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The comparison of physic-chemical properties of chitosan/collagen/hyaluronic acid composites with nano-hydroxyapatite cross-linked by dialdehyde starch and tannic acid

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ABSTRACT

Scaffolds based on chitosan, collagen and hyaluronic acid, cross-linked by dialdehyde starch with hydroxyapatite were obtained with the use of the freeze-drying method. Scaffolds were cross-linked by tannic acid or dialdehyde starch addition. Composites were characterized by different analyses, e.g. SEM images, porosity, density, liquid uptake, and mechanical tests. In addition, the adhesion and proliferation of human osteosarcoma SaOS-2 cells were examined on the obtained scaffolds.

The results showed that the properties of the scaffolds based on chitosan, collagen, and hyaluronic acid can be modified by cross-linkers addition. The compressive modulus for the scaffolds cross-linked by dialdehyde starch was higher than for those cross-linked by tannic acid. The porosity of scaffolds cross-linked by starch was higher than those in which tannic acid was applied. However, the former presented lower density. SEM images showed the homogeneous scaffold structure with interconnected pores. Scaffolds cross-linked by tannic acid exhibited higher biocompatibility than those cross-linked by dialdehyde starch. However, the results showed that both scaffolds, cross-linked by dialdehyde starch and by tannic acid can provide the support required in tissue engineering and regenerative medicine. The scaffolds presented here may be easily operated to fit such small bone defects without causing adverse reactions.

1. Introduction

Porous structures widely applied in tissue engineering are called scaffolds. Cells attach to their surface as well as to deeper parts of material. The interconnected pores enable the nutrients flow and cells are able to proliferate. However, the biocompatibility of scaffolds has to be improved in order to promote cells attachment and proliferation inside the material.

Hydroxyapatite, an inorganic compound, is the main type of apatite present in the native human bone. The addition of commercially available hydroxyapatite improves the scaffolds biocompatibility [1–3]. It is the factor which indicates deeper penetration of cells into the material, their attachment, proliferation and, as a result, the improvement of the regeneration process.

The properties of scaffolds with hydroxyapatite addition have already been studied [4]. It was observed that they degrade easily and have poor mechanical parameters. The properties of the biomaterial can be improved by the cross-linker addition to the polymeric mixture. Such a method is called chemical cross-linking and is more effective than other methods such as the physical or enzymatic modification [5].

Numerous cross-linkers have already been studied for chitosan and collagen mixtures. Synthetic and natural compounds can be applied to modify the material properties. The improvement of scaffolds properties was noticed after e.g. *N*-(3-dimethylamino propyl)-N'-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) mixture [6] or glutaraldehyde [7] addition. However, the addition resulted in the necessity to remove unreacted substances due to their toxicity. Natural cross-linkers application, e.g. starch [8], tannic acid [9], genipin [10], was also reported. Even if the unreacted substrates remain in the scaffold structure, the immunological reaction after the material implantation was not noticed.

Studies of scaffolds based on chitosan, collagen, and hyaluronic acid with hydroxyapatite were previously carried out [4]. Calcium

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release from the samples was detected. It initiates the mineralization process which is related to the bone regeneration. Thereby, in this paper, cross-linkers such as dialdehyde starch and tannic acid are proposed to improve the physicochemical and biological properties of scaffolds. Due to soft character of the obtained scaffolds and the presence of collagen, a natural component of bone matrix, we presume these scaffold may find applications in non load-bearing bone regeneration strategies.

2. Materials and methods

Collagen (Coll) was isolated from rat tail tendons under laboratory conditions [11]. Chitosan (CTS) (DD = 77%, $M_v = 5.4 \times 10^5$ g/mol [12]), hyaluronic acid (HA) (1.8×10^6 g/mol [13]), nano-hydroxyapatite (particle size<200 nm (BET); nHAp) and tannic acid (TA) were purchased from Sigma-Aldrich company (Germany). Dialdehyde starch (ST) was purchased from Chemos GmbH&Co. KG (Germany).

2.1. Samples preparation

CTS and Coll, each separately, were dissolved in 0.1M acetic acid at 1% concentration. Hyaluronic acid was prepared at 1% concentration in 0.1M hydrochloric acid. Solvents were chosen based on previous miscibility studies [14]. Dialdehyde starch have already been reported as safe and effective cross-linker [8]. ST was dissolved in distilled water at 3% concentration. Chitosan and collagen were mixed in 50/50 weight ratio with 1, 2, and 5% addition of hyaluronic acid. Then two types of cross-linkers were added separately– dialdehyde starch at 5% or tannic acid at 20% addition. Such ratios were chosen based on the previously reported studies [8,13]. The nano-hydroxyapatite was added to such mixtures in the 50 and 80 w/w% ratio. All the mixtures were frozen and lyophilized (ALPHA 1–2 LDplus, CHRIST,–20 °C, 100 Pa, 48 h). The scheme of the scaffolds preparation is shown in Scheme 1. The statistical analysis was made in the Excel program by the standard deviation and *t*-Student test calculation. The value of $p < 0.05$ was considered as significant.

2.2. 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 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.

2.3. Porosity and density

The porosity and density of the obtained scaffolds were measured by the liquid displacement [15]. In this study, the scaffold of the known weight (W) was immersed in the determined volume of isopropanol (V_1) for 5 min. The total volume of isopropanol with the impregnated scaffold was V_2. After the scaffold removal, the volume of isopropanol was measured (V_3). The density (d) and porosity (ε) were calculated by using the equations:

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

$$\varepsilon = \frac{V_1 - V_3}{V_2 - V_3} * 100\% \quad (2)$$

2.4. Liquid uptake

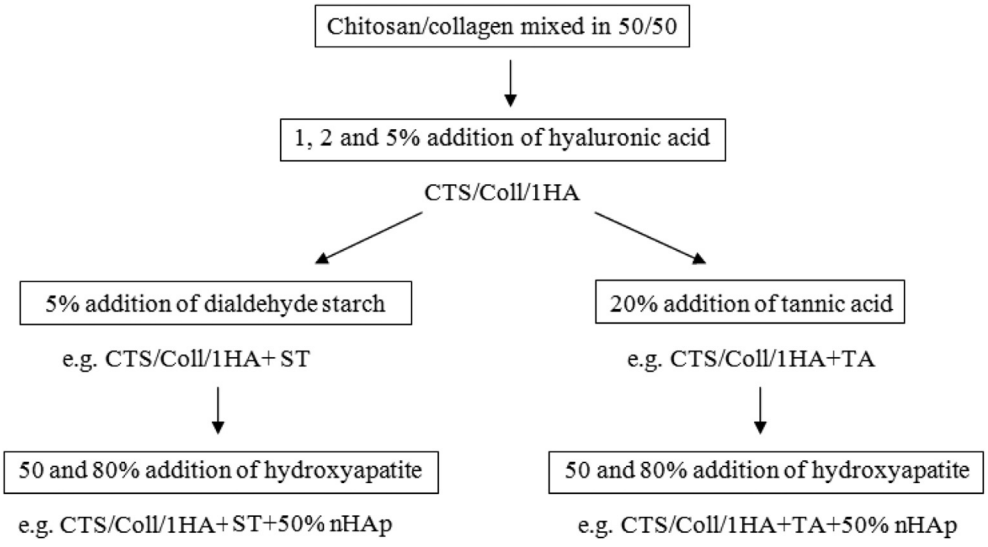
Scaffolds were immersed in 15 ml phosphate buffer solution (PBS) and incubated at 37 °C for 24 h. After the time intervals, the samples were removed. The weight values of with the immersed sample (m_0) and after the scaffold removal (m_t) were determined. The water uptake was then calculated:

$$\text{Liquid uptake} = \frac{m_t - m_0}{m_0} * 100\% \quad (3)$$

Liquid uptake for each type of scaffolds was carried in triplicate.

2.5. Mechanical testing

Mechanical properties were measured by a mechanical testing machine (Z.05, Zwick/Roell, Germany) for each kind of sample with and without calcium phosphate. Cylindrical samples sized 20 mm in diameter and 13 mm high were prepared for mechanical testing. The samples were introduced between two discs and compressed. The measurements were carried out for the scaffolds immersed in



Scheme 1. The scaffolds preparation scheme.