Polymer Testing 62 (2017) 132–136

Contents lists available at ScienceDirect

Polymer Testing

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

Collagen-based scaffolds enriched with glycosaminoglycans isolated from skin of *Salmo salar* fish

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ARTICLE INFO

Article history:
Received 1 June 2017
Received in revised form
22 June 2017
Accepted 22 June 2017
Available online 24 June 2017

Keywords:
Collagen
Glycosaminoglycans
Scaffolds
Osteosarcoma SaOS-2 adhesion
Tissue engineering

ABSTRACT

In this study both collagen and glycosaminoglycans were isolated from biodegradable waste. Namely collagen was isolated from rat tail tendons and glycosaminoglycans (GAGs) from fish skin. Porous materials were then obtained based on the isolated collagen with 1 or 5% addition of GAGs by freeze-drying process. The scaffolds were studied by infrared spectroscopy, mechanical testing and examined for the porosity and density. The scaffolds structure was observed by scanning electron microscope. The adhesion and proliferation of human osteosarcoma SaOS-2 cells was examined on prepared scaffolds to assess their biocompability.

The results showed that the addition of glycosaminoglycans improves the properties of collagen-based scaffolds. Mechanical strength was increased by GAGs addition as well as the porosity of studied materials. Each scaffold with and without GAGs displayed porous structure with interconnected regular shaped pores. The attachment of cells was better for pure collagen scaffold, however, GAGs additive promoted the cells proliferation on the scaffold.

1. Introduction

Tissue engineering is an interdisciplinary field aiming at developing biological substitutes or improving materials functions [1]. Biomimetic materials are designed to mimic native tissue and display better biocompatibility and bioactivity compared to traditional artificial biomedical implants [2]. Porous 3D structures (scaffolds) are primarily used to provide the support for cells and allow for their deep penetration within the scaffolds for tissue regeneration purposes.

Functional materials for tissue engineering can be obtained from natural polymers, e.g. collagen or glycosaminoglycans (GAGs), such as hyaluronic acid (HA) and chondroitin sulfate (CS) [3]. Materials for the biomedical application can be prepared from ternary blends or polymer mixtures because the use of such macromolecule complexes improves physicochemical properties of scaffolds [4].

Natural polymers can be isolated from different natural sources. The main collagen sources are bovine skin [5], fish skin [6] and scales [7] or rat tail tendons [8]. Glycosaminoglycans (GAGs) can be isolated from rooster combs [9], fish skin [10] or eyeballs [11].

In biomaterials science, it is extremely important to use safe substrates which will not cause any immunological reactions after implantation. The compounds isolated from natural sources are biocompatible and non-toxic. Thereby, the use of collagen or glycosaminoglycans to obtain biomaterials is beneficial compared to synthetic materials due to their natural biological origin.

Commercially available collagen and glycosaminoglycans (hyaluronic acid or chondroitin sulfate) are expensive. Their use as substrates for biomaterials science increases the final production costs. The application of food industry waste is eco-friendly action due to the management of harmful wastes for the environment.

The aim of the study was to isolate collagen and glycosaminoglycans from biodegradable wastes and use them for the development of the scaffolds for the potential tissue engineering application.

2. Materials and methods

2.1. Sample preparation

Collagen and the mixture of glycosaminoglycans (GAGs) were isolated in our laboratory. Collagen was isolated from rat tail

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http://dx.doi.org/10.1016/j.polymertesting.2017.06.022
0142-9418/

tendons, washed in distilled water, dissolved in 0.1 M acetic acid and lyophilizated. The procedure of glycosaminoglycans (GAG) isolation was performed according to the procedure reported elsewhere [12]. The skin from *Salmo salar* fish (from Koral s. c., Tychy, Poland) was cut on a small pieces and defatted in acetone (250 g of tissue per 300 ml acetone) for 48 h. The obtained precipitate was dried at 60 °C for 24 h. 100 mM sodium acetate buffer (pH = 5.5) and added (20 ml per 1 g of precipitate) containing 5 mM EDTA and 5 mM cysteine. Papain was added (100 mg per 1 g of precipitate) to the prepared solution, and it was incubated for 1 h at 60 °C. After boiling for 10 min, the obtained mixture was centrifuged at 10 000*g* for 15 min. Three volumes of saturated ethanol with sodium acetate were added and mixture was incubated at 4 °C for 24 h. The precipitate was centrifuged at 10 000*g* for 15 min and dried at 60 °C.

The 1% concentration solution of collagen was prepared in 0.1 M acetic acid and GAGs in distilled water. To the collagen solution the mixture of GAGs was added in weight ratio 1 and 5% and mixed on magnetic stirrer. The mixtures were then placed in sterile polystyrene plates, frozen and lyophilizated. As a results 3D porous structures, called scaffolds, were obtained.

