocompatibility and biodegradability. These materials provide analogous environment to the extra cellular matrix (ECM) and provide an induced rate of synthesis or growth of new tissues. Several natural polymers viz collagen, chitosan, gelatin, and keratin possess the ability to induce the proliferation of cells and hence find their use as a biomaterial for a wide range of biomedical applications [1].

Amongst all biopolymers, collagen is a widely accepted material for tissue engineering applications. In view of its low antigenicity, excellent biocompatibility and biodegradability [2], collagen and collagen composites are widely used for soft tissue engineering applications such as wound healing [3,4], corneal implants [5], nerve regeneration [6] and drug delivery [7].

Chitosan is (1–4)-linked 2-amino-2-deoxy-b-glucan, a byproduct of N-deacetylation of chitin. It is a major constituent of crab and shrimp shells, and cuticles of insects [8,9]. Studies have shown the potential of chitin and chitosan for being used as novel biomaterials. These carbohydrate polymers have high biocompatibility [10,11], wound healing capability [8,12] and anti microbial activity [13,14].

Gelatin is the denatured form of collagen. Gelatin has low antigenicity and it promotes cell adhesion, differentiation and proliferation [15]. Gelatin also possesses high cytocompatibility, which makes it a potential candidate as a biomaterial for various tissue engineering applications [16,17].

Keratin is a family of fibrous proteins, which is found abundantly in nature. It forms the main constituent of hair, wool, nail, horn and hooves of mammals, birds and reptiles [18]. Keratin contains cysteine amino acid residues (7–20%) [19]. The oxidation of these cysteine residues leads to inter- and intra-molecular covalent bonds which are responsible for the toughness of the keratin fibers. Keratin contains cell adhesion sequences RGD (Arg-Gly-Asp), and LVD (Leu-Asp-Val), which are also found in extra cellular matrix proteins like fibronectin [18,20]. Keratin also possesses cellular binding motifs like native ECM, which mimic the cellular attachment sites. Due to the presence of such properties, keratin can be used for the development of different tissue engineering constructs. Although keratin based scaffolds and films have been prepared without using any additive, the major drawback of these constructs is their brittleness [20]. Hence, no single polymer is considered best to meet all the requirements of tissue engineering constructs [21].

In the present work, we report the preparation of a new composite scaffold comprising keratin, gelatin and chitosan, which is comparable

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http://dx.doi.org/10.1016/j.msec.2014.09.021
0928-4931/

to collagen scaffold in terms of physico-chemical and biological properties.

2. Material and methods

2.1. Materials

For extraction of keratin, we purchased sodium dodecyl sulfate (SDS) from Sisco Research Laboratories Pvt. Ltd., urea (mol. wt. 60.6), 2-mercaptoethanol (mol. wt. 78.13) and dialysis membrane (molecular weight cutoff — 14,000 Da) from HiMedia. For fabrication of scaffolds, chitosan with ≥75% degree of deacetylation, and gelatin were obtained from Sigma Aldrich, whereas acetic acid was purchased from Merk.

2.2. Methods

2.2.1. Extraction of keratin from bovine hooves

The raw hooves obtained from animal slaughter house were washed thoroughly with distilled water, dried and pulverized. The extraction process reported by Yamauchi et al. [22], was adopted to extract keratin from the pulverized hooves. Defatting of the hooves was done with the help of soxhlet apparatus using a mixture of hexane and dichloromethane in the ratio of 1:1 v/v. About 10 g of defatted hooves was taken in a 500 ml conical flask and a mixture of 7 M urea, 6 g SDS and 15 ml β-mercaptoethanol was added into it and incubated for 12 h in an orbital shaker at 60 °C. The resulting mixture was centrifuged at 6000 rpm for 15 min. Finally, the filtrate was collected and dialyzed against water for 7 days to obtain pure keratin with predominantly two molecular fractions at 45 and 60 Da.

2.2.2. Fabrication of keratin composite scaffold

To prepare keratin composite scaffold, about 120 mg of chitosan was dissolved in acidified water (using 50 mM acetic acid) to prepare chitosan solution. Gelatin solution was prepared by dissolving 240 mg of gelatin in hot water. Equimolar amount of chitosan and gelatin solutions were homogenized for 5 min. The extracted keratin solution (containing about 120 mg of keratin) was added drop by drop to the above mixture under continuous stirring to obtain a scaffold containing keratin, chitosan and gelatin in the ratio of 1:1:2 (w/w) respectively. The solution (about 48 ml) was homogenized for 30 min using Ultra Turrax IKA T25. The homogenized solution was frozen at −40 °C overnight and lyophilized for 48 h to form a porous scaffold with dimensions of $10 \times 10 \times 0.9$ cm^3. The final concentration of keratin and chitosan in the homogenized solution was 2.5 mg/ml each and gelatin was 5 mg/ml.

2.2.3. Fabrication of collagen scaffold

To isolate type I collagen, the procedure given by Tanaka et al. [31] was followed. Isolated collagen was dialyzed against 0.05 M acetic acid and lyophilized to obtain a powder. To prepare the collagen scaffold, lyophilized collagen was dissolved in 0.05 M acetic acid to obtain 3 mg/ml concentration of collagen. To this solution, Triton X 100 was added and homogenized for about 20 min using Ultra Turrax IKA T25. This was then kept at −40 °C and lyophilized to get a porous scaffold.

2.3. Fourier Transform Infrared Spectra

Fourier Transform Infrared (FTIR) spectra of the composite scaffold, keratin, gelatin and chitosan were measured with ABB-FTIR, Model MB 3000 in the range of 500–4000 cm^{-1} with a resolution of 8 cm^{-1}, at a scan rate of 32 scans/sample. Potassium bromide (KBr) was mixed with the composite scaffold, lyophilized keratin, chitosan and gelatin respectively to prepare the pellet for FTIR measurements.

2.4. Thermal analysis of composite scaffold

Differential scanning calorimetric (DSC) analysis of prepared composite scaffold was performed using Universal V4.4A TA Instrument to observe the thermal degradation behavior. The sample was heated to a temperature range from 25 °C to 300 °C, at the rate of 5 °C/min. The calibration of the instrument was done by Indium standard and liquid nitrogen was flushed at the rate of 50 ml/min to the calorimetric cell.

Thermo gravimetric analysis (TGA) of prepared composite scaffold was done using Universal V4.4A TA Instruments to check the thermal stability of the scaffold. The sample was heated at a temperature range from 20 °C to 800 °C at a rate of 20 °C/min in nitrogen atmosphere.

2.5. Porosity of the scaffolds

The porosity (P) of the keratin composite and collagen scaffolds was measured by ethanol infiltration method [23]. Briefly, weight of the dry scaffolds (W_o) was recorded and then they were soaked in ethanol by exhausting the air bubbles. Then the scaffolds were taken out, surface ethanol wiped and were weighed immediately (W_e). The porosity of the scaffolds was defined as

$$\%\,\text{porosity} = [(W_e - W_o)/\rho V_s] \times 100$$

where, ρ represents the density of ethanol at room temperature (0.789 mg/ml) and V_s is the volume of the scaffold which was calculated from the geometry of the scaffold.

2.6. Compressive strength and tensile strength analysis

The compression and tensile strength of keratin composite and collagen scaffold were analyzed using Texture Analyzer Pro CT V1.4 Build 17 (Brookfield Engineering Labs, Inc). The respective samples were taken in triplicates. To analyze compression strength in dry state, circular punches were cut with thickness of 7–10 mm and diameter of 15 mm and compressed till the thickness of the sample reduced to 50% of the original thickness. For wet state analysis compression strength was determined for the sample immersed in phosphate buffer saline (pH-7.4) for 1 h and then analyzed in the same way as mentioned above.

For tensile strength analysis, rectangular sections were cut with a cross-sectional area of approximately 7×4 mm^2. For the analysis in dry state, the samples were clamped vertically, with a gauge length of 10 mm and tested with a trigger load of 7 g, at the test speed of 0.5 mm/s. All samples were stretched until failure. For the analysis in wet state, samples were immersed in phosphate buffer saline (pH-7.4) for about 1 h followed by the determination of tensile strength.

2.7. Water uptake studies of the composite scaffold

The specimens of dimension 1×1 cm^2 were cut in triplicate from each of the scaffolds (keratin composite scaffold and collagen as control) and dried under vacuum at 60 °C for 4 h. Subsequently, the weight of each sample (W_{dry}) was recorded and then immersed into PBS. After 24 h of incubation, the scaffolds were taken out, wiped on the surface and immediately weighed (W_{wet}). The water uptake capacity of the scaffold was calculated by using the following formula:

$$\text{Water uptake}\,\% = \left[\left(W_{wet} - W_{dry}\right)/W_{dry}\right] \times 100.$$

2.8. Scanning Electron Microscopy

The surface morphology, structural integrity and interconnectivity of the pores in the scaffold were observed using Scanning Electron Microscopy (SEM HITACHI-S3400N) at a magnification of 300×. The samples were prepared by sputter coating the scaffold surface with a thin layer of gold. The surface of the samples was scanned at 15 kV.