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Scaffolds based on chitosan and collagen with glycosaminoglycans cross-linked by tannic acid

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ABSTRACT

Scaffolds based on chitosan, collagen, and glycosaminoglycans-enriched ones, cross-linked by tannic acid can be obtained with the use of the freeze-drying method. Composites were characterized by different analyses, e.g. SEM images, porosity and density measurements, swelling, liquid uptake, mechanical tests in wet conditions, and enzymatic degradation by collagenase and hyaluronidase. In addition, the viability of human osteosarcoma SaOS-2 cells was examined on the obtained scaffolds.

The results showed that the scaffolds based on chitosan, collagen, and glycosaminoglycans cross-linked by tannic acid are not cytotoxic. Scaffolds are stable in aqueous environment and show high swelling behavior. Each material porosity is above 90% which is appropriate for the tissue engineering applications. The mechanical parameters of the scaffolds decrease with increasing immersion time in PBS. SEM images showed the homogeneous scaffold structure with interconnected pores. Due to their stability and biocompatibility, the scaffolds presented here may be easily operated to fit such small bone defects.

1. Introduction

Glycosaminoglycans (GAGs) are polyanionic linear polysaccharides with a repeating disaccharide units structure. GAGs are components of various structural and connective tissues such as skin, cartilage, and cornea [1]. Each tissue produces GAGs with specific polymeric chains binded with proteins, enzymes, or ions [2–4]. The connective tissues of marine organisms are rich in sulfated glycosaminoglycans [5,6]. The isolation of GAGs from marine organisms, e.g. the *Aetobatus narinari* [7], *Cyclopterus lumpus* [8], *Anodonta cygnea* [9], or *Salmo salar* [10] has already been reported.

Glycosaminoglycans can be identified by the spectrophotometric assay in the absorption spectrum of 1,9-dimethylmethylene blue (DMB) [11,12]. The DMB complex can be stabilized by the use of formate buffer. Another dye type for GAGs identification is Alcian blue [13]. However, the DMB use is a more sensitive method than Alcian blue use [14].

The obtainment of scaffolds based on collagen with glycosaminoglycans has already been reported [15–17]. Such materials present appropriate biocompatibility and are not cytotoxic. GAGs are also added to chitosan-based materials [18,19]. The obtained composites were characterized as biocompatible and bioresorbable. Moreover, the appropriate biological properties of chitosan/collagen scaffolds with GAGs were reported previously [10]. However, materials based on chitosan, or their mixture, exhibit low stability in aqueous environment and need to be cross-linked to improve their properties.

Tannic acid is a glucose and gallic acid polyphenol. It can be applied to scaffolds as a cross-linker due to the hydrogen and electrostatic interactions formation [20]. Tannic acid addition to the polymeric scaffolds results in the obtainment of materials biocompatible and stable in aqueous conditions [21,22].

The aim of the study was to obtain scaffolds based on chitosan and collagen, glycosaminoglycans-enriched ones, cross-linked by tannic acid. The novel materials were characterized by physicochemical methods as well as an *in vitro* experiment. Such scaffolds can find a potential clinical application to fill small bone or cartilage defects.

2. Materials and methods

2.1. Sample preparation

Collagen was isolated from rat tail tendons in our laboratory. Chitosan (DD = 77%, M_v = 5.4 × 10^5 g/mol) and tannic acid were purchased from Sigma-Aldrich Company (Germany). Polymers were dissolved in 0.1M acetic acid at 1 wt.% concentration each. The procedure of glycosaminoglycans (GAGs) isolation was according to the

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procedure reported previously and the presence of hyaluronic acid and chondroitin sulfate in the isolated GAGs mixture was noticed by the spectrophotometric method [10]. The GAGs solution at 1% concentration in distilled water was prepared.

2.2. Scaffolds preparation

Chitosan and collagen solutions were mixed in the 50/50 wt ratio [23]. During stirring, 1 and 5 wt.% of the isolated glycosaminoglycans solution was added. Then, tannic acid was added as a cross-linker in the 20% weight ratio based on the previous studies [20,22]. The obtained solutions were frozen and lyophilizated at −55 °C and 5Pa (ALPHA 1-2LD plus, CHRIST, Germany) for 24 h. As a result, 3D porous structures (scaffolds) were obtained.