2.2. Infrared spectroscopy (IR-ATR)

FTIR analysis was made for each kind of scaffold. Dried scaffolds were scanned with 4 cm^{-1} resolution by Nicolet iS10 equipped with an ATR device with diamond crystal. The characteristic peaks of IR transmission spectra were recorded. Each recorded spectrum was the average of 64 scans.

2.3. Scanning electron microscopy (SEM)

The morphology of the samples was studied using Scanning Electron Microscope (SEM) (LEO Electron Microscopy Ltd, England). Scaffolds were frozen in liquid nitrogen for 3 min and gently cut with a razor scalpel for the interior structure observation. Samples were covered by gold and scanning electron microscope images were made with resolution 500 μm.

2.4. Porosity and density

The density and porosity of scaffolds were measured by isopropanol displacement, because it does not wet the sample [13]. Samples were put into the known volume of isopropanol (V_1). After 5 min the change in volume of isopropanol-impregnated scaffold was measured. The sample was removed from the solution and again the difference in isopropanol volume was determined. The density of the porous sample (d) was calculated as follows:

$$d = \frac{W}{V_2 - V_3} \quad (1)$$

where W is sample weight, V_2 is the total volume of isopropanol and isopropanol-impregnated scaffold, V_3 is the isopropanol volume after sample removing. The porosity (p) of the scaffold was calculated using the equation:

$$p = \frac{V_1 - V_3}{V_2 - V_3} \quad (2)$$

where V_2, V_3 as above, V_1 is the initial volume of isopropanol.

2.5. Mechanical testing

Mechanical properties were measured using a static mechanical testing machine (Z.05, Zwick/Roell, Germany). Stress–strain curves were recorded under room conditions for scaffolds with a dry surface. Scaffolds were placed between two discs and compressed. The slope of the linear region of the compression curve gives the Young's modulus (E_{mod}). The stress needed to compress scaffold to 20% of its height is taken as maximum force (F_{max}).

2.6. Attachment and proliferation of human osteosarcoma SaOS-2 cells

For cell culture, scaffolds (0.5 cm height, 0.12 mm diameter) were soaked in 70% EtOH (water solution) followed by washing in sterile phosphate buffer solution (PBS; pH = 7.4). The scaffolds were then transferred to 24-well plates and left for 24 h to dry out. For the biocompatibility studies human osteosarcoma cell line SaOS-2 was used. These cells closely resemble the osteoblast phenotype and respond similar manner to normal human osteoblasts [14]. Cells were seeded at the density of 15×10^4 cells/scaffold and cultured for total of 4 days in alpha-MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. At day 1 culture, cell-seeded scaffolds were transferred to new culture plates and covered with fresh culture medium. The initial plates were examined with the CellTiter96Aqueous One Solution Cell Proliferation Assay (MTS, Promega, Poland) for any remaining cells that had not adhered to the scaffolds. Briefly, MTS solution was diluted 10x in phenol-free alpha-MEM and 200 μl aliquots were added per well. The absorbance at 490 nm was measured after 30 min incubation at 37 °C in the dark. At day 4 culture, the scaffolds were examined for cell viability using similar procedure except that the scaffolds were covered with 400-μl aliquots of 10x diluted MTS. This assay was previously successfully used by us to examine cell viability on polyurethane [15]. Results were expressed as % change in cell viability on collagen-GAG scaffolds compared to results obtained for pure collagen scaffold. For statistical analysis, the value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Infrared spectroscopy (IR-ATR)

The IR-ATR spectra in the range of 1200–1700 cm^{-1} is shown (Fig. 1). Amide I and II bands can be clearly observed. The Amide I is the most sensitive peak to consider the secondary structure of proteins [16]. It arises from C=O and N-H vibrations of peptide bonds. Its position at 1668 cm^{-1} for pure collagen is shifted to higher wavenumbers (1655 and 1654 cm^{-1}) as well as the peak shape was changed after the addition of glycosaminoglycans. It justifies the hydrogen bonds formation between collagen and GAGs what indicates the structural rearrangement in protein conformation [17]. The Amide II localization (around 1556 cm^{-1}) and shape was not changed after the addition of glycosaminoglycans to the collagen. It suggests that between Amide II band and GAGs interactions were not formed.

3.2. Scanning electron microscopy (SEM)

Materials should be porous to be considered in tissue engineering application. Scanning electron microscope images shown that obtained scaffolds have porous structure (Fig. 2). The pores are interconnected and their diameter is lower than 500 μm. Such decrease is related with increasing amount of glycosaminoglycans in the sample. New interactions between polymeric chains are formed and the pores diameter is lower than for pure collagen. Furthermore, for each pore of scaffolds are open with regular shape. The material is homogenous in the whole volume.