2.3. Scaffolds porosity and density

The density and porosity of scaffolds were measured by isopropanol displacement, because it does not wet the sample [24]. 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.

2.4. Scanning electron microscope

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 gentry 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.

For the cells observation on the scaffold the samples were dried in the CO_2 critical point dryer, attached to the microscope adhesive holders and covered with a gold layer. The SEM images were taken under JEOL JSM5410 scanning electron microscope (JEOL, Tokyo, Japan).

2.5. Swelling properties and liquid uptake

Swelling behavior was measured by immersing the scaffolds in a phosphate-buffer saline (PBS) solution (pH = 7.4) for 2, 24, 48, 72 h. After each period of time, the immersed materials were gently dried and weighed. The swelling ratios were then calculated using equation (3) [22]:

$$swelling\ [\%] = \frac{m_{S(t)} - m_{S(0)}}{m_{S(0)}} * 100\% \quad (3)$$

where $m_{s(t)}$ is the weight of the scaffolds after immersion in PBS for the period of time and $m_{s(0)}$ is the weight before immersion.

Liquid uptake is the percentage liquid content after the scaffolds immersion. It is related with the scaffolds weight changes. Scaffolds are placed in the determined PBS mass (m_0) and are then removed without squeezing. The rest of the PBS solution was weighed (m_t) and the liquid uptake was calculated [22]:

$$Liquid\ uptake = \left| \frac{m_t - m_0}{m_0} \right| * 100\% \quad (4)$$

2.6. Mechanical testing

Mechanical properties were measured by mechanical testing machine (Z.05, Zwick/Roell, Germany) for each kind of sample. Scaffolds were immersed in PBS for 2, 4, 24, 48 and 72 h. After those periods of time samples were put between two discs and compressed. Measurement was carried out in PBS solution (pH = 7.4). The compressive modulus is a Young modulus for the compression process, where it determines the stiffness of an elastic composite. It was calculated from the slope of the stress-strain curves in the linear region (strain from 2 to 5%). The compressive stress is the force on the surface area which has to be applied to compress the scaffold to 20% of its height [22]. For each kind of composite at least ten samples were tested and the standard deviation was calculated.

2.7. Enzymatic degradation

2.7.1. Collagenase

For the enzymatic degradation study, the scaffolds with a determined mass were immersed in 1 ml of 0.1M Tris-HCl (pH = 7.4) containing 50 mM $CaCl_2$ and incubated in 37 °C for 30 min. 1 ml of 0.1M Tris-HCl containing 50 units of collagenase from *Clostridium histolyticum*, Type I (Sigma-Aldrich, Poland) was added to the solution. The scaffolds were incubated at 37 °C for 1 h. The reaction was stopped by the addition of 0.2 ml 0.25M EDTA. The samples were rinsed with distilled water and immersed in methanol for 3 h. Then, they were rinsed with distilled water again, frozen, and lyophilized. The percentage of weight loss was determined by the calculation of mass difference between scaffolds before and after degradation [24].

2.7.2. Hyaluronidase

The samples with the known weight were immersed in PBS and placed at 37 °C for 30 min. 1 ml of PBS containing 50 units of hyaluronidase from bovine testes (Sigma-Aldrich, Germany) was added to the solution and incubated for 1 h. Then, the samples were rinsed with distilled water three times and immersed in methanol for 3 h. They were rinsed with distilled water again, frozen, and lyophilized. The percentage of weight loss was calculated [25].

2.7.3. In vitro tests

The *in vitro* testes were carried out with the procedure published previously [17,22]. Scaffolds (0.5 cm height, 0.12 mm diameter) were soaked in 70% EtOH (water solution) and washed in sterile phosphate buffer solution (PBS; pH = 7.4). For the preliminary assessment of scaffolds suitability for cell growth, human osteosarcoma cell line SaOS-2 was used [26]. These cells are often the first choice to assess scaffolds biocompatibility, especially if they are aimed at bone tissue engineering application [27,28]. 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. Cell-seeded scaffolds were examined with the CellTiter96Aqueous One Solution Cell Proliferation Assay (MTS, Promega, Poland). MTS solution was diluted 10x in phenol-free alpha-MEM and 400 μl aliquots were added per well per scaffold. The absorbance at 490 nm was measured after 30 min incubation at 37 °C in the dark [27,28]. Results were expressed as % change in cell viability compared to results obtained for unmodified scaffolds